1

44433

National Library
of CanadaBibliothèque nationale
du CanadaCANADIAN THESES
ON MICROFICHETHÈSES CANADIENNES
SUR MICROFICHE

NAME OF AUTHOR/NOM DE L'AUTEUR Douglas Mills Templeton

TITLE OF THESIS/TITRE DE LA THÈSE Studies on the multifunctionality of the flavoprotein
lipoamide dehydrogenase.

UNIVERSITY/UNIVERSITÉ Carleton University.

DEGREE FOR WHICH THESIS WAS PRESENTED/
GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE Ph.D.

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE DEGRÉ 1979

NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Dr. C.S. Tsai

Permission is hereby granted to the NATIONAL LIBRARY OF
CANADA to microfilm this thesis and to lend or sell copies
of the film.

*L'autorisation est, par la présente, accordée à la BIBLIOTHÈ-
QUE NATIONALE DU CANADA de microfilmer cette thèse et
de prêter ou de vendre des exemplaires du film.*

The author reserves other publication rights, and neither the
thesis nor extensive extracts from it may be printed or other-
wise reproduced without the author's written permission.

*L'auteur se réserve les autres droits de publication; ni la
thèse ni de longs extraits de celle-ci ne doivent être imprimés
ou autrement reproduits sans l'autorisation écrite de l'auteur.*

DATED/DATE

July 6, 1979

SIGNED/SIGNÉ

Doug Templeton

PERMANENT ADDRESS/RÉSIDENCE FIXE

RR 5Ottawa, OntarioK1G 3N3

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

AVIS

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

**THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED**

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

**LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE**

STUDIES ON THE MULTIFUNCTIONALITY
OF THE FLAVOPROTEIN-
LIPOAMIDE DEHYDROGENASE

by

■ Douglas Mills Templeton, B.Sc.

A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Department of Chemistry
Carleton University

Ottawa, Canada

June, 1979

The undersigned recommend to the Faculty of Graduate
Studies acceptance of the thesis

"Studies on the Multifunctionality of the
Flavoprotein Lipoamide Dehydrogenase"

submitted by Douglas Mills Templeton in partial fulfillment
of the requirements for the degree of Doctor of Philosophy.

Thesis Supervisor

Chairman, Department of Chemistry

External Examiner

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the invaluable and enthusiastic guidance of Professor C.S. Tsai throughout the course of this research. Thanks are also due to Dr. B.R. Hollebone for helpful discussions, and the availability of the MCD facilities, and to Drs. J. Neelin and H. Yamasaki of the Biology Department of this University for the use of their equipment. The assistance of Drs. D. Rayner and M. Yaguchi of NRC, in supervising the fluorescence decay measurements and providing amino acid analyses, respectively, is also gratefully acknowledged. Mr. Karl Diedrich of this department was always helpful. Special thanks are due to Eva Gudgin and Roberta Fulthorpe, particularly for their aid in preparation of the manuscript, and finally to my parents for their continuing moral support throughout the course of my education.

ABSTRACT

A definition of enzyme multifunctionality is proposed and a classification of multifunctional enzymes given. Enzymes with separable, inseparable, and single active centres are distinguished, as well as those displaying multifunctionality at a single active site. Multifunctionality conferred by a bound cofactor is distinguished from that due to a single protein site. Experimental criteria for the demonstration of each type of multifunctionality are outlined, and do not require that reactions be of demonstrated physiological significance. Within this framework an investigation of the multifunctionality of the flavoprotein lipoamide dehydrogenase is undertaken. Lipoamide-linked dehydrogenase (DHase), nicotinamide nucleotide transhydrogenase (THase), single-electron transferase (ETase), quinone dye diaphorase (DPase), and NADH oxidase (OXase) activities are discussed. Six isozymes are separated by isoelectric focusing and shown by kinetic analysis to display the same mechanism for each reaction, ruling out the existence of isozymes as the source of multifunctional activity. Absorption spectra and fluorescence decay measurements indicate similar flavin environments in isozymes arising from the pyruvate dehydrogenase and α -ketoglutaric dehydrogenase complexes. Diimidoester cross-linking of the dimeric protein indicates that the dimer displays all activities, and that while the monomer lacks DHase activity, the presence of monomeric and dimeric species in equilibrium is ruled out as the source of the multifunctionality. Results of a series of chemical modifications of the enzyme are presented which produce unique alterations in the pattern of multifunctionality. Reaction

with excess methyl acetimidate modifies 19 lysine residues and results in a species with selectively enhanced DHase activity. Reaction with phosphoramidate has a similar effect, tentatively ascribed to phosphorylation of a histidine residue. Alkylation with iodoacetamide of a single sulfhydryl residue resulting from reduction of the active site disulfide produces a species with a dialyzable flavin and enhancement of all activities but DHase, which is lost. Extensive carboxymethylation with iodoacetic acid following pretreatment with β -mercaptoethanol produces a monomeric enzyme species with no tendency to dimerize. DHase and THase activities are lost. ETase remains and DPase is greatly enhanced. FAD is readily removable from this species and spectrally appears as free flavin, although several independent lines of reasoning argue that it is still stoichiometrically associated with the active centre. In contrast to native enzyme, treatment of this species with cuprous ion has an adverse effect on the DPase activity. Modification of the reduced enzyme with cobaltous sulfate selectively enhances the ETase activity, possibly due to stabilization of a flavin semiquinone intermediate. Co(II) is not chelated by the active centre disulfide, but rather, it is argued, has a flavin ligand. Magnetic circular dichroism (MCD) spectroscopy indicates that the cobalt atom is in a tetracoordinate hydrophobic environment. Blockage of the disulfide with arsenite results in a loss of DHase activity and enhanced DPase activity, THase and ETase being affected to lesser extents. The factors affecting the multifunctionality of the enzyme are summarized. Increasing polarity of the flavin environment, loosening of the flavin, blockage

or destruction of the active site disulfide, conformational changes, and monomerization all favour DPase activity but inactivate DHase. The activities follow the order of sensitivity DPase < ETase < THase < DHase. Conformational changes in modified enzymes have been followed by Attenuated Total Reflectance infrared spectroscopy, which is shown to be a sensitive method for this purpose. In all cases, including apoenzyme formation, conformational alterations are in the direction of increasing α -helical content. The OXase reaction has been less fully investigated, but appears to be similar to ETase. In addition, low pH selectively enhances ETase and OXase activities. This is thought to arise from a greater concentration of reduced flavin in the half-reduced EH_2 intermediate, which then forms a relatively stable free radical and effects splitting of the electron pair originating from NADH. Both reactions are also cooperatively activated by addition of catalytic amounts of free flavin, which may produce the semiquinone radical free in solution. Two NAD^+ binding sites per subunit are found by difference spectroscopy, and are identified with unique spectral features. Site I is the normal catalytic site and results in quenching of flavin absorption and fluorescence by enhancing flavin-protein or flavin-flavin interactions. Binding of NAD^+ at site II results in flavin-adenosine charge transfer complex formation, which may prevent full reduction of the enzyme to EH_4 . Site II does not bind thio- NAD^+ (TNAD^+) and is missing for all analogs (NAD^+ , TNAD^+ , and 3-acetylpyridine- NAD^+) in the mono-alkylated enzyme. Site II is a conformationally sensitive site and displays both catalytic and regulatory functions. The pH dependence of the dissociation constants of NAD^+

analogs at both sites is investigated. Loss of site II is a factor which can be added to the multifunctional sensitivity scale. Contrary to previous reports, the fully reduced EH_4 species has been found to be active in all reactions. The observed inactivity and avoidance of formation of EH_4 in the normal catalytic cycle is thought to reflect the instability of this species. The flavin is reduced in EH_4 and becomes non-planar. It then is easily lost from its binding site. Fluorescence energy transfer measurements show that the flavin is at $14 \text{ \AA}$ from a tryptophan residue, and that this distance is invariant under subtle conformational changes of the active centre. Thus tryptophan does not appear to be involved in the hydrophobic stabilization of bound flavin. A second tryptophan residue is exposed on monomerization, and may be involved in dimer association. Theoretical calculations show that hydrophobic effects alone cannot account for the difference in binding energies of FAD and FMN to the apoenzyme, and a more specific protein-adenosine interaction is indicated. Several lines of evidence, including increased fluorescent quantum yield of the isoalloxazine chromophore indicate that the flavin is bound in an unfolded conformation. Fluorescence decay measurements indicate non-equivalent environments for FAD in the two subunits of the native dimer. The possibility of flavin-flavin interaction is considered. An MCD study of free and bound flavins is reported. In unmodified enzyme, it is argued that donation of electron density from disulfide to flavin is observed. In the reduced enzyme, a feature attributable to a thiolate anion is observed, ruling out previously proposed covalent or biradical structures. The

red colour of this enzyme is thought to result from thiolate-to-imidazolium charge transfer, and not thiolate-to-flavin as had also been suggested. Destruction of 5 histidine residues by rose bengal sensitized photooxidation eliminates the charge transfer feature. Evidence is presented that an active centre histidine involved in the charge transfer with thiolate also acts as a base catalyst in the DHase reaction. Photooxidation reduces this activity exclusively to 35% the normal value. Mono-alkylation affects the substrate binding sulphydryl, leaving the catalytic thiolate free to form. A model of the active centre of lipoamide dehydrogenase is presented, and a reaction scheme accounting for the observed multifunctionality is proposed.

TABLE OF CONTENTS

Acknowledgements	i
Abstract	ii
List of Tables	viii
List of Figures	x
List of Schemes	xiv
List of Abbreviations	xv
Introduction:	
Part A - Enzyme Multifunctionality	1
Part B - Flavins and Flavoproteins	33
Part C - Lipamide Dehydrogenase	72
Materials and Methods	101
Results:	
I - Purity	131
II - Chemical Modifications	132
III - Isozymes	207
IV - Structural Studies	218
V - Properties of Reduced Enzyme Species	256
VI - Nicotinamide Nucleotide Binding Studies	279
VII - THase Reaction	306
VIII - ETase Reaction	310
IX - OXase Reaction	325
X - DPase Reaction	331
Discussion	347
Conclusions	415
Appendix I	417
Appendix II	420
References	428

LIST OF TABLES

1. Classification of Multifunctional Enzymes	21
2. Spectral Characteristics of the Flavins	39
3. Kinetic Data for Lipoic Acid Analogs	73
4. Amino Acid Composition of Lipoamide Dehydrogenase	81
5. Absorption Ratios Indicative of Enzyme Purity	133
6. Activities of E_{RCM} and Related Preparations	135
7. Summary of Molecular Weights of Native RCM-Enzymes	142
8. Partial Amino Acid Analyses of E_{RCM} and Related Species	146
9. PCMB Titration of E_{RCM} and Related Species	147
10. Characteristic Polypeptide IR Frequencies	149
11. Effects of Cu^{2+} on Native and RCM-Enzymes	160
12. Effects of Alkylating and Reducing Reagents on Activities	162
13. Activity Alterations Following Photooxidation	171
14. Amino Acid Analyses of Control Enzyme, E_{amid} , and E_{hv}	172
15. Duplicate Amino Acid Analyses of Table 14	173
16. Multifunctional Activities of E_{amid}	176
17. Multifunctional Activities of E_{phos}	176
18. Degree of Cross-linking	192
19. Cross-linking under Various Conditions	195
20. Multifunctional Activities of E_{As}	199
21. Multifunctional Activities of E_{Co}	202
22. Fluorescence Data of Native Enzyme and E_{Co}	204
23. Isozyme Separation Data	210
24. Kinetic Parameters of Isozymes	216
25. Fluorescence Decay Fitting Parameters	219
26. Fluorescence Decay Data, Summary	221
27. Summary of Fluorescence Energy Transfer Data	227
28. Quantum Yields of Enzyme, FAD, and E_{RCM}	

29. MCD Signs of Flavins in Various Solvents	228
30. Multifunctional Activities of EH_4 (Dithionite)	258
31. Multifunctional Activities of Reduced E_{ma}	258
32. Effects of Possible Inhibitors Arising from the NADase Reaction	264
33. Multifunctional Activities of EH_4 (NADH/NADase)	264
34. Redox Potentials	266
35. Dialysis of Flavin from Reduced Enzyme Forms	278
36. Dissociation Constants for Pyridine Nucleotide Binding	297
37. Fluorescence Data of $\epsilon\text{-NAD}^+$ and Enzyme	305
38. Kinetic Parameters of THase with Analogs	309
39. Multifunctional Effects of H_2O_2 and Riboflavin	322
40. NADH and Dihydrolipoamide OXase Activities	329
41. Summary of Isozyme Properties	348
42. Summary of Multifunctional Variations	398

LIST OF FIGURES

1.	Nomenclature of the flavins	35
2.	Charge redistributions in excited flavins	38
3.	π -Electron densities of various flavins	41
4.	Jablonski diagram for riboflavin	43
5.	Dipoles of isozlloxazine	45
6.	Redox states of flavins	48
7.	Apparatus for measuring dissolved O_2	119
8.	Progress of incorporation of ^{14}C into E_{RCM}	137
9.	Gel filtration of dimer and E_{RCM}	138
10.	Standard curve for SDS gel electrophoresis	141
11.	Isoelectric focusing of E_{RCM}	143
12.	Effect of FAD on E_{RCM} activity	145
13.	ATR-IR spectra of native enzyme samples	150
14.	ATR-IR spectra of E_{RCM} and related species	152
15.	Absorption spectra of native enzyme and E_{RCM}	155
16.	ATR-IR spectrum of phenylglyoxal-modified enzyme	165
17.	Methylene blue-sensitized photooxidation	167
18.	Anaerobic photolysis of enzyme and FAD	169
19.	Rates of anaerobic photolysis of FAD and E_{RCM}	169
20.	DHase kinetics of E_{amid}	177
21.	ATR-IR of E_{amid}	178
22.	Time dependence of E_{amid} formation	179
23.	Standard curves for TNBS determinations	181
24.	Time dependence of E_{phos} formation	183
25.	Phosphorylation of E_{amid}	185
26.	ATR-IR of E_{phos} and E_{amid}	186
27.	DHase kinetics of E_{phos}	188
28.	Effect of hydroxylamine on E_{phos}	190
29.	Densitometer scan of cross-linked mixtures	193
30.	Activities of cross-linked species	196
31.	Absorption spectra of E_{As}	198

32. Absorption spectra of E_{Co} preparation	201
33. Fluorescence of FAD in native enzyme and E_{Co}	203
34. MCD spectrum of E_{Co} (cobalt region)	206
35. Isoelectric focusing pattern of Sigma enzyme	208
36. Kinetics of Isozyme I	211
37. Kinetics of Isozyme VI	213
38. Absorption spectra of Isozymes I-VI	217
39. Fluorescence emission of tryptophan, FAD and enzyme	222
40. Fluorescence emission of native enzyme, E_{ma} and E_{RCM}	225
41. MCD of FAD in phosphate buffer	232
42. MCD of FAD in methanol	233
43. MCD of lipoamide dehydrogenase	235
44. MCD of enzyme reduced with dithionite	236
45. MCD of FAD in water, reduced with dithionite	238
46. MCD of enzyme reduced with NADH	239
47. MCD of enzyme reduced with dihydrolipoamide	240
48. MCD of E_{hv} reduced with NADH	243
49. MCD of glutathione reductase	245
50. MCD of glutathione reductase reduced with NADPH	246
51. MCD of E_{ma}	248
52. MCD of E_{ma} reduced with NADH	250
53. MCD of E_{Co} (flavin region)	253
54. MCD of enzyme with 2 mM NAD^+	254
55. Absorption spectrum of enzyme reduced with dithionite	257
56. Reduction of E_{ma} with dithionite and NADH	260
57. Reoxidation of EH_4 by lipoamide	262
58. Reduction of enzyme by NADH/NADase	265
59. EDTA-catalyzed photo-reduction of enzyme	268
60. Dithionite titration of enzyme	269
61. Reduction of enzyme by dihydrolipoate and dihydrolipoamide	271
62. Interconversion of EH_2 produced by NADH and dihydrolipoamide	273

63. Interconversion coupled to an LADH system	275
64. Interconversion of EH_2 forms at pH 5.7	276
65. Difference spectra of NAD^+ binding	280
66. Titration of enzyme with NAD^+	281
67. Difference spectra of NAD^+ binding at pH 7.0 and 5.5	282
68. Difference spectrum of NAD^+ binding to E_{ma}	284
69. Titration of E_{ma} with NAD^+	285
70. pH Dependence of NAD^+ binding sites I and II	286
71. Stockell plots of NAD^+ binding to native enzyme at pH 7.0	291
72. Stockell plot of NAD^+ binding to native enzyme at pH 5.5	292
73. Klotz plot of NAD^+ binding to E_{ma}	294
74. Fluorescence titration of E_{ma} with NAD^+	295
75. Harbury-Foley plot of NAD^+ binding to E_{ma}	296
76. Difference spectrum of TNAD^+ binding to enzyme	300
77. Stockell plot of TNAD^+ binding to enzyme	301
78. Difference spectrum of 3-AP- NAD^+ binding to enzyme	302
79. Fluorescence of enzyme in the presence of $\epsilon\text{-NAD}^+$	304
80. THase kinetics of native enzyme and E_{ma} with TNAD^+	307
81. THase kinetics of native enzyme and E_{ma} with 3-AP- NAD^+	308
82. pH Profile of the ETase reaction	311
83. Stoichiometry of the ETase reaction	313
84. ETase kinetics of E_{Co}	315
85. ETase kinetics of E_{RCM}	316
86. ETase kinetics of native enzyme	317
87. ETase kinetics activated by riboflavin	319
88. Difference spectra of riboflavin reduction by enzyme	323
89. pH Profiles of OXase activity of native enzyme and E_{ma}	326
90. OXase kinetics of E_{ma}	328

91. Absorption spectra of DCIP species	333
92. NMR spectrum of DCIP	334
93. Rate ratio of DCIP species	339
94. pH Dependence of 2,6-dibromindophenol reduction potential	340
95. DPase kinetics of enzyme denatured by repetitive freezing	342
96. DPase kinetics of E_{RCM}	343
97. DPase kinetics of E_{RCM}	344
98. pH Profiles of DPase with DCIP species	346
99. Flavin charge transfer complexes	384
100. Proposed active centre structure of lipoamide dehydrogenase	389
101. Activity scale of the multifunctional reactions	399
102. Mechanistic pathways of lipoamide dehydrogenase	410

LIST OF SCHEMES

I	- Proposed transition states for the DHase reaction	95
II	- Possible catalytic role of an active centre base	97
III	- Optical arrangement for difference spectroscopy	124
IV	- Reaction of arginine with α -dicarbonyls	164
V	- Mechanism of amidine formation with lysine	175
VI	- Proposed arsenite adduct	197
VII	- Equilibria of DCIP	331
VIII	- Transition states for flavin photochemistry	355
IX	- Two-site model of THase activity	393

ABBREVIATIONS

3-AP-NAD ⁺	3-Acetyl pyridine analog of NAD ⁺
ATR-IR	Attenuated Total Reflectance infrared spectroscopy
DCIP	2,6-Dichloroindophenol
DMA	Dimethyladipimide dihydrochloride
DMIM	Dimethyl malonimide dihydrochloride
DMIS	Dimethyl succinimide dihydrochloride
DMS	Dimethyl suberimide dihydrochloride
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FlH•	Flavin reduced by one equivalent
FlH ₂	Flavin reduced by two equivalents
Fl _{ox}	Oxidized flavin
α-KGDC	α-Ketoglutarate decarboxylase complex
LADH	Horse liver alcohol dehydrogenase
MCD	Magnetic circular dichroism
NAD(P) ⁺	β-Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced form of NAD(P) ⁺
ε-NAD ⁺	1,N ⁶ -ethenoadenine analog of NAD ⁺
PDC	Pyruvate decarboxylase complex
SDS	Sodium dodecyl sulfate
TNAD ⁺	Thio analog of NAD ⁺
TNBS	Trinitrobenzene sulfonic acid

Enzyme species:

E _{amid}	Enzyme modified with methyl acetimidate
E _{hv}	Enzyme illuminated in the presence of rose bengal
E _{ma}	Enzyme mono-alkylated from EH ₂ with ICH ₂ CONH ₂
E _{phos}	Enzyme modified with phosphoramidate
E _{RCM}	Enzyme treated with ICH ₂ CO ₂ H in the presence of β-mercaptoethanol
E _{As,Co,Cd,Cu}	Enzyme modified with the indicated metal
E _{ox}	Oxidized enzyme
EH ₂	Intermediate reduction by 2 equivalents per FAD

EH₄

Full reduction by 4 equivalents of FAD

Reactions:

DHase

NADH:lipoamide dehydrogenase

THase

NADH:TNAD⁺ transhydrogenase

ETase

NADH:K₃Fe(CN)₆ electron transferase

DPase

NADH:DCIP diaphorase

OXase

NADH:O₂ oxidase

INTRODUCTION

PART A - ENZYME MULTIFUNCTIONALITY

1) Perspective

Over the past few years, the terms multifunctional protein and multifunctional enzyme have come into general usage. In addition, a general discussion (1) and a more thorough review (2) of the topic have appeared. There is, however, no acceptable definition of multifunctionality which is adhered to by most authors. In some instances the definition is so general as to include complexes of quite distinct and independently isolable polypeptide units; in others, so restrictive as to ignore a chemical richness displayed often by a single protein site. Current concepts may be of significance to genetic and evolutionary studies. It is the aim of the present author, however, to both tighten the biological aspects of the definition of multifunctionality, and at the same time relax the chemical criteria, with the hope of developing a definition which will aid in attempts to unravel the underlying chemical complexity of many biological catalytic units.

At the outset, a distinction must be drawn between multi-enzyme complexes and multifunctional enzymes, although such a distinction has not always been preserved, even in recent years. The simplest criterion is to demand that a multifunctional enzyme exhibit more than one type of reaction within a single polypeptide chain, whereas in multienzyme complexes, two or more polypeptides

held together by non-covalent interactions are required for the multiple reactions. Separate chains held together by disulphide linkages, *in vitro* or *in vivo*, might present a problem in the application of this definition, but as yet no such examples are known (1). Perhaps the term "non-covalent" as used above should be qualified to negate only covalent peptide bond linkages.

In the literature, confusion has understandably often arisen due to incomplete separation or failure to disrupt a complex upon isolation. This problem is compounded by the fact that the enzymes of a complex are almost always associated so that they may more efficiently catalyze sequential reactions of a particular metabolic pathway. If the multistep sequence is not understood in biochemical detail, a single step may well be assumed, and a single enzyme expected. As the pathway is elucidated, this "enzyme" may be considered multifunctional until further resolved. Such multi-enzyme complexes have been recently reviewed (3), although the distinction is still not always maintained. In a short review of "multienzyme clusters", Gaertner treats multifunctional enzymes under the heading of multienzyme conjugates (4). Such mistakes should be more infrequent with current improvements in biochemical separation techniques, although the problem of nicking of multi-domain multifunctional enzymes (defined below) by endogenous proteases may still cause this type of multifunctionality to be overlooked. The difficult to purify, insoluble membrane proteins also present problems, however, especially those functioning in a redox capacity. The number of intermediates between ultimate

donor and acceptor species is generally best explained *post facto*, when all these intermediates have been isolated and their redox potentials determined. An extremely important example of this situation is the cytochrome P-450 system, where necessary intermediates are still being sought, and different classes of substrates may be oxidized by a single enzyme of interconvertible forms, by isozymes of a single enzyme, or by a number of distinct, inducible enzymes (5-8). At present, "system" appears sufficiently ambiguous to describe what may prove to be an extremely flexible multifunctional enzyme.

Of more interest are the polypeptides which in some species form non-covalently interacting complexes carrying out multistep reactions (generally biosynthetic), and in others are covalently linked to form a single polypeptide with discrete catalytic domains. The domain concept of multifunctional enzymes will be considered below. For the time being, we will anticipate that discussion and point out that this concept is of evolutionary significance, since it can best be thought of in terms of independent genes fusing to produce a new cluster of genes or genome coding for a single, multidomain protein. It is also clear that while mapping of genetic mutations has in the past been one of the most powerful tools for elucidating the number of steps in a given metabolic reaction, it can seldom remove the confusion of the multienzyme complex *versus* the multiple-domain polypeptide in a given instance.

Before proceeding to the more strictly biochemical considerations, it seems appropriate to point out here a particularly powerful tool

which has thus far seen only limited use, and that by geneticists. By artificially induced gene mutations, gene fusions have been produced which create artificial multiple-domain proteins (9,10). Such techniques are potentially useful in the mechanistic study of the biochemical multifunctionality of proteins, both by allowing production of new enzymes for study, and by allowing observation of one activity in the enforced presence of one or several others. They also mirror a leading theory of the evolution of multi-domain proteins (11).

Type A:

Biochemically, multifunctionality of enzymes is considered as a macromolecular property, i.e. the distribution of sites throughout the macromolecular structure, and their kinetic consequences, are considered. At this point, three terms must be defined as they are used in the present discussion.*

*Once again, there is a discrepancy in the current literature concerning the precise usage of these terms. Finn Wold (12) considers the active centre of an enzyme to be a region into which an inhibitor can compete with substrate for entry, or where a reaction can be blocked by chemical modification. In 1974, Light (13) defined three terms as follows:

That region of an enzyme that takes part in the interaction with the substrate is called the active centre. The active site is that part of the active centre that is concerned with the catalytic event. The specificity site is the region of the enzyme, close to the active site, that binds the substrate and properly orients the molecule "to fit" at the active site.

Zeffren and Hall in 1973 (14) have defined active site as "a specific localized, and relatively small three dimensional region on or near the enzyme surface within which all enzyme-substrate interactions take place". In 1978, authors have variously used

By active site will be meant residues and/or bound cofactors directly involved in the chemical mechanism of the catalytic event.

By active centre will be meant the somewhat arbitrary collection of residues in the neighbourhood of the active site, which may be indirectly involved in substrate or cofactor binding, or maintenance of active site integrity, or may serve as sites for chemical modifications which affect enzyme activity indirectly.

By domain will be meant a more general structural feature, which will generally contain an active or binding centre, complete with all the attendant protein necessary for maintenance of the observable property. This may represent an entire subunit of a multisubunit enzyme, a ubiquitous structural feature which may be incorporated into a range of individual enzymes (such as the NAD⁺ binding domain of Rossman *et al.* (19)), or may be used to distinguish between the juxtaposed units of the enzyme displaying catalytic and binding roles (*vide infra*).^{*} In addition, we shall require that a domain be independently separable (as in mutant or proteolysed enzymes) without loss of distinguishing property.

the terms binding domain, structural domain, and catalytic domain (15), folding domain (16), and interface domain (17), by the latter meaning the region of a multisubunit enzyme specialized for subunit association.

^{*}Much of the mass of a protein molecule is thought to be present to maintain a desirable conformation, and to aid in the folding of the protein to that conformation (for example, see 19,20), and in this case a domain may represent a protein segment of molecular weight in excess of 2×10^4 daltons (16).

The necessity for this distinction is not strictly academic. For instance, as will be seen in Section (iii) below, the kinetics of multidomain multifunctionality will differ observably from those of single centre multifunctionality. This thesis concerns an enzyme displaying single site multifunctionality, and different chemical criteria must be used in its demonstration, and so on.

When a single active centre or active site gives rise to the multiple activity, the situations will be treated below. Here, two situations are discussed; where the sites of the multiple functionality are separated and either (i) in distinct domains, or (ii) not in distinct domains. This distinction will become clear.

(i) Distinct domains, fragmentable

This situation is best illustrated with several examples. Kirschner and Bisswanger (2) have suggested that genetic mutations which map some distance apart and cause loss of particular activities, provide evidence for autonomous active domains. There is clearly a problem in this view. If the protein chain folds back on itself, as it must certainly do in maintaining its tertiary structure, then residues well separated in the primary structure may find themselves in close proximity, and single centre multifunctionality is not ruled out.* Clearly a demonstration of multi-domain

*This evidence is more convincing when, for example, none of the twenty missense mutants of the bifunctional aspartokinase I: homoserine dehydrogenase I cause loss of both activities (21).

multifunctionality requires fragmentation and identification of particular activities with specific homogeneous fragments. As Kirschner and Bisswanger (2) point out, this is commonly done by introduction of nonsense mutations in the structural gene, and/or mild proteolysis.

In *Escherichia coli*, fatty acid synthesis is accomplished by six distinct enzymes with little affinity for each other (1). However, in yeast and higher animals, the reaction sequence has been shown to be associated with a single dimeric enzyme composed of two dissimilar polypeptide chains. Of the eight activities present in the yeast enzyme, many complementary missense mutations cause loss of a single one (2). Endogenous proteolysis has proven the multi-domain structure of this enzyme, as original isolations not preventing such nicking lead to preparation of separable enzymes carrying various activities, and the multifunctionality of the subunits was in question (22). Both protease treatment and genetic mutations have been used to isolate fragments of anthranilate synthase, independently displaying glutamine amido transferase and phosphoribosyl transferase activities (2,23,24). A final example is the arom complex of *Neurospora crassa* (see Table 1). Much work had been done on the structure (25) and kinetic properties (26) of this "multienzyme complex" when Lumsden and Coggins (27) showed in 1977 that the five activities are in fact associated with identical pentafunctional subunits, very sensitive to proteolysis and capable of retaining full individual domain-associated activities upon fragmentation. Other examples of multifunctional enzymes

where active domain fragments have been isolated are included in Table 1 and are labeled as type A1, according to the terminology explained below.

(ii) Multiple centres, not fragmentable

We now consider a class of multifunctional enzymes where distinct active centres occur, yet where no fragmentation of active domains is possible. Of course the placing of an enzyme in this category is a somewhat tentative process. We think here of enzymes where the multiple active centres reside at spatially separated points, yet require a common mass of protein for the maintenance of their respective activities. Experimentally, the demonstration of this requirement may reflect simply the inability of the methods employed to dissect out active fragments, and an enzyme of this type (which will be referred to as type A2 below), may well eventually be shown to be of type A1.

For demonstration of this type of multifunctionality, short of an X-ray structure with active centres identified, the minimum chemical criterion must be the demonstration of inhibition and selective chemical inactivation or activation of the multiple activities. Often two or more of the centres are in juxtaposition, and the process of direct substrate transfer between them (more common in enzymes catalyzing sequential steps of a metabolic pathway) is known as channeling (28). Davis (29) presented a discussion of this phenomenon in *Neurospora* in 1967, although the term has only more recently seen general usage. Duggelby *et al.* (30) have recently used the concept of channeling as a

basis for a classification of multifunctional enzymes. In the presence of channeling, we would be surprised to observe the fragmentability of type A1 enzymes, since a criterion for release of product from one centre is a concerted transfer to a second centre, and hence the normal mechanism is dependent upon the presence of this second centre.

However channeling need not occur in type A2 enzymes. Duggelby *et al.* (28) have shown that ^{14}C -chorismate scrambles completely with unlabeled prephenate in the medium, proving release of the intermediate product prephenate from the first centre (cf. Table 1). The presence of two centres has been shown by selective inhibition and chemical inactivation of the two activities (30). These activities have not been fragmented, and good evidence that the two centres are structurally, inseparably linked comes from the observation that both activities are inhibited by binding of a single phenylalanine molecule to a third site (31). A similar bifunctional enzyme, chorismate mutase-prephenate dehydrogenase, may be a type A2 enzyme with at least partial channeling of prephenate (31). Other enzymes displaying type A2 multifunctionality are listed in Table 1.

(iii) Kinetic consequences of types A1 and A2

When the multiple reactions catalyzed by types A1 and A2 enzymes are completely independent, the kinetics of these activities can be treated independently. An examination of the entries in Table 1 will reveal, however, that in general these types of enzymes have evolved to deal with at least linked, if

not sequential, reactions. Storer and Cornish-Bowden (32) have presented a kinetic analysis of coupled enzyme reactions, where the product of a first reaction is the substrate for that of a second, distinct enzyme. Unless the concentration of the second enzyme is high, keeping that of the intermediate product very low, the second reaction will in general be described by Michaelis-Menten kinetics, as will the first. Only in the limit of vanishing intermediate concentration will the second reaction be first order. A lag time is introduced, dependent on the diffusion time of the first product to the second enzyme. In addition there is a time delay while the concentration of the intermediate product builds up to a steady state level, dependent on the relative velocities of the first and second reactions. The extension of these considerations to multicentre enzymes is immediate. Furthermore, in channeling the intermediate, the enzyme eliminates the diffusion time to the second site, and makes any lag phase in appearance of the final product dependent only on the kinetic parameters of the two (or more) sites involved.

In keeping with this view of multifunctionality is expression of the properties of compartmentation and coordinate effects (4), both of obvious selective advantage. By the former is meant the containment of a series of pathway intermediates at one cellular site, either by channeling or merely by proximity effects. Even for unrelated multi-activities there may be an advantage to localizing reactions of metabolic intermediates to reduce cellular clutter. By coordinate effects is meant the various means by which

a chain of reactions (linear or branching) may be controlled (eg. allosterically regulated) by action at a single point in the chain. In a single multifunctional protein of type A1 or A2, information may be mechanically communicated to two or more distinct active centres simultaneously *via* conformational effects. Again, even for non-sequential reactions, group control may be advantageous.

The lag phase discussed above for sequential enzyme reactions may be overcome or reduced by two factors in a multi-centre enzyme; by reduction of both transient and transit times (4). By transit time is meant the time of transit of an intermediate from its production centre to its reaction centre. In the special case of channeling, this time is virtually eliminated, but even if the transit is completely reliant on diffusion, proximity effects will shorten the process. In large cells of high viscosity diffusion of intermediates could become rate limiting (4). Transient time is the name given to the lag arising from Michaelis-Menten kinetics as described above. In a reaction sequence $A \rightarrow B \rightarrow C$, release of B to the cell in general has a diluting effect: even independently of any activating effect of B, the K_m/V_{max} ratio of the second enzyme determines the concentration of B which must be built up at the second active centre. In other words, the effective concentration of B is higher near its centre of production. This effect has been observed for the shikimate kinase reaction of the arom complex (33), for instance.

Two additional mechanisms for increasing K_m and/or V_{max} which have been suggested for multienzyme complexes (34) are also applicable

to types A1 and A2 multifunctional enzymes catalyzing sequential reactions. Firstly, an internal "energy transfer" scheme could couple reactions to derive activation energies from direct intra-complex activation rather than random thermal activation. Secondly, in cases of channeling, binding and/or catalysis at a second site could be favored by delivery of substrate in a sterically optimal orientation from a first site.

Type B:

We now turn to a level of consideration of multifunctionality which has not been clearly recognized in previous work. The reasons for this are three-fold. Until recently the number of enzyme mechanisms understood in sufficient chemical detail to allow sensible discussion of this type of multifunctionality has been comparatively small, and has included even fewer examples of 'complicated' multifunctional systems. Secondly, the multifunctionality is often, but not always, displayed despite a single biological activity, through reactions that can have at best limited significance in the living cell. Thirdly, the different activities do not always involve reactions traditionally considered to be of chemically distinct types. This will be further elaborated below.

The type of multifunctionality under consideration here is that displayed by a single active centre, and I shall refer to it as type B (1 or 2). The active centre of an enzyme may be visualized as, in the absence of a cofactor, a region of richness of chemical

functionality supplied by the amino acid residues of which it is constituted. It is reasonable to expect that stereo-electronic requirements aside, this functionality will display the same range of chemical reactivity that is observed in conventional (i.e. non-macromolecular) organic chemistry. When the steric and coulombic restrictions of the active centre are taken into account, an additional selectivity is usually imposed. Thus, regarding the first point above, it is clear that the prediction, at least, of this type of multifunctionality requires an understanding of the chemical mechanism and structures involved, and this is available in relatively few cases.

With regard to the second point, it is noted that the present definition of multifunctionality does not necessitate that the various reactions be of demonstrated or even likely physiological importance. To attain a satisfying level of understanding of enzymatic processes, it can often be useful to consider the enzyme molecule as an independent chemical entity displaying various catalytic properties. It is hoped that the present study will illustrate the utility of investigating non-biological reactions, and using the criteria of this more general definition of multifunctionality as a probe of structure-function relationships.

Lastly, we do not demand that the reactions catalyzed be of chemically distinct types (say redox *versus* non-oxidative group transfer) before the functionalities be considered distinct. An enzyme may perform one type of reaction on two quite distinct classes of substrate molecule, and do so quite selectively. This

flexibility of recognition by the enzyme is as important a feature of its behaviour as the flexibility to perform multiple reaction types.

It may be objected at this point that the above definition is so general as to include almost any enzyme, if only some new molecule could be found to react catalytically with some functionality at the active centre. Admitting this to be the case, it should be pointed out that the number of instances where this has actually been done is still relatively few. This definition is, however, intended more to suggest an approach to the investigation and understanding of enzyme mechanisms than to serve as a tool for classification. A deeper chemical insight into the workings of an active centre can often be gained by consideration of independent activities, and before giving a few examples, we will briefly discuss the criteria for, and experimental advantages of, this situation.

For single centre multifunctionality, we shall require that (a) at least two separate functional groups (generally on two separate amino acid side chains) be involved catalytically with different classes of substrates (type B1), or (b) that if a single site is involved, it have activity with chemically distinct classes of substrates at two or more selective binding sites (type B2).

For two reactions of different substrate classes to be distinct, we shall allow a common binding site, but require distinct active sites (B1). A shared active site is reserved for type B2 enzymes. Proof of this type B multifunctionality requires ultimately

correlated structural (X-ray) and mechanistic detail. However, strong evidence is provided by independent chemical modification, selective partial denaturation, or selective genetic mutation, of the activities when the residues involved can be shown to be constituents of a single active centre. A kinetic display of competing reaction pathways for independently variable reactions is also relevant. A full investigation of this chemical multifunctionality serves to uncover, by the converse of this reasoning, the functionality of the active centre. Additional functionality can also serve as an internal standard for mechanistic probes and techniques which selectively investigate single activities. By using artificial (i.e. non-biological) activities, this logistic principle has been used extensively and with success in the work to be reported in this thesis. The investigation of seemingly artificial reactions may also uncover hitherto overlooked biological functions. It is with these considerations in mind that some of the results obtained with horse liver alcohol dehydrogenase (LADH) can be understood.

It is well known that LADH displays both dehydrogenase and dismutase activities (35,36), and these involve acyl enzyme formation at an active site sulfhydryl residue (35). The active site also involves a catalytic role for zinc (35,37). It has recently been shown (38) that the formation of acyl enzyme implies inherent esterase activity. This activity does not necessitate a catalytic role of polarization for the zinc atom, and this site may not then be involved in the esterase activity, although the

sulfhydryl may be common to all reactions. Differential deactivation of the three activities by chemical modification with iodoacetamide and iodoacetate, and by heat treatment, has been shown(38).

Recently Pocker *et al.* (39) have demonstrated the hydrase and esterase activities of carbonic anhydrase in the single bifunctional substrates, pyruvate esters. A single binding site is indicated for expression of both activities, but different "modes of binding" were not ruled out, consistent with re-orientation to different catalytic sites. A single molecule of acetazolamide stoichiometrically inhibits the esterase activity, by binding tightly to an enzyme group, but has a much smaller effect on the hydrase activity. This is good evidence that the group affected by the inhibitor has a catalytic role in the esterase activity, and is in the neighbourhood of, but not required for, the hydrase activity. The group has a pKa of about 7, and is possibly an imidazole.

There are occasional instances where any plausible mechanisms for two or more reactions suggest placement of the enzyme displaying them in category B2, even in the absence of further structural evidence. For example, bacterial NADase catalyzes the hydrolysis of the bond between the nicotinamide and adenosine diphosphoribose moieties of NAD⁺. In addition, the mammalian enzyme catalyzes a transglycosidation reaction between nicotinamide and analogs of NAD⁺ (40,41). As well as the uni-substrate hydrolytic site, a transglycosidation transfer may occur to a second binding site at the active centre for nicotinamide, in the

bi-substrate reaction, where condensation occurs specifically.

II) Classification and Examples

In this section we shall summarize the types of multifunctionality and give a table of examples gleaned from the literature. A unifying definition will be attempted.

We shall define a multifunctional enzyme as one which displays catalytic activity involving multiple substrate specificity. Specificity is multiple if it is shown towards two or more distinct classes of chemical species which cannot be interconverted by simple chemical means without losing specificity towards the intermediates of such a conversion. This definition is intended to reflect either binding or catalytic specificity, but unlike multifunctional proteins in general, multifunctional enzymes must have catalytic activity with the various species bound.

The following types of multifunctionality are distinguished:

- Type A - distinct active centres
 - A1 - active centres in distinct domains
 - A2 - active centres not in distinct domains

- Type B - a single active centre
 - B1 - different active sites
 - B2 - a single active site for different selective binding sites

- Type C - multifunctionality of a bound cofactor
 - C1 - inorganic cofactor
 - C2 - organic cofactor

Note that class C has not been discussed above, and examples are not included in Table 1. It will be discussed below, briefly, and throughout the remainder of this thesis. The inclusion of class C represents, admittedly, an artificial distinction. If the cofactor is considered the active site, we are dealing with a special case of B2. The distinction will be justified below.

Multifunctionality due to isozymes or multiple enzyme forms has been omitted here. Each isozyme should necessarily display all activities (and thus fall into one of the above categories), or we would consider that different enzymes were being dealt with. There can at most be selective activations or deactivation amongst a group of isozymes, each of the same type as the entire enzyme mixture, and displaying the same activities. This point may be modified as our knowledge of multifunctional systems increases, but is true for the present.

Duggelby *et al.* (28) have recently provided a partially structural, partially mechanistic classification for multifunctional enzymes, according to whether or not they catalyze sequential or non-sequential reactions along a metabolic pathway. The sequential examples are further subdivided according to single active site, non-interacting multiple sites, or channeling. This classification is both incomplete (in view of the active site/active centre distinction), and carries no mechanistic or structural information for the large class of non-sequential multifunctional enzymes. In addition, the rather specialized criterion of channeling is often difficult to demonstrate, and assignments based on the

literature are uncertain. However, where possible, the enzymes in Table 1 will be classified in this system as well, and identified as Duggelby types (i) - (iv) according to the following scheme:

- (i) sequential, single active site
- (ii) sequential, non-interacting multiple sites
- (iii) sequential, with channeling
- (iv) non-sequential, not linked

In Table 1 are shown many of the more well known examples of multifunctional enzymes, which fall into categories A1, A2, B1, and B2. The list is not exhaustive, but is greatly expanded from any previously published. It is intended, by example, to serve as a guideline for the classification of such enzymes, and the assignment of category has been by application of the criteria given above, based on the references included in the table. These criteria are summarized here, and are intended for comparison and contrast with those given in the following section for types C1 and C2, relevant to the remainder of the thesis. These are intended as guidelines for investigating chemical aspects of the enzymes involved. It is stressed that the main goal of the present scheme is not the rigorous classification of multifunctional enzymes, but rather the establishment of a set of guidelines for the experimental investigation and chemical understanding of these species, best taking advantage of the handle offered by the presence of multiple activities.

Type A1 - Fragmentability, with retention of activities,

must be demonstrated, usually by proteolysis or genetic means. Failing this, the multiplicity of domains must be shown by X-ray structural analysis revealing discrete structural units with identifiable catalytic activities.

Type A2 - X-ray structure must reveal multiple active centres which are not separable by chain cleavage. This could result, for instance, from a common segment of the polypeptide chain folding back into each centre. Failing this, a tentative assignment to this class can be made when all attempts at fragmentation fail, as long as multiple centres are indicated. Support for multiple centres comes from kinetic measurements (demonstration of transient and transit times for sequential reactions), chemical means (selective chemical modifications affecting activities, accompanied by peptide analyses), and independence of activities revealed by genetic point mutations which consistently effect single activities. A common site of allosteric control may suggest an absence of independent domains.

Types B1 and B2 - For rigorous demonstration, these types require structural information (regarding a single centre), and a mechanistic implication of the various sites involved within that centre. Again independent variation of activities with chemical modifications and environmental changes, and inhibition studies, are useful tools in investigating the mechanistic basis of the assignment.

Table 1: Classification of Multifunctional Enzymes
Lacking Bound Cofactors

(a) Enzyme commission number for the enzyme or
single reaction, as applicable (see 42)

(b) See text for explanation of symbols

(c) Where applicable

EC no. (a)	Name	Reactions Catalysed	Type (b)	Duggleby Ref. Type
EC 1.1.1.23	Histidinol dehydrogenase: imidazole-acetol-phosphate amino transferase	1. L-histidinol $\xrightarrow{2\text{NAD}^+}$ Histidine 2. imidazole acetol phosphate + glutamate → histidinol phosphate + oxoglutarate	A1	(iv) 43,2
EC 2.3.1.38	Fatty acid synthetase	1. acetyl-CoA + ESH → acetyl-S-E + CoASH	A1	(ii) or 2,44,
EC 2.3.1.39	(liver)	(<i>E. coli</i>): malonyl-CoA + ESH → malonyl-S-E + CoASH 2. acetyl-S-E + malonyl-S-E → acetyl-S-(malonyl-S-E) 3. acetyl-S-(malonyl-S-E) → acetoacetyl-S-E + CO ₂ (<i>E. coli</i>): acetyl-S-E + malonyl-S-E → 3-oxobutyl-S-E + CO ₂)	(iii) 45	
EC 2.3.1.41		4. acetoacetyl-S-E $\xrightarrow{\text{NADPH}}$ β-hydroxybutyl-S-E (<i>E. coli</i>): 3-oxobutyl-S-E $\xrightarrow{\text{NADPH}}$ D-3-hydroxybutyl-S-E)		
EC 1.1.1.100		5. β-hydroxybutyl-S-E → trans-crotonyl-S-E + H ₂ O (<i>E. coli</i>): D-3-hydroxybutyl-S-E → trans-crotonyl-S-E + H ₂ O)		
EC 4.2.1.58		6. trans-crotonyl-S-E $\xrightarrow{\text{NADPH}}$ butyl-S-E		
EC 1.3.1.10		7. butyl-S-E → butyl-S-E-SH 8. palmityl-S-E + CoASH → palmityl CoA + ESH (The eight activities of the enzyme of higher animals are here compared with the well-known pathway in <i>E. coli</i> , which is catalysed by individual enzymes.)		

EC no.	Name	Reactions catalyzed	Type	Duggleby Type	Ref.
EC 2.7.2.4	Aspartokinase I:homoserine	1. L-aspartate ^{ATP} 4-phospho-L-aspartate	A1	(iv)	2,46-49
EC 1.1.1.3	dehydrogenase I (<i>E. coli</i>)	2. L-aspartate- β -semialdehyde NAD(P) ⁺ L-homoserine			
EC 2.7.2.5	Pyrimidine nucleotide biosynthesis (early enzymes in <i>E. coli</i> + eukaryotes)	1. Carbamyl phosphate synthetase 2ATP + NH ₃ + CO ₂ + H ₂ O → 2ADP + P _i + carbamoylphosphate	A1	(ii) or (iii)	1,46
EC 2.1.3.2		2. Aspartate transcarbamylase carbamoyl phosphate + L-aspartate → N-carbamoyl-L-aspartate + P _i			
EC 3.5.2.3		3. Dihydroorotase N-carbamoyl-L-aspartate → L-4,5-dihydro-orotate			
EC 2.7.7.7	DNA polymerase I (<i>E. coli</i>)	1. Polymerization using SS-DNA as template → DS-DNA 2. exonuclease of SS-DNA (3' → 5') 3. exonuclease of DS-DNA (5' → 3')	A1	(iv)	2,46,50
EC 3.2.1.33	Glycogen debranching enzyme	1. Dextrin-6- α -glucosidase(hydrolysis of α -1,6-glucan links in dextrans)	A1 (?)	(iv)	46,51
EC 2.4.1.25		2. 4- α -glucanotransferase (phosphorolysis of α -1,4-linked glucose units to 4 before α -1,6-branch)			

EC no.	Name	Reactions catalysed	Type	Duggleby Type	Ref.
EC 4.1.3.27	Anthranilate synthase: anthranilate-phosphoribosyl- transferase. (<i>Salmonella, E. coli</i>)	1. Chorismate + L-glutamine → pyruvate + L-glutamate + anthranilate 2. phosphoribosyl-diphosphate + anthranilate → phosphoribosylanthranilate + PP _i	A1	(ii) or (iii)	2,53, 44
EC 5.4.99.5	Chorismate mutase: Prephenate dehydratase	1. Chorismate → prephenate 2. Prephenate → phenylpyruvate + CO ₂	A1	(ii)	2,28
EC 6.3.4.3	Formyl-methenyl-methylene- tetrahydrofolate synthetase	1. Formyltetrahydrofolate synthetase Tetrahydrofolate $\xrightarrow[\text{CO}_2]{\text{ATP}}$ 10-formyl-tetrahydrofolate	A1	(ii) or (iii)	54,55
EC 3.5.4.9		2. Methenyl-tetrahydrofolate cyclohydrolase 10-formyl-tetrahydrofolate → 5,10-methenyl- tetrahydrofolate			
EC 1.5.1.5		3. Methylene tetrahydrofolate Dehydrogenase 5,10-methenyltetrahydrofolate → 5,10-methylene-tetrahydrofolate (NB. Activities 2 & 3 probably of Type B1 cf. 54,56)			
EC 6.4.1.2	Acetyl CoA carboxylase	1. HCO ₃ ⁻ + biotin-E $\xrightarrow{\text{ATP}}$ CO ₂ -biotin-E 2. CO ₂ -biotin-E+acetyl CoA → malonyl CoA+biotin-E	A1	(ii) or (iii)	2,57
-	Threonine biosynthesis enzyme	Steps in Threonine biosynthesis	A1	(ii) or (iii)	1

EC no.	Name	Reactions catalyzed	Type	Duggieby Type	Ref.
-	Arom complex (<i>Neurospora crassa</i>)	1. Dehydroquininate synthetase 3-deoxy-D-arabino-heptulosonate-7-phosphate → dehydroquininate	A1	(ii) or (ii)	1,25- 27,46, 58
EC 4.2.1.10		2. Dehydroquininate dehydratase dehydroquininate → dehydroshikimate			
EC 1.1.1.25		3. Dehydroshikimate reductase dehydroshikimate → shikimate			
-		4. Shikimate Kinase Shikimate → shikimate-5-phosphate			
-		5. 3-enolpyruvylshikimate-5-phosphate synthetase shikimate-5-phosphate → 3-enolpyruvyl- shikimate-5-phosphate			
EC 1.1.1.53	Cortisone Reductase	1. 3 α -hydroxysteroid dehydrogenase	A2 (?)	-	59,46
EC 1.1.1.51		2. 20 β -hydroxysteroid dehydrogenase			
-	Phosphoribosylanthranilate isomerase: Indo-3-glycerol phosphate synthase (<i>E. coli</i>)	1. N-(5'-phosphoribosyl)-anthranilate → 1-(2'-carboxyphenylamino)-1-deoxyribulose- 5-phosphate (X) 2. X → 1-C-(3'-indolyl)-glycerol-3-phosphate + CO ₂ + H ₂ O	A2	(iii)	2,44
EC 4.2.1.20	Tryptophan Synthase (<i>V. crassa</i>)	1. Indoleglycerol phosphate → Indole + 3-phosphoglyceraldehyde	A2	(iii)	2,46

EC no.	Name	Reactions catalysed	Type	Duggleby Type	Ref.
Tryptophan Synthase cont'd.					
		2. Indole + serine $\rightarrow$ Tryptophan + H ₂ O			
		3. Indoleglycerolphosphate + serine $\rightarrow$ Tryptophan + 3-phosphoglyceraldehyde			
EC 5.4.99.5	Chorismate mutase:	1. Chorismate $\xrightarrow{H_2O}$ Prephenate	A2(?)	(iii)	(?)2,46
	Prephenate dehydrogenase	2. Prephenate $\xrightarrow{NAD(P)^+}$ 4-hydroxyphenylpyruvate + CO ₂			44,31
EC 6.3.1.3	Purine biosynthesis enzymes (Yeast) (ade-1 gene product)	1. Phosphoribosyl glycine amide synthetase	A2		60
		Phosphoribosylamine $\rightarrow$ Phosphoribosylglycine-amide. (monomer and multimer active)			
		2. Phosphoribosylaminoimidazole synthetase			
		Phosphoribosyl-formylglycineamide $\rightarrow$ phosphoribosylaminoimidazole (only multimer active)			
EC 5.4.99.5	3-deoxy- δ -arabinoheptulosonate-7-phosphate synthase:	1. Phosphoenolpyruvate + erythrose phosphate $\rightarrow$ arabinoheptulosonate-phosphate	A2 (?)	(iv)	61
	Chorismate mutase	2. Chorismate $\rightarrow$ Prephenate			
	(<i>Bacillus subtilis</i>)				

EC no.	Name	Reactions catalysed	Type	Duggleby Type	Ref.
-	ϕ X174 cistron A protein	<ol style="list-style-type: none"> 1. Nicking DNA(+) strand to initiate replication 2. Unwinding DNA to create replicating fork 3. Excision to ϕ-X unit length DNA after replication 4. Ligation to close circle. 	A2	(iv)	62
EC 1.1.1.1	Alcohol Dehydrogenase (Liver)	<ol style="list-style-type: none"> 1. Dehydrogenase $RCH_2OH \xrightarrow{NAD^+} RCOH \xrightarrow{NAD^+} RCO_2H$ 2. Dismutase $2RCOH \xrightarrow{H_2O} RCH_2OH + RCO_2H$ 3. Esterase $RCO_2R' \rightarrow RCO_2H + R'OH$ 	B1	(i) (?)	35,36
EC 1.1.1.41	Isocitrate dehydrogenase	<ol style="list-style-type: none"> 1. Isocitrate dehydrogenase (oxidative decarboxylation; normally irreversible) $\text{Isocitrate} \xrightarrow{NAD^+} \alpha\text{-Ketoglutarate} + CO_2$ 2. Reductive carboxylation (CO_2 fixation) $\alpha\text{-Ketoglutarate} \xrightarrow{NADH, CO_2} \text{isocitrate}$ 3. Oxalosuccinate reductase $\text{Oxalosuccinate} \xrightarrow{NADH} \text{isocitrate}$ 4. Oxalosuccinate decarboxylase (no cofactor required) $\text{Oxalosuccinate} \rightarrow \alpha\text{-Ketoglutarate} + CO_2$ 	B1	(i)	63

EC no.	Name	Reactions catalysed	Type	Duggleby Type	Ref.
EC 2.7.1.40	Pyruvate Kinase: Malic Enzyme	1. Pyruvate Kinase $\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{Mg}^{2+}} \text{Pyruvate} + \text{ATP}$ 2. Oxaloacetate decarboxylase $\text{Oxaloacetate} \xrightarrow{\text{Mn}^{2+}} \text{pyruvate} + \text{CO}_2$ 3. Hydrolase $\text{Phosphoenolpyruvate} \rightarrow \text{pyruvate} + \text{P}_i$	B1	(i)	64-66
EC 4.2.1.1	Carbonic Anhydrase	1. Esterase $\text{RCOCO}_2\text{R}' \xrightarrow{\text{H}_2\text{O}} \text{RCOCO}_2^- + \text{R}'\text{OH}$ 2. Hydrase $\text{RCOCO}_2^- \xrightarrow{\text{H}_2\text{O}} \text{RC(OH)}_2\text{CO}_2^- + \text{H}^+$ or $\text{RCOCO}_2^- \xrightarrow{\text{H}_2\text{O}} \text{HCO}_3^- + \text{H}^+$ 3. Hydration of aldehydes $\text{Ar-COH} \rightarrow \text{Ar-CH(OH)}_2$	B1	(i)	39 46 67
	Aminoglycoside antibiotic inactivating enzyme	1. 6'-acetylation of kanamycin, tobramycin 2. 2"-phosphorylation of gentamycin	B1	-	68
EC 1.2.1.3	Aldehyde Dehydrogenase (Liver)	1. Dehydrogenase $\text{RCOH} \xrightarrow{\text{NAD}^+} \text{RCO}_2\text{H}$ 2. Esterase $\text{p-NO}_2-\phi-\text{OCOR} \xrightarrow{\text{H}_2\text{O}} \text{p-NO}_2-\phi-\text{OH} + \text{RCO}_2\text{H}$	B2	(i)	69,70

EC no.	Name	Reactions catalysed	Type	Duggleby Ref. Type
EC 2.7.5.4	Phosphoglyceromutase	1. Biphosphoglyceromutase 1,3-di-P-G + 3-P-G $\rightarrow$ 2,3-di-P-G + 3-P-G	B2	(i) (?) 71,72
EC 3.1.3.13		2. 2,3-Biphosphoglycerate phosphatase 2,3-di-P-G $\rightarrow$ 3-P-G + P_i		
EC 2.7.5.3		3. Phosphoglyceromutase 3-P-G + 2,3-di-P-G $\rightarrow$ 2-P-G + 2,3-di-P-G (P-G = phospho-glycerate)		
EC 3.2.2.5	NADase	1. Hydrolase $NAD^+ + H_2O \rightarrow$ nicotinamide + adenosine-diphosphoribose 2. Transglycosidase $NAD^+ + (nicotinamide)^* \rightarrow (NAD^+)^* + nicotinamide$	B2 (?)	- 40
EC 4.1.1.32	Phosphoenol pyruvate carboxykinase	1. Oxaloacetate $\xrightarrow{GTP}$ phosphoenolpyruvate + CO_2 (reversible) 2. Oxaloacetate $\rightarrow$ pyruvate + CO_2 (irreversible)	B2 (?)	(i) (?) 73,74
-	Modulator-dependent protein kinase	1. Phosphorylation of histones 2. Phosphorylation of myosin light chains 3. Phosphorylation of phosphorylase kinase	B2 (?)	(iv) (?) 75
-	2,3-diphosphoglycerate regulating enzyme (erythrocytes)		(?)	(?) 76

Type C:

When we deal with an enzyme which contains no cofactor, and only amino acid functional groups are present, the catalytic possibilities of a single site are limited. A new spectrum of chemical reactivity is offered, however, by the presence of a cofactor, and the multifunctionality arising from such a non-proteinaceous enzyme group is in a class quite by itself. While this class may be seen as a special case of type B2, several factors make its independent definition practical. Both the metal ion and small organic cofactor display different reactivity from that usually seen for amino acid functionality. In general the free solution chemistry of the cofactors is extensively studied, and may be exploited by the investigator (and the enzyme!) in approaching the multifunctional problem. The cofactor is furthermore often removable, allowing dissection of the "active site" out of the holoenzyme. Rapid exchange with the environment may even function in the catalytic event. A greater proportion of non-biological activities are displayed by enzymes with cofactors, again the enzyme being seen as harnessing something of a generally reactive entity. Thus the goals of investigation of class C are also unique. A primary concern is understanding the perturbations induced on the cofactor by the protein, and *vice versa*, so that the harnessing process may be appreciated. Perhaps all enzymes with cofactors can be considered multifunctional to some degree; thus their inclusion in Table 1 is not practical.

Here type C multifunctionality will be considered exclusively for enzymes containing, in their native state, a cofactor which may be covalently or non-covalently associated with the protein. The multifunctionality is then intended to involve the cofactor as the active site, and arises from the chemical reactivity inherent in that cofactor. The cofactor is generally part of the protein for the very reason of this reactivity, and may belong to two categories. It may be a metal ion (type C1) or a small organic cofactor (type C2). It is to be noted that, whereas in a proteinaceous site, active site refers to a single functional group, here the entire cofactor acts as an independent unit, and is considered a site although it may contain many points of functionality.

The advantages of seeking both natural and artificial functionalities of class C enzymes are those of doing so for class B examples. Additionally, there is an opportunity to probe not only the possibilities of the enzyme system, but also the effects, often startling, of the protein environment on the chemistry of the cofactor. The utility of this approach with metallo-enzymes has enriched both inorganic and bioinorganic chemistry, and nothing further will be said on this topic.

The case of an organic cofactor is perhaps epitomized by the flavoproteins, those which contain a flavin as cofactor. The chemical flexibility of this species, its sensitivity to protein environment, the quantity of pertinent data in the literature, and the fundamental importance of a number of flavoproteins,

all make an investigation along the above lines particularly rewarding. It is upon the flavoproteins that we will ~~focus~~ focus our attention.

PART B - FLAVINS AND FLAVOPROTEINS

Perhaps the most challenging and most widely studied class of enzymes displaying the type C2 multifunctionality are those involving a flavin cofactor. The flavins display high reactivity in a variety of electron transfer processes, and are sensitive to various environmental factors. The investigation of this multifunctionality can be fruitful in providing insights into the physical basis of flavin chemistry by correlation of flavin reactivity with perturbations induced by the peptide environment. The flavin serves as a sensitive probe of enzyme structure, mechanism, and conformational flexibility. Many of these principles are illustrated in the present thesis. To begin with, some basic principles underlying flavin-solution chemistry will be introduced.

I) The Flavins

An understanding of flavoproteins demands some familiarity with chemical and electronic structure, chemistry, photochemistry, and redox properties of flavoproteins. Since a comparison of the properties of flavoproteins with free flavins is the logical (and traditional) approach to an understanding of flavoprotein mechanisms, it seems appropriate to commence with a discussion of free flavins. This is also the effective framework for the exploration of type C2 multifunctionality as displayed by flavoproteins.

The flavins are derivatives of the benzpteridine structure commonly known as alloxazine. When the N(10) position is alkylated, the N(10) - C(10a) double bond isomerizes to the C(10a) - C(1) position, forming the so-called isoalloxazine. When alloxazine is dimethylated at C(7) and C(8), the resultant structure is known as lumichrome, and N(10) alkylation of lumichrome produces the series of compounds known as flavins. In biological systems the simplest occurring flavin has a ribitol side chain as the N(10) substituent, and is called riboflavin. Phosphorylation of the ribitol sugar produces the flavin nucleotides. The structures and nomenclature of these alloxazine derivatives are summarized in Figure 1 (see 77,78).

We are here primarily concerned with the flavin-adenine dinucleotide (FAD). The ribitol phosphate side chain is sufficiently long and flexible to allow an intramolecular stacking interaction of adenine with the isoalloxazine moiety. Interaction of flavin and purine derivatives is well established (79-82) and such an intramolecular interaction has been used to account for the low quantum yield of FAD fluorescence in aqueous solution (79,81). Addition of alcohol favors unfolding and increases the quantum yield (83). Proton and ^{31}P - NMR have been used by Kainosho and Kyogoku (84) to show a rapid equilibrium between adenine stacked over the "front" and "back" of isoalloxazine, and a coplanar isoalloxazine-adenine hydrogen-bonded interaction has also been identified (82,85,86). Polar, protic solvents such as water favour charge-transfer complex formation with isoalloxazine

Figure 1: Nomenclature of the flavin ring systems (77,78).

and a suitable donor (87,88). De Kok *et al.* (89) have estimated from fluorescence lifetimes that FAD is 70% folded in neutral aqueous solution.

In discussion of the electronic states of flavins, the terminology of Platt (90) will be used.* The flavin chromophore is yellow, and has four absorption bands around 220, 260, 375 and 450 nm (78). The two higher energy absorptions are usually obscured in flavoproteins by aromatic amino acid absorptions, and it is the two lower energy ones which are most useful. These two transitions are assigned as ${}^1L_a \leftarrow {}^1A$ and ${}^1L_b \leftarrow {}^1A$ for the 375 and 450 nm bands respectively (91), and should be polarized in the transverse and longitudinal

*In this terminology, a free-electron model is used for the π -electrons of the periphery of an aromatic system. For a polyaromatic hydrocarbon where no carbon atom is shared by more than two rings (i.e. of formula $C_{4n+2}H_{2n+4}$) all π -electrons are free to circulate around the perimeter, and will have angular momenta quantized as a plane rotor; $E = q^2 h^2 / 2ml^2$, where q is an integer, 0,1,2,..., h is Planck's constant, m is the electron mass, l is the length of its path, and E is the energy of the system. The q 's are added algebraically to give a total Q for the system. The states $Q = 0,1,2...$ are designated A, B, C..., and the states $Q = 2n, 2n+1, 2n+2...$ are designated K, L, M, The highest filled shell will have $q = n$ (for $4n+2$ carbons) and the first empty shell $q = n+1$. The two possible transitions between these two levels then have $Q = (n+1) \pm n = 1$ or $2n+1$, and are due to states B and L. These states are doubly degenerate, as the Q may be in either direction around the ring, and when breakage of symmetry removes the degeneracy, the two resultant states are designated by subscripts a and b. Each may be singlet or triplet, for a total of eight states; ${}^1,3B_{a,b}$ and ${}^1,3L_{a,b}$. By Hund's rules the 3L_a should be of lowest energy, 1B_b of highest. The ground state is of course 1A .

directions respectively (78)*, although asymmetry and substituent effects alter the cleanliness of these polarizations. These polarizations are in qualitative agreement with the charge redistributions (Fig. 2) postulated by Kosower in 1962 (92). Experimental absorption data are given in Table 2. In aqueous solution, neutral, oxidized flavins have a fluorescence emission at 536 nm, with quantum yields of 0.25 for riboflavin and flavin mononucleotide (FMN), and 0.025 for FAD (81).

The earliest molecular orbital calculations on the flavin chromophore were carried out by Pullman and Pullman in 1959 (95), using an LCAO method with parameterized coulomb and exchange integrals. While their energy levels were in poor agreement with experiment, the π -electron densities obtained (Fig. 3) serve to explain many of the anomalies of flavin chemistry, notably the high reactivity of the C(8) α -methyl group, and of C(4a) in the reduced species (*vide infra*).

Fox *et al.* (96) have done complete self-consistent field (SCF) calculations using the Pariser-Parr-Pople treatment of π -systems, and find transitions of 407, 326, 307, 277, and 268 nm. Examination of fully reduced flavin allows prediction of a non-planar system, with N(5) and N(10) sp^3 hybridized and tetrahedral. Song (97) has calculated the structure of the $\sigma + \pi$ framework of isoalloxazine by CNDO methods, and has

*These polarizations are argued from the effects of substituents upon the corresponding transitions of acridine.

Figure 2: Charge redistributions in flavins upon absorption of light; after Kosower (92).

Table 2: Spectral characteristics of the flavins, collected from references 77, 78, 91, 93, 94. Values are absorption maxima in nm, for water as a solvent unless otherwise specified. The extinction coefficients ($\text{mM}^{-1}.\text{cm}^{-1}$) are given in parantheses. Fl_{ox} , Fl , Fl_{red} are oxidized, semiquinone and fully reduced flavins respectively.

- a) Riboflavin unless otherwise stated
- b) "buffer" is potassium phosphate, 0.1 M, pH 7.0
- c) shoulder
- d) with 0.02 M triethylamine

Redox state and species ^a	Solvent ^b	Absorption maxima (nm) ^c (ϵ in parentheses; $\text{mm}^{-1}\text{cm}^{-1}$)
$\text{Fl}_{\text{ox}}^+ \text{H}_2$	6N HCl	222 364, 390
$\text{Fl}_{\text{ox}}^+ \text{H}$		
Riboflavin	H_2O	223(30.1) 268(31.4) 374(10.8) 448(12.3)
FMN	H_2O	223(30.5) 268(32.8) 374(10.6) 448(12.6)
Lumiflavin	H_2O	225.5(30.3) 265.5(32.4) 369(8.4) 444(10.9)
Riboflavin	buffer	- 266(32.5) 373(10.6) 445(12.5)
FMN	buffer	- 266(31.8) 373(10.4) 445(12.5)
FAD	buffer	- 263(38.0) 375(9.3) 450(11.3)
Riboflavin	98% dioxane(H_2O)	224(31.0) 344(8.4) 440(12.1)
lumichrome	CHCl_3	- 350, 387
Lumiflavin	cyclohexane: dioxane 1:1	223(27.1) 271(32.3) 332(6.6) 440(10.2)
Fl_{ox}^+	2N NaOH	230 270 350 444
FlH_3^+	6N HCl	- 258 358 488
FlH_2	buffer	
	CHCl_3	
FlH^-	CHCl_3^d	
$\text{Fl}_{\text{red}}^+ \text{H}_4$	6N HCl	- 250 316
$\text{Fl}_{\text{red}}^+ \text{H}_3$	sulfate, pH 2.0	- 250, 280(sh) $\sim$ 400(sh; 0.78)
$\text{Fl}_{\text{red}}^+ \text{H}_2$	borate, pH 9.0	- 256, 288 $\sim$ 350(sh)

570(sh)^c, 622
590(sh)
612

Figure 3: π -electron densities of the isoalloxazine ring system (ref. 95) (a) FMN, (b) FMNH₂, (c) distribution of the unpaired electron density in FMNH•.

determined the spin density of the lowest energy triplet state.

This $\pi^* \leftarrow \pi$ triplet is quite similar in charge distribution to the flavin semiquinone radical, as proposed by Lhoste *et al.* (98), especially regarding a concentration of spin density on N(10).

The singlet-to-triplet transition is predicted to be at 620-730 nm.

With refinement of the Pariser-Parr-Pople SCF method, Song (99)

has calculated orbital energies in good agreement with experimental results, and has shown the $\pi^* \leftarrow \pi$ nature of several of the

lowest transitions. Transition dipoles have also been calculated,

and $\pi^* \leftarrow n$ transitions sought. The knowledge of the electronic

structure and excited states of flavins was summarized by Song

in 1969 (100). We shall consider the various photophysical

processes in the context of the Jablonski diagram for riboflavin

shown in Figure 4.

Process 1: Singlet transitions of the $\pi^* \leftarrow \pi$ type have been found both theoretically and experimentally, as discussed above, for the 445 and 365 nm absorptions. The low energy band appears as a single (asymmetric) absorption band in aqueous solution, but as solvent polarity is decreased, increasingly resolves into a peak at 450 nm with less intense shoulders at 425 and 475 nm (88,94).

It has been argued, however, both from theoretical results (96,99, 100) and from constancy of fluorescence polarization across the

band (101-103) that this absorption represents a single electronic transition with resolution of groups of vibrational levels

increasing in non-polar solvents. This low energy transition

is polarized along the long molecular axis with the 365 nm

transition dipole at 30° - 40° to it, again experiment (102,104) and theory (99) being in agreement. Figure 5 shows these transition dipoles as well as the dipole moment orientation of isoalloxazine (100). Transitions of the $\pi^* \leftarrow n$ type have not been observed spectroscopically, although one predicted at 371 nm (99) would lie under the stronger corresponding $\pi^* \leftarrow \pi$ band (100,105).

Processes 2,3,5: Quantum yields of 0.26 and 0.7 for fluorescence and intersystem crossing, in aqueous solution, indicate very low internal conversion to the lowest singlet and ground states (100). Quantum yields of fluorescence increase markedly in organic solvents, presumably due to depression of intersystem crossing, although this is not well understood.

Processes 6,7: Phosphorescence from flavins is weak, the quantum yield of riboflavin in ethanol at 77°K being only 0.0012 (83). Little is known about radiationless transition from the lowest triplet to ground state, and the question of the weak phosphorescence remains unresolved (100).

Processes 4,8: The photochemistry of flavins has been extensively studied (106), and may be divided into reactions of four types; anaerobic photoreductions (inter- and intramolecular), aerobic photoreductions, and photoadditions (78). Anaerobically, illumination of flavins containing a ribitol side chain results in photodegradation accompanying reduction of the isoalloxazine ring (107). Following reoxidation after photolysis of riboflavin, a product distribution of lumichrome

Figure 5: Dipole moment and transition dipoles of the isoalloxazine ring system, after ref. 100.
 1 - transition moment of 450 nm absorption.
 2 - transition moment of 370 nm absorption.
 3 - ground state dipole moment.
 4 - lowest triplet state dipole moment.

(16.8%), 2'- and 4'-ketoriboflavin (17%), formylmethylflavin (58%), plus minor products, was found (108). Lumichrome is thought to arise from Norrish type II hydrogen abstraction from the 2'-carbon by singlet excited flavin (106). The ketoriboflavins are thought to arise from a similar mechanism, while the formylmethyl derivative implies abstraction of hydrogen from the 2'-OH group (78). Lumichrome and 4'-ketoriboflavin, furthermore, are thought to result from singlet flavin, 2'-ketoriboflavin and formylmethylriboflavin from a triplet state (108,78).

Electron donors such as amines and amino acids photo-reduce flavins anaerobically (78). In the presence of ethylenediamine tetraacetate (EDTA), illumination results in the transient production of semiquinone radicals (109). Massey *et al.* (110) have recently explained this as dark reaction disproportionation following formation of an EDTA adduct and subsequent two-electron reduction of flavin. Most photo-reductions are thought to involve triplet flavin states due to the short lifetimes of the singlet (78,91). Radda (111) has found that photoreduction by EDTA is inhibited by species which complex with and quench the triplet state. A general mechanism of photoreduction by many types of species has been suggested to involve $H^{\bullet}$ abstraction to form a flavin semiquinone as the initial post-excitation step (106).

An important anaerobic reductant is NADH. More will be said of this below, the photochemical aspects being stressed

here. Frisell and MacKenzie (112) found rapid oxidation of NADH upon illumination in the presence of riboflavin. Anaerobically, the flavin was reduced, but in the presence of oxygen, rapid reoxidation of flavin allowed no buildup of the reduced species. Radda and Calvin (113) have confirmed this to be the case with FMN and FAD, and in addition found a slow ($k_2 = 0.16 \text{ M}^{-1} \text{ s}^{-1}$) second order dark reaction for FMN and NADH (*vide infra*).

Photoadditions occur anaerobically, notably with α -ketoacids and phenylacetic acid, again involving the triplet state (78,106). Addition is to N(5), in keeping with the higher spin density seen on this centre in the theoretical studies (97).

Triplet flavin will transfer energy to ground state $^3\Sigma_g^- \text{O}_2$, converting it to $^1\Delta_g$ (114,100,78). The singlet oxygen is then a powerful oxidant, its reaction with closed shell organic molecules no longer being spin forbidden. The reaction is rapid, and in the presence of excess O_2 the flavin semiquinone is rapidly reoxidized. In light of results to be presented, it is interesting to note that flavin-sensitized photooxidation of histidine has recently been reported (115).

Before proceeding further, the various reduced flavin structures should be summarized. Figure 6 shows the species which have been identified, along with the pK_a values at the three oxidation levels.

The physical organic chemistry of the flavins has been studied extensively, notably by Bruice, who has provided an excellent review (117). This work will concern us here only

Figure 6. Redox scheme of the flavin ring system.
Taken from refs. 77,78,116.

in so far as it directs attention to the reactivity of the flavin nucleus. Reduction by NADH and dithionite, as well as reactions with oxygen and sulfite, will be considered as pertinent to the present work, but other aspects of flavin reactivity will be very briefly treated now.

Nucleophilic attack under solvolytic conditions generally leads to ring opening (77). For example, hydroxide ion attacks the C(4) carbonyl and results in a rearranged product, and hydrolytic attack at the C(10a) position is also known (117). The C(4a) position is also susceptible to nucleophilic addition. The molecular orbital calculations of Sun and Song (118) have shown an ordering of reactivity to electrophilic attack of $N(1) > N(10) > N(3) > C(9) > C(7) \approx C(4a)$. The question of attack and/or addition at N(1) *versus* N(5) *versus* C(4a) is central to flavin biochemistry, and will be dealt with when considering sulfite addition and NADH oxidation. Dithiol oxidation has been suggested to proceed *via* a C(4a) adduct (117). Alcohols are oxidized *via* N(5) addition to the carbonyl carbon (119), and the reverse reduction of carbonyls by FH_2 is stepwise (i.e. free radical) in nature (120,117). Oxidation of 9-hydroxy- and 9-methoxyfluorene carbanions by flavins proceeds *via* a free radical mechanism, even in the dark (121).

The C(8)- α -methyl group has been shown above by theoretical calculation to be unusually reactive, and this is born out by its exhibition of nucleophilic attack upon aldehydes (122). Indeed, when covalent adducts to proteins are observed, they

always involve histidinyl or cysteinyl linkages to this methyl group (78,123-126), and it may be considered a functional group of the flavin nucleus (77). Presumably this reactivity results from a methylenelike resonance structure. In D_2O the C(8)-methyl group exchanges H for D with a rate constant of $2.4 \times 10^{-6} \text{ s}^{-1}$ at pH 6.8 (117).

We turn now to the question of sulfite addition (117), a reaction which, as will be seen, has been very useful in classifying the reactivity of flavoproteins. Sulfite adds to N(5) of a series of flavins, with a rate constant proportional to the half-wave potential of the flavin. When the C(8)- α -methyl group is missing, 6,8-disulfonic acid derivatives occur, and these may be further reacted to give adducts at the C(4a) or N(5) positions. An important distinction is found. Addition to N(5) occurs without catalysis. However before addition, the $pK_a < 0$ for this imine, and upon addition at C(4a), N(5) becomes a secondary amine with a $pK_a \approx 14$. Thus along the reaction coordinate of C(4a) addition, protonation of N(5) occurs, and this addition is subject to general acid catalysis. This change in basicity of over 10^{14} must be born in mind when N(5) *versus* C(4a) addition is considered in flavoprotein mechanisms, where a protein residue may be the acid catalyst. Dithionite reduces flavins rapidly, supposedly by nucleophilic substitution of SO_2^- or $S_2O_4^{2-}$ at N(5). A two-electron process is thought to be involved, as semiquinone appears more rapidly from comproportionation of reduced and oxidized flavin than from dithionite

reduction, and reduction of the semiquinone by dithionite is slow (78,127).

The nature of interaction and reaction of free oxidized flavin with reduced nicotinamide cofactors is a crucial problem in biochemistry, and it has sparked controversy for at least two decades. In 1959, Isenberg and Szent-Gyorgyi (128) reported low temperature spectroscopic evidence supporting a charge transfer complex between NADH and FMN. Later Isenberg's room temperature ESR measurements supported this view (129). Radda and Calvin (113) failed to observe changes in fluorescence or absorption, and questioned this view, explaining the earlier results on the basis of the second order dark reduction (*vide supra*) and subsequent comproportionation of FMNH_2 and FMN to semiquinone. Sakurai and Hosoya (130) observed complexation between NAD^+ and FMNH_2 , which they concluded to be of a charge transfer nature, but saw no such complexation with oxidized flavin. Porter *et al.* (131) then observed a charge transfer band ($\lambda_{\text{max}} \approx 550 \text{ nm}$) in a stopped flow spectrophotometer, which appeared rapidly upon mixing lumiflavin and N-methyl-1,4-dihydronicotinamide (NMNH), $K_d \approx 0.1\text{M}$, concomitant with competing (i) intra-complex reduction of flavin followed by dissociation and regeneration of Fl_{ox} by O_2 , and (ii) direct oxidation of "activated" NMNH by O_2 . Blakenhorn (132) has observed saturation (indicative of complex formation) in reaction of NMNH with flavin analogs. Complex formation depended not only on steric factors, but from the energy of an associated long wavelength absorption was seen also

to depend upon the redox potential of the flavin (consistent with charge transfer). The rate of reduction of the flavin (shown to occur within the pre-equilibrium formed complex) was also redox potential dependent. Blakenhorn and coworkers (133) have also prepared flavin derivatives with N(10) linked to the pyridine nitrogen of nicotinamide, and observed intramolecular charge transfer, prior to reduction of the flavin moiety, when the nicotinamide moiety was selectively reduced with ferricyanide (134). Charge transfer complex formation was maximum with three bridging methylene groups, which allowed stacking of dihydronicotinamide over the central ring of flavin, with a hydrogen of C(4) of the pyridine juxtaposed for transfer to N(5) of the flavin. Recently Porter *et al.* (135) have obtained the X-ray structure of this "biscoenzyme" molecule in various oxidation states, and find no evidence for intramolecular stacking in the crystal, which they argue must be favoured by hydrophobic interaction in aqueous solution.

Regardless of the presence of complex formation, non-enzymatic reduction of flavin by NADH may proceed *via* hydride transfer, group transfer, or single electron (free radical) reduction. A kinetic isotope effect of $k_H/k_D = 3.2$, and the ionic strength dependence of the rate, indicate C-H bond cleavage as the rate determining step in an ionic transition state (136). Traditionally two-electron (i.e. hydride) transfer has been proposed (137,138), though at least one widely used textbook favours a sequential one-electron process (139), as does Kosower

(140). All elementary texts agree on a 1,5-reduction mechanism as opposed to 4a-5, however (e.g. see 138,139). Both Hemmerich *et al.* (127) and Massey *et al.* (141) have proposed a group transfer mechanism involving a covalent pyridine adduct to N(5). It should be noted that borohydride reductions of flavins are slow (78,127), and some irreversibility in flavin reductions has been taken to implicate a 4a-5 reduction with slow cleavage of the resultant C(4a)-H bond (127). Bruice (117) favours single electron transfer followed by formation of what he terms an intimate radical pair with hydrogen radical. Kill and Widdowson (142) have demonstrated the possibility of such a mechanism for NADH. In reaction of N-benzyl-1,4-dihydro-nicotinamide with geminal bis-nitroalkanes, reduction occurred via rate limiting initial electron transfer followed by hydrogen radical transfer, with intermediates visualized by ESR. Jones and Taylor (143,144) have reported rate limiting hydrogen transfer in an NAD^+ recycling scheme involving flavin. While borohydride reduces the C(4) carbonyl, the more specific tri-tertiary-butoxy lithium aluminum hydride (TBAH) was suggested to complex with the carbonyl and enforce reduction by hydride transfer to N(5) (145). Reaction of N(3) methylflavin with TBAH gave immediate reduction and the spectrum of the 1,5-reduced product, with retention of both carbonyl signals in the infrared, but an ESR signal typical of the semiquinone was also present. It appears as if even these strong hydride reductants would reduce flavins in one-electron steps. Walsh *et al.* (146) have evidence for

initial proton transfer to flavin followed by carbanion reduction to FADH_2 as the mechanism of (enzymatic) FAD oxidation of 2-chloroalanine to pyruvate. It may safely be said that both the mechanism and initial products of reduction of free flavins by NADH are far from resolved, and a variety of competing routes may obtain.

The half-reduced semiquinone flavin is in general an unstable free radical species. The comproportionation equilibrium

is very rapid in solution ($k_2 = 2.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ for riboflavin (147)) and lies far to the left (148,110). In other words, when formed, the semiquinone will rapidly disproportionate. Anticipating the discussion of flavins in proteins, we note that three means of stabilizing flavin semiquinones have been discussed.

i) Resonance stabilization of the radical results from the delocalization allowed by the extensive π -system (149).

ii) Oxidized flavins have very low affinities for transition metal ions (77). However, flavosemiquinone states show different behaviour. The disproportionation reaction of equation 1 is inhibited by metal ions (77) and the stabilization of $\text{FADH}^\cdot$ by metal ions has been reported (150,151).

iii) Stabilization of free radicals by complexation with aromatic compounds has been suggested to occur through participation of the unpaired electron density in $2p \sigma$ bonds (152), and

this has been proposed as relevant for flavosemiquinones (137), particularly where aromatic protein residues may be involved.

The main spectral characteristics of the various forms of flavin at both semiquinone and hydroquinone oxidation levels have been included in Table 2. An early study of FMNH[•] produced by dithionite titration was carried out by Beinert (153). Fully reduced flavin produces a broad, structureless band extending from 300 to 500 nm. The naturally occurring flavins show very low fluorescence in their reduced states (154). Recent developments in the chemistry and spectral properties of fully reduced flavins have been discussed by Hemmerich and Haas (155). The fully conjugated oxidized system is of course planar, but sp³ hybridization of N(5) in the fully reduced species causes bending. Looking edge-on, a shallow V is formed with N(5) -N(10) axis as the pivot (the so-called "butterfly" structure). The internal angle of the V is about 159° (117). This has been confirmed by X-ray structural analysis (156).

The reactions of the reduced flavins with molecular oxygen are of biological importance, and also lead to a deeper understanding of the reactivity of these species. The reoxidation reaction is kinetically complex (141), but it seems commonly accepted that the initial step is formation of a perhydroxyflavin. This may form at either the C(4a) or C(10a), and further clarification of these possibilities has been difficult due to rapid decomposition of the intermediates (116,141). A bridged C(4a)-C(10a) singlet

peroxide collapsing to the epoxide has also been suggested (157). H_2O_2 , $\text{O}_2^- \rightleftharpoons \text{HO}_2^\cdot$, $\text{HO}^\cdot$, and HO^+ have all been suggested as decomposition products, and it has been proposed that flavoprotein oxygenases yield H_2O_2 , dehydrogenases O_2^- and semiquinone, and hydroxylases HO^+ or $\text{HO}^\cdot$ (78). Glucose oxidase has recently been shown to be reoxidized in a step involving O_2^- formation (158). Massey *et al.* (141) and Bruice and coworkers (117,159) favor the C(4a) peroxide adduct, and recent work appears to bear them out (see Walsh, 160). Adducts at C(6), C(8), C(9a) and C(10a) are still being considered (161,162), although Ghisla *et al.* (163) have most recently used ^{13}C -enriched FMN to show by ^{13}C -NMR that the intermediate oxygen adduct in the bacterial luciferase reaction is at position C(4a) of the flavin. Chemiluminescence accompanying decomposition of mixed peroxides formed from 4a-hydroperoxy-flavin has been observed (164), a finding of importance to the bacterial luminescence. Mechanisms of oxygen fixation by flavins and flavoproteins have been reviewed (165-167).

II) Immobilized Flavins

As a logical transition from free to enzyme bound flavins, investigation of the properties of flavin cofactors immobilized in various synthetic polymers and supports has become an active area of research in the past couple of years. A "synthetic flavo-enzyme" has also been prepared (168,169).

Shinkai *et al.* in 1978 (170) have provided the first example

of facilitated reduction of flavin by NADH in a non-enzymatic (and non-photochemical) system. An N(3)-benzylated derivative of 2',3',4',5'-tetra-O-acetyl riboflavin was incorporated into polystyrene. The polymer was made cationic by use of N,N,N-trimethylbenzylamine derivatives of styrene, and hydrophobic by replacement of one of the N-methyl groups with a dodecyl side chain. In comparison with 3-methyl-tetra-O-acetyl riboflavin, various polymers gave enhancements of 460-4650-fold in catalytic efficiency of reduction by NADH. Michaelis constants were comparable to those of alcohol dehydrogenases, i.e. $(0.2 - 1.0) \times 10^{-5}$ M. Higher incorporation of the dodecyl group markedly enhanced the catalytic efficiency. Flavin spectra were bathochromically shifted ($\lambda_{\text{max}} = 453-455\text{nm}$) indicating the flavin was in a hydrophobic environment created by this dodecyl group. The K_m values indicate that reaction is at least in part enhanced by strong binding of NADH, probably as an anion, to specific sites in the polycationic polymer. With disulfide flavoproteins such as lipoamide dehydrogenase and glutathione reductase in view, it is interesting that rapid oxidation of glutathione by the flavin-containing polymer was also observed.

Tetra-O-acetyl riboflavin brominated at the C(8)- α -methyl position was condensed to polyethylenimine by Spetnagel and Klotz (171), who partially quaternized amino groups with dodecyl chains. Again a bathochromic shift of the low-energy flavin absorption was observed ($\lambda_{\text{max}} = 455\text{ nm}$), as well as a hypsochromic shift of the next band ($\lambda_{\text{max}} = 352\text{ nm}$), characteristic

of flavins in protein environments (*vide infra*). NADH was catalytically oxidized in the presence of O_2 , with Michaelis-Menten kinetics where O_2 was non-rate limiting. The reaction produced peroxide, and was not photo-dependent. An increase in catalytic efficiency of 120-fold over free riboflavin was observed, but it is noted that this is comparable to the rate of O_2 -linked NADH oxidation of several flavoproteins in the absence of their natural substrate acceptor. The authors (171) cite the work of Blakenhorn (132,134) on NADH-flavin charge transfer complexes. They find NADH binding to the polymer to be two orders of magnitude greater than that of $K_d = 0.1$ M found by Blakenhorn for binding to free flavin. The cationic polymer matrix is thus seen as serving to increase the concentration of NADH in the region of the flavin; and also stabilize the charge transfer complex in which the redox process is thought to take place. The hydrophobic environment also increases the redox potential of the flavin, which relates directly to the rate of oxidation of NADH (see 133 and Table 34).*

Shinkai *et al.* (173) have also observed an enhancement of 10^6 times in the rate of oxidation of nitroethane carbanion by their cationic polymeric flavin system (170). It is suggested that this may be an electron transfer reaction catalyzed by the poly-electrolyte environment. The enhancement is 10^3 -fold over

*Tabulations of the results of polarographic studies on flavins and flavoproteins (172) and of half-wave reduction potentials (106) have been given.

flavin in cationic micelles, and is increased by dodecyl group content. The reaction is insensitive to the redox potential of the flavin, although the relevance of this to NADH oxidation is uncertain, as the mechanism of nitroalkane carbanion oxidation in the polymer is unclear.

Perhaps the transition to flavoproteins is completed by the work of Kaiser and coworkers (168,169), who have introduced tetra-O-acetyl riboflavin into papain *via* a cysteinyl sulfide or sulfone linkage to the C(8)- α -methyl group. Papain is a non-flavoprotein proteolytic enzyme, and the flavin binds to an active site sulfhydryl group without preventing binding of peptide substrates. The resultant flavopapain, termed a "semi-synthetic enzyme" by the authors (168), had less than one per cent of its proteolytic activity, but this activity was returned upon decomposition of the flavin adduct by addition of cysteine. The bound flavin is immobilized in a hydrophobic substrate binding cleft, and displays rates of oxidation of N-benzyl-1,4-dihydronicotinamide comparable to those of natural flavoproteins, with Michaelis-Menten kinetics similar to the system of Shinkai *et al.* (170). Here, however, saturation is not due to charge transfer complex formation ($K_d \approx 0.1$ M; *vide supra*), as concentrations of reduced nicotinamide derivative and flavin are low ($\sim 10^{-3}$ M). Rather, the Michaelis-Menten complex is a natural enzyme-nicotinamide nucleotide complex.

III) Flavoproteins

In 1964, Dixon and Webb (174) listed forty known flavo-enzymes, and since that time at least as many more have been discussed in the literature. The complexity of flavin chemistry is reflected in the vast range of metabolic activities of these enzymes, which include mono- and di-oxygenases, dehydrogenases of a broad range of substrates, and metallo-flavoenzymes, and include many examples of both soluble and insoluble enzymes. Indeed, the literature of flavoproteins is so vast that no attempt to survey it completely has been made, although as will be seen, several authors have sought trends in an attempt to gain some unified theory of flavoprotein mechanisms. It is primarily the non-metallo-dehydrogenases which concern us here.

In order to understand the similarities and differences between the chemistry of flavins in enzymes and that of free or artificially immobilized flavins, one must have some understanding of the nature of binding of the flavin cofactor. At present, only ten flavoproteins are known where the flavin is covalently bound (123-126, 175-177), always through a cysteinyl or histidyl linkage at the C(8)- α -methyl group. In the majority of flavoproteins, the cofactor (riboflavin, FAD, or FMN; see 174) is non-covalently, though tightly, bound. Apparent dissociation constants are typically in the range of 10^{-8} - 10^{-10} M, though D-amino acid oxidase is more readily dissociable ($K_d = 2.5 \times 10^{-7}$ M) and some flavoenzymes exhibit no detectable dissociation under physiological conditions (78). Various lines of physical evidence indicate a

great diversity of binding sites, and this may reflect a role of flavin binding in maintenance of protein structure as well as catalysis (e.g. see 178). Indeed, in some flavoenzymes, such as glyoxylate carboligase, the catalytic participation of the flavin is in doubt, despite its obligatory presence for activity (179, and cf. also 78). In many instances, removal of flavin has a destabilizing effect on protein conformation. This point will be taken up in some detail with regard to lipoamide dehydrogenase (but see 78,178,180,181). It is important here to note that in order to understand a particular flavoprotein, this synergism must be considered: the protein environment profoundly effects flavin chemistry, and the flavin can profoundly effect the protein conformation.

Perhaps our major probes of flavin environment in proteins are spectroscopic, as sometimes drastic changes in both absorption and fluorescence are seen to occur on binding. As stated above, decreased solvent polarity causes resolution of vibrational structural of the 450 nm absorption band, accompanied by a red shift of 5-10 nm, and a blue shift of as much as 40 nm in the 370 nm band (88). Similar spectral changes are seen in flavo-proteins (103), and are generally interpreted as arising from desolvation (aqueous) of the isoalloxazine chromophore. D-amino acid oxidase has been well studied (103,182), and addition of several compounds which hydrogen bond with the protein causes conformational changes and increases the magnitude of these spectral alterations, presumably by burying the flavin more

deeply in a hydrophobic pocket. A similar interpretation of the spectra of flavins immobilized in synthetic polymers may be advanced (*vide supra*).

Hemmerich *et al.* (183) have noted four classes of half-reduced flavoprotein. They are:

- i) stoichiometric amounts of blue semiquinone (the neutral semiquinone spectrum)
- ii) stoichiometric amounts of red semiquinone (the anionic semiquinone spectrum)
- iii) no paramagnetic (semiquinone) intermediate, and
- iv) non-stoichiometric amounts of semiquinone.

It is suggested (183) that class (i) flavoproteins have a carboxylic acid residue stabilizing the semiquinone by hydrogen bonding to N(5) in a $\text{RCOO}^- \cdots \text{HF1}^\bullet$ structure, while in class (ii) the stabilization is by a protonated amino acid residue, such as $\text{R-NH}_3^+ \cdots \text{F1}^\bullet$, again hydrogen bonding to N(5). Class (iv) flavoproteins may "discharge" the flavin radical by interflavin contact (i.e. disproportionation), or by flavin-metal contact (183).

Lipoamide dehydrogenase is perhaps the best example of a class (iii) flavoprotein, and the nature of its half-reduced form is discussed in some detail in this thesis.

The fluorescence of the flavin fluorophore may be statically quenched, that is quenched by formation of a non-fluorescent ground state complex (81). Complex formation between flavin and a number of substances has been observed by Weber (184), and has been used to account for the low quantum yield of FAD in terms of intra-molecular quenching by adenine in the folded conformation (81).

Many of Weber's complexes give charge transfer spectra (80). Among the static quenchers are indoles and aromatic and phenolic species, and it is thus not surprising that the fluorescence of the flavin cofactor is often quenched on binding to a protein site (81,103,154,185) where binding hydrophobicity may be maintained by the presence of aromatic amino acid residues. A few flavoproteins retain some fluorescence, and once again the notable exception of lipoamide dehydrogenase will be discussed below, where it will be seen that the FAD fluorescence is greatly enhanced upon binding.* The fully reduced flavin was said to be non-fluorescent, and it has been argued (154,185) that this is due to enhanced radiationless energy dissipation to the environment by facile (vibrational) inversion processes of the "butterfly" structure of the flavohydroquinone. In keeping with this view, Ghisla *et al.* (154,185) have observed fluorescence emission from fully reduced flavins held rigid in a glassy matrix at 77°K, and also in some flavoproteins. The authors have argued that red shifted (5-25 nm) absorptions of the lowest energy transitions of the fully reduced flavoproteins indicate a more planar Fl_{red}, which is thus held rigidly in this strained conformation. The flavin may also be constrained by hydrogen bonding. In the absence of complete quenching, this enforced

* An interesting sidelight to this discussion is the recent work of Visser *et al.* (186) who have used increases in fluorescence to monitor release of flavin from a series of flavodoxins at increased pressure, internal collapse of the proteins at 5-10 kbar presumably destroying the flavin binding site.

rigidity will decrease the efficiency of the vibrational radiationless transfer mechanism, and fluorescence may be observed.

Massey and Palmer (187) have applied the technique of anaerobic photo-irradiation in the presence of EDTA to flavoproteins, and demonstrated production of flavoprotein semiquinones. The rates of reduction amongst a group of enzymes were variable, and consistently slower than for free flavins. Elliott and Bruce (188) have shown formation of a C(4a) adduct of unknown structure to occur upon reaction of acetates with flavins, and recent work by Massey and Hemmerich on flavin (110) and N(5)-deazaflavin (189,190) catalysis of EDTA-catalyzed photoreduction of flavoproteins has determined that photodecomposition of such an adduct is the mechanism of reduction. The product is fully reduced flavin. In the enzyme systems, blockage of the C(4a) site from attack by EDTA prohibits reduction by this mechanism. However, addition of a catalytic amount of free flavin causes rapid production of the semiquinone form of some flavoproteins, and it is suggested that this results from comproportionation of fully reduced free flavin and oxidized enzyme-bound flavin (110). It is further suggested that the slow reduction in the absence of added catalyst may be due to the small equilibrium concentration of free flavin present.

In general, whereas well resolved ESR spectra can be obtained for free flavin radicals (e.g. see 191), the low correlation times of proteins prohibit similar resolution in

flavoprotein semiquinone spectra (103,192,193).. Molecular tumbling averages anisotropic contributions to electron-nuclear magnetic dipole contributions to the magnetic field, and allows observation of hyperfine structure which is otherwise masked under one broad signal. This is called inhomogeneous broadening as it results from local inhomogeneity of the magnetic field due to orientation effects. Theoretical calculations show that while the rotational correlation time, τ_c , for a free flavin is of the order of 10^{-9} s, that of a globular protein of molecular weight of only 1.5×10^4 is 10^{-7} s, and thus whereas in the free flavin anisotropic terms of up to 100 MHz are averaged out, terms of only about 1 MHz will be averaged in the protein (193). The broad, unresolved structures observed are an indication that in the protein the flavin is fairly rigidly held and shares the τ_c of the protein as a whole. In a survey of seventeen flavoprotein semiquinones, Palmer *et al.* (192) showed that red (anionic) semiquinones have a linewidth of 15 ± 1 gauss, while in neutral blue semiquinones this value rises to 19 ± 1 gauss, due in part to the 8 gauss coupling with the additional proton on N(5). Thus in principle this technique can serve to determine the nature of the semiquinone even when the solution concentration of it is low, although again in the special case of lipoamide dehydrogenase complications will be seen.

In an electron-nuclear double resonance (ENDOR) experiment, the height of a partially saturated ESR signal is monitored as a

function of radio-frequency irradiation. For instance, ENDOR has confirmed that the unpaired electron of the lumiflavin anionic radical is strongly coupled to the protons of the C(8)- α -methyl group (193). In proteins, stabilization of the radical by an exchangeable protein proton is seen as a "matrix ENDOR" signal, and differential loss of this signal in D₂O has been taken to reveal varying degrees of hydrophobicity (the more hydrophilic, the more complete the exchange) in flavin binding sites at the semiquinone level of reduction (193). Significantly, it is concluded that the hydrophobicity of the site need not be the same at oxidized and half-reduced levels, arguing for control of protein conformation by flavin redox state.

Production of the flavoprotein semiquinone by free flavin catalyst in the EDTA-photo-irradiation sequence points to a central question of flavin-biochemistry. Flavins can dis- and com-proportionate, and many flavoproteins have multiple flavin binding sites, often on distinct subunits. To what degree is flavin-flavin cooperativity biologically significant in enzyme mechanisms? A related point for consideration is that flavins have a common occurrence in metabolic pathways where they must couple electron pair donors to one-electron acceptors (e.g. see 138,139), such as the coupling of NADH or quinones to Fe(III) or Cu(II) metalloenzymes in electron transport chains. According to Shaffer's "Equivalence Change Principle" (194), such reactions would be prohibitively slow without an intermediate capable of facile two-electron reduction and one-electron oxidation. With

two reduction levels open to the flavin, it serves well as such an intermediate, and the disproportionation reaction obviously suggests the possibility of flavin cooperativity in such "electron pair splitting" (183).^{*} The title enzyme of this thesis displays its (type C2) multifunctionality in part by reaction with both one and two electron oxidants, and we shall provide physical evidence that interaction of two flavin cofactors on two separate subunits is a distinct mechanistic possibility. Hemmerich *et al.* (183) have also discussed the unlikelihood that an acceptor such as a cytochrome would accept electrons sequentially from both Fl_{red} and FlH^+ states which differ by as much as 260 mV in redox potential. They suggest that com- and/or dis-proportionation may get rid of the undesirable state. The interflavin contact could involve π -complex formation (as it would in solution), but, given the steric requirements of the protein environment, could also reflect N(5)-N(5) σ -interaction (183). Note also that this interaction could be mediated by a second chemical structure, including a protein disulfide. Kamin (195) provides evidence that at least two enzymes display flavin cooperativity. In an elegant experiment, FMN was selectively removed from NADPH-sulfite reductase (EC 1.8.1.2), an enzyme containing four FAD, four FMN, and four heme molecules, and an unknown number of iron sulfides. It was shown that FMN mediates the transfer of one electron from FAD to "Fe-S₂" (195).

^{*}Note, however, that many, but not all, "step-down" (i.e. two-electron oxidizing - one-electron reducing) flavoproteins contain more than one flavin (see for example 195).

Palmer and Massey (103) have thought it more likely, however, that with a few exceptions, possession of two flavins merely reflects multiple binding sites with no more significance than the several independent coenzyme binding sites of many pyridine nucleotide-linked dehydrogenases.

Whether $\pi-\pi$ or σ (N(5)-N(5)) in nature, the flavin-flavin contact model may suffer from severe steric hindrance. It was mentioned above that blockage of the C(4a) site in proteins is generally held responsible for their inability to form flavin-EDTA adducts. The presence of enzyme blocking groups in this region was also significant with regard to semiquinone stabilization. It is also important, however, that the protein may both protect this reactive flavin site and make it available for intramolecular reactions. Hemmerich *et al.* (183) have pointed out an additional mode of steric control over flavin chemistry which is available to the enzyme: chemistry at the N(5) imine site of the oxidized flavin clearly involves in-plane attack, while attack at C(4a) must be out-of-plane.

Sulfite and O_2 were shown above to react with free flavins forming what are generally accepted to be N(5) and C(4a) adducts respectively. Massey *et al.* (196) have shown that a number of flavoprotein oxidases form stable sulfite adducts, whereas dehydrogenases do not. This work was extended (197) and spectral indications are that the adducts form at N(5). The correlation of unstable sulfite adducts with slow oxidation by O_2 in the reduced state indicates that the flavoprotein dehydrogenases may

have their flavin(s) blocked both in-plane and out-of-plane, consistent with residence of the flavin in a hydrophobic pocket.

Palmer and Massey (103) have provided a classification for non-metallo-flavopenzyme mechanisms which is more useful as a framework than it is rigorous. Their scheme is summarised here, and it should be recognized that the examples (taken also from reference 103) are likely, but in few cases proven. The flavo-enzymes may then behave in the following ways:

i) Functioning between oxidized and fully reduced forms.

Examples: glucose oxidase
glycollic acid oxidase
L- α -hydroxyacid oxidase

ii) Functioning between oxidized and semiquinone forms.

Example: L-amino acid oxidase (snake venom)
Note: This mechanism probably involves further reduction of $2(\text{FADH}^\bullet) \rightarrow 2(\text{FADH}_2)$. No clean example is known.

iii) Functioning between fully reduced and semiquinone forms.

Examples: NADPH:cytochrome c reductase
NADPH:cytochrome b_5 reductase

iv) Functioning through a substrate-complexed semiquinone.

Examples: D-amino acid oxidase
acyl CoA dehydrogenases

v) Functioning with flavin semiquinone and another redox group in the enzyme.

The best examples of this are the disulfide-containing enzymes, such as lipoamide dehydrogenase, glutathione reductase, and thioredoxin reductase. The mechanism of lipoamide dehydrogenase will subsequently be discussed in detail (Part C), and will be somewhat modified by the results presented here. A recent review of the flavin-disulfide enzymes has appeared (198).

Whatever the nature of the flavin binding site or NADH oxidation mechanism, the fact that nearly all flavoproteins are reduced by NAD(P)H can, by analogy with the work done on

flavins in synthetic polymers, be predicted from their ability to bind the pyridine nucleotide in a centre containing an immobilized flavin. A great insight into the multifunctionality of the subsequently reduced flavoprotein has come with the largely ignored work of Dixon. He has shown (199-201) that with few exceptions the reductions carried out by flavoproteins occur only with substrates that would also be reduced by free reduced flavin (FlH^+ or Fl_{red}), and furthermore at similar or slower rates than those occurring with free flavin. Thus we view the apoenzyme of flavoproteins as a natural means of a) immobilizing the flavin for reduction, b) harnessing the reactivity of the reduced flavin, and c) controlling cellular chemistry by quenching certain reactivities some or all of the time. It is with this perspective that we turn now to the title flavoprotein as a model of type C2 multifunctionality, and we will present evidence gleaned from the investigation of this multifunctionality that will allow us to develop the above view of the role of apoenzyme in the specific case.

PART C - LIPOAMIDE DEHYDROGENASE

In 1939, Straub (202) isolated from porcine heart muscle an enzyme which he showed spectrally to be a flavoprotein of unknown flavin content. The biological function of the enzyme remained unknown, but it was found to catalyze the oxidation of NADH in the presence of a suitable acceptor such as methylene blue (203), and was termed a diaphorase. There was controversy over the Straub's diaphorase for some years, resulting in part from the fact that it often copurified with another flavoprotein having cytochrome c reductase activity (e.g. see 204,205), though lacking this property itself. It was not until 1957 that Savage (206) was able to purify the enzyme to electrophoretic homogeneity and calculate a molecular weight of 8.0×10^4 on the basis of diffusion and sedimentation. Spectral comparison indicated one mole of FAD per mole of enzyme. Massey (207) reported differential extraction of diaphorase and cytochrome reductase activities, and suggested that a low molecular weight electron carrier might couple diaphorase to cytochrome c reductase allowing efficient reduction of cytochrome c by NADH. His initial report (207) noted that lipoic acid could serve such a coupling role, and the importance of such a lipoic acid dehydrogenase activity soon became apparent when diaphorase was purified as a highly active lipoate reductant (208). Cytochrome c reductase was shown to be quite independent of Straub's

diaphorase (209), and the identity of the latter with lipoyl dehydrogenase was established (210). Lipoyl dehydrogenase was found to reduce lipoic acid analogs, $K_3Fe(CN)_6$, and 2,6-dichloro-indophenol (DCIP), and was found to show an eighty-fold increase in catalytic efficiency when lipoamide replaced lipoic acid as the acceptor (210). Henceforth this enzyme will be referred to as lipoamide dehydrogenase, and has the enzyme commission designation EC 1.6.4.3. It was concluded that a negative charge on the substrate hindered approach to the enzyme, only the naturally occurring L-isomer was an active substrate, and further that the forward reaction (reduction of lipoamide) was favoured over the reverse (oxidation of dihydrolipoamide). Some data of Massey (210) are given in Table 3.

Table 3: Comparative data of lipoamide dehydrogenase, from reference 210

Substrate	pH optimum	K_m (mM)	Maximum turnover no.
DL-lipoic acid	5.9	2.0	1,000
DL-lipoamide	6.5	5.0	80,000
DL-dihydrolipoamide	7.9	0.4	9,400

Subsequently arsenite was shown to inhibit the lipoamide reaction only in the presence of reducing substrate, and the role of an active centre disulfide-dithiol redox couple was thus demonstrated (211).

Searls and Sanadi (212) independently confirmed the

identity of lipoamide dehydrogenase and Straub's diaphorase.

Their enzyme was isolated from the α -ketoglutaric dehydrogenase complex, and the metabolic function of this enzyme was established.

We now digress to elucidate this function.

The discovery of the metabolic role of lipoic acid has lead to the elucidation of the pathway of a key metabolic transformation, the decarboxylation of α -keto acids with acylation of coenzyme A (CoA). Thus in the Krebs cycle, α -ketoglutarate is decarboxylated to form succinyl-CoA, and acetyl units are recovered from pyruvate upon its decarboxylation to form acetyl-CoA. Both decarboxylations are carried out by a multistep process (213,138, 139) beginning with a attack of the α -carbonyl of substrate by a thiazole ylide of thiamine pyrophosphate. Decarboxylation of the adduct by pyruvate or α -ketoglutarate dehydrogenase is followed by acyl group transfer to enzyme-bound oxidized lipoic acid (L-6,8-thioctic acid). The formation of the acylated lipoic acid is catalysed by dihydrolipoyl transacetylase or transsuccinylase to which the lipoic acid is bound *via* an amide linkage to the ϵ -amino group of a lysine residue. Subsequent acylation of the sulfhydryl group of CoA leaves reduced lipoic acid (6,8-dithio-octanoic acid) which is reoxidized at the expense of NAD^+ . This latter step is catalyzed by the title flavoprotein. The dehydrogenase, transacetylase, and flavoprotein occur together in multi-enzyme complexes, forming an α -ketoglutarate dehydrogenase complex (α -KGDC) or a pyruvate dehydrogenase complex (PDC). More will be said of these complexes below.

Our mechanistic understanding of lipoamide dehydrogenase really began in 1960 with the work of Massey *et al.* (214) who showed that both NADH and dihydrolipoamide reduce the enzyme to a red intermediate with a visible absorption spectrum resembling that of a flavin semiquinone. Since each of these substrates transfer two equivalents per flavin, it was assumed that the flavin accepted one while the active centre disulfide accepted the other, splitting to form a sulfur radical and a new sulfhydryl group. It was suggested that partial overlap of the sulfur and flavin radicals helped to stabilize the red intermediate, and this was supported by the observation that addition of p-chloromercuriphenyl sulfonate, a specific sulfhydryl reagent, discharged the red colour. In the normal catalytic cycle, the enzyme oscillated between oxidized and red forms (E_{ox} and EH_2) with no full reduction to EH_4 . Reactions of E_{ox} with NADH and of EH_2 with NAD^+ were both complete within the dead time of a stopped flow apparatus, and the rate limiting steps of both forward and reverse reactions were reaction of lipoamide with EH_2 and dihydrolipoamide with E_{ox} respectively. It was proposed that NADH reduced the disulfide to a dithiol which then transferred hydrogen radical to FAD.

Searls *et al.* (215) supported this view for dihydrolipoamide as reductant, but proposed that NADH reduces flavin directly, with subsequent transfer of H^+ to the disulfide. They showed in addition that whereas incubation with both arsenite and $CdCl_2$

following reduction with NADH inhibited the dehydrogenase (DHase) activity, the diaphorase (DPase) activity was enhanced, and therefore did not require the disulfide. Thus when reduction is by a sulfhydryl group, e.g. dihydrolipoamide, it may remain bound to the active centre and cause FAD to remain in the oxidized state. Significantly (though misunderstood at the time), it was also shown that Cd^{2+} or arsenite treatment caused disappearance of the broad band at ~ 530 nm associated with EH_2 . Massey and Veeger (211) had found that a lag sometimes occurred in the DHase reaction which was eliminated by addition of NAD^+ . They concluded that their biradical EH_2 transferred electrons to oxidized substrate stepwise, and that NAD^+ was more efficient at accepting an electron from the enzyme than was lipoamide. Searls *et al.* (215) proposed a pairwise reduction as the general mechanism, and observed the lag only below pH 6.0.

Massey and Veeger then modified their mechanism when they noted (216) that excess NADH caused only formation of EH_2 . Exclusion of the resultant NAD^+ with *Neurospora* NADase caused full reduction to EH_4 by two equivalents of NADH. Whereas dihydrolipoamide in excess would produce only EH_2 (presumably by reaction with the disulfide, leaving a flavin which could be further reduced by dithionite), NADH could react directly with flavin (for instance when the disulfide was blocked by arsenite), and could produce EH_4 if NAD^+ was excluded (216), consistent with the proposal of Searls *et al.* (215). The requirement for NAD^+

in the DHase reaction was then considered to result from protection of the enzyme from full reduction (216), rather than its ability to accept a single electron (211). The observed transhydrogenation reaction between oxidized and reduced NAD(H) analogs, as well as protection by NAD^+ from full reduction, was taken to suggest the existence of two pyridine nucleotide binding sites (216). The first molecule of NADH would then bind covalently to, and split, the disulfide, producing EH_2 . A second, regulatory site, called the Y-site, could either bind NAD^+ and result in stabilization of the enzyme at the EH_2 oxidation level, or bind a second molecule of NADH, with the result that EH_4 would be formed. Thus, while FADH_2 could be produced, it was considered catalytically significant by some authors (215) and not so by others (216,217). In addition, the two groups differed on their proposed structures of the red EH_2 form. Searls and Sanadi (218,219) proposed that the red colour was due to a charge transfer complex between FAD (oxidized) and a vicinal dithiol. They saw an absorption at 535 nm on mixing of dihydrolipoic acid and FMN (219). Massey and Atherton (220) found that dihydrolipoic acid slowly reduces FMN, which is then reoxidized by O_2 with peroxide formation. Photodecomposition of the unidentified products of the peroxide reaction then accounted for the spectral features observed by Searls and Sanadi. The semiquinone nature of EH_2 was reasserted. Gascoigne and Radda (221) later showed that indeed dihydrolipoic acid reduces free flavin, but they proposed, on the basis of

2

substituent effects, half-wave potentials, and a similarity to the NADH-flavin reaction (considered hydride in nature by them) an ionic rather than radical mechanism.

Incubation with Cu^{2+} ion caused catalytic oxidation of an essential sulfhydryl group, with almost complete loss of ability to reduce lipoamide (DHase) and 3-acetyl-pyridine-adenine dinucleotide (THase with 3-AP-NAD), a reduction in the rate of $\text{K}_3\text{Fe}(\text{CN})_6$ reduction (ETase), and a large increase in the rates of DCIP and methylene blue reductions (DPase) (217). This was attributed to over reduction of the Cu^{2+} - treated enzyme by NADH to EH_4 , which would result from loss of the protective NAD^+ site, and this view was supported by the observation that phosphate was a competitive inhibitor of NAD^+ , and also helped protect against modification by Cu^{2+} . It was then restated that EH_4 was inactive in the DHase and THase reactions, but active (though less so) in the ETase reaction, and was the favoured reductant in the DPase reaction. It was also noted, however, that cupric ion treatment caused a slight change to a more polar FAD environment, evidenced by a decrease in λ_{max} from 455 to 452 nm. It was later shown (222) that Cu^{2+} -treatment resulted initially in oxidation of two sulfhydryls to form a new, second disulfide per flavin, which eventually resulted in loss of flavin and denaturation.

The red colour of EH_2 , which develops rapidly on anaerobic reduction by NADH, disappears to be replaced by a new band at ~ 700 nm under some circumstances, and gives the enzyme a blue-green appearance (223). Thus both Cu^{2+} and arsenite treatments give

the altered spectrum at room temperature, as does the unmodified enzyme at 4°C. The phenomenon occurs only when NADH is the reductant, and is removed by addition of NADase. Thus the new absorption was considered to result from a complex between NAD^+ and reduced flavin. It has been mentioned that EH_4 forms by NADH reduction at low temperature, and of course when the disulfide is blocked by arsenite the flavin is reduced preferentially. The copper enzyme is also susceptible to over reduction. That the blue-green complex is of a charge transfer nature with reduced flavin as donor and oxidized pyridine nucleotide as acceptor was supported by several lines of evidence (223). The energy of the absorption maximum varies with the redox potential of the analog used, and its intensity with temperature. Such charge transfer complex formation was also demonstrated in model (i.e. free reduced flavin) systems (223). NADPH is not a substrate for the enzyme, and no complex was observed with NADP^+ , probably due to blockage of binding by the 2'-phosphate of the adenosine moiety. NADP^+ also fails to reoxidize EH_2 (224).

Changes of a non-charge transfer type were also shown to be caused by NAD^+ in the EH_2 spectrum produced by NADH (224). Addition of NAD^+ to EH_2 caused slight changes of the 450 nm and 530 nm regions, and these changes did not require fully reduced flavin (the donor; 223). Formation of the new species was complete within the dead time of a stopped flow machine, and thus could be a catalytic intermediate. It was proposed that it represented binding of NAD^+ to the disulfide or Y-site, and was also characterized by a shoulder at 565 nm.

The enzyme was first reviewed by Massey in 1963 (225); and previously unpublished structural results were presented. The enzyme was a dimer consisting of two subunits of molecular weight of approximately 5.5×10^4 daltons, each containing one active centre with one molecule of FAD. Interestingly enough, these active centres were suggested to function independently. In support of this view were advanced the stoichiometric reduction to EH_2 by NADH, and the inability of the supposed semiquinoid EH_2 to disproportionate, even when stabilizing NAD^+ was removed with NADase. The two subunits were believed to be held together by an interpeptide disulfide bridge (226). The published amino acid composition is given in Table 4 for future reference.

The FAD prosthetic group was known not to be covalently linked, but tightly bound all the same. Its removal was inefficient and involved partially denaturing (strongly acidic) conditions, and the resultant apoenzyme was unstable (227,226). The enzyme is unique amongst flavoproteins in having greatly enhanced FAD fluorescence (45% of free FMN; 185), and is non-fluorescent in the reduced state (185,154). FAD is bound in the open conformation and may experience some dynamic quenching by a protein sulfhydryl group (89). The enzyme is inactive towards sulfite. Its reduced form is reoxidized slowly by O_2 (196).

In the forward direction (NADH oxidation), the DHase reaction was reported to be activated by phosphate, and also dependent on increasing ionic strength (225). Most importantly, the inactivity of EH_4 , the semiquinoid ("strongly interacting biradical")

Table 4: Amino acid composition of lipoamide dehydrogenase (pig heart), expressed in moles per mole of flavin. Data from reference 198.

Amino Acid	no. of moles	Amino Acid	no. of moles
{ Cysteic acid	10	Ile	36
($\frac{1}{2}$ cys)	2	Leu	31
Asp	44	Tyr	8
Thr	26	Phe	15
Ser	24	Lys	36
Glu	47	His	11
Pro	18	Arg	14
Gly	53	Trp	2
Ala	46	FAD	1
Val	44	Total residues	474
Met	9		

Minimum MW = 51,000

NH₂-terminal : Ala

nature of EH_2^* , and the mechanism proposed by Massey and Veeger (216) were reasserted, and became the basis for future discussions. It will be shown that the arguments of Massey (225) on each of these points are incorrect. It should be stressed that here, we are considering only the porcine heart enzyme. Although lipoamide dehydrogenase has been isolated from a variety of other sources (198,229) many fundamental properties are variable,[†] and no other species shall concern us.

More detailed attempts to understand the molecular basis of the DHase mechanism followed Massey's early picture (225), but for the most part the other catalytic activities were ignored or poorly treated. Thus Guilbault *et al.* (230) investigated the DPase reaction as a model system for fluorometric kinetics, and erroneously[‡] concluded that it proceeds *via* a ping pong mechanism.

*Preliminary work had indicated an ESR signal from the reduced enzyme in the presence of NAD^+ , but its appearance was much slower than that of the 565 nm spectral feature (224). Later a signal of linewidth 15 gauss (identifiable with a red, anionic semiquinone) was reported (192). This is puzzling in light of the later assertion by the authors that no ESR signal is seen upon reduction by substrate or dithionite (228) or by the EDTA-photoreduction process (187). It was later put forward (103) that this argues for the "strongly interacting biradical" structure, in which either (a) a magnetic dipole-dipole interaction couples the two electrons forming an $S=1$ triplet state, which then suffers inhomogeneous broadening as the triplet state energies split anisotropically, or (b) an electrostatic exchange interaction pairs the two spins. It has been reported (183), with no details, that lipoamide dehydrogenase can be made to display a blue semiquinone structure.

[†]For instance, the EH_2 form of the *E. coli* enzyme is quite unstable (cf. 198)

[‡]Present work; and Dr. C.S. Tsai, personal communication.

The two areas most concentrated upon during this period were the role of protein (i.e. conformation) in structure-activity relationships, and the nature of pyridine nucleotide binding.

Phenyl mercuric acetate distinguishes amongst two rapidly reacting, two slowly-reacting, and two very slowly reacting sulfhydryl residues (per flavin) in oxidized lipoamide dehydrogenase (231). These authors considered that rapid reaction of the mercurial at one sulfhydryl placed a conformational strain on the active centre, so that when the disulfide was reduced in the catalytic sequence the nascent thiols separated. With this destruction of the reversible disulfide redox couple, the DHase activity was lost. In addition, increased exposure of the flavin was thought to favour formation of FADH_2 and cause the observed enhancements of DPase activity.

The specifics of protein conformation, and the related problem of the monomer $\rightleftharpoons$ dimer equilibrium were attacked by Veeger and coworkers (232-239). In an early report (232) an apoenzyme was prepared by ammonium sulfate precipitation at pH 1.4-1.5 and high ionic strength (3M KBr). The resultant protein had a molecular weight of 5.5×10^4 daltons, contradicting the previous view (226) that an interpeptide disulfide maintained the dimer. The apo-monomer contained < 10% residual flavin and displayed < 5% of the native DHase activity, yet variably normal or increased DPase activity (233). FAD reassociated with the apoenzyme ($\Delta H = -8.3 \text{ kcal.mol}^{-1}$, $\Delta S = -4 \text{ e.u.}$), and at 5°C caused a 20-fold increase in DPase activity over original holoenzyme, while DHase remained at $\sim 5\%$. Fluorescence

polarization returned in a two-step process, the second step being slower than the return of DPase activity. In conjunction with a second order rate constant for return of DHase activity at room temperature, and sedimentation studies, this was taken to support the view that, while DPase activity is enhanced when FAD binds to monomer, a second, slower conformational change then ensues. This conformational change allows redimerization with return of DHase activity and reduction of DPase activity to normal levels. For the overall reconstitution of holoenzyme, the activation energy is 21 kcal.mol^{-1} (233). Rapid initial increase of FAD fluorescence on binding, followed by a slow rise to a maximum value can be attributed to initial opening of FAD on binding, followed by conformational rearrangement affecting the interaction of the isoalloxazine moiety and a protein residue (234,89). Freezing of the diluted holoenzyme also caused partial denaturation, FAD becoming dissociable and displaying a more polar (blue-shifted) spectrum in the 450 nm region. This also resulted in enhanced DPase and reduced DHase activities. It was suggested that conformational changes at the active centre of the DCIP-active enzymes prohibit disulfide-substrate binding (see ensuing discussion of reference 232). A puzzling aspect is that while both Cu^{2+} -modified protein and initial monomer from apoenzyme reconstitution display enhanced activity with DCIP, reconstitution of an apo-monomer from Cu^{2+} -treated holoenzyme produces no such effect (233). In addition, apoenzyme treated with Cu^{2+} will no longer reassociate. Clearly the conformational strain of Cu^{2+} modification (see 222) must require at least partial stabilization of tertiary structure by flavin to avoid complete,

irreversible denaturation. The conformational flexibility of the enzyme must be great. Visser and Veeger (235) proposed at least nine different conformational states to explain the DPase/DHase activity ratios and molecular weight of different species produced by aerobic and anaerobic freezing, cold incubation, or FAD removal. The stabilizing role of flavin is further supported by the instability of apoenzyme preparations, and by the binding constant of FMN to apoenzyme. Initially this association constant is only about 1/20 that of FAD, but after two hours has increased to a value comparable to that of FAD, strong evidence for an induced fit mechanism (236).

The picture was further complicated by investigations of the monomer $\rightleftharpoons$ dimer equilibrium in the native enzyme, and the existence and interconvertibility of dissociable and non-dissociable forms (237-239). The monomer $\rightleftharpoons$ dimer equilibrium was shown to be temperature dependent, and the association of a hydrophobic nature (233), but various factors affecting the position of the equilibrium were found to be irreproducible with different samples (237). A dissociable form of the enzyme was found which monomerized on dilution, but a heat treatment (70°C for 5 minutes; 240, 241) in the isolation procedure favoured a much more stable dimeric form (237). This non-dissociable form could be converted into the dissociable form by several months of storage frozen. Increased salt concentrations stabilized the dimer at higher protein concentrations, and phosphate buffer (30 mM) specifically protected against monomerization upon dilution. Only the dimer (indicated by light

scattering) had DHase activity, and loss of this activity upon monomerization is not due to denaturation (as shown by ORD measurements; 238). Lowering the dielectric constant of the medium also favoured dissociation, even of the (relatively) non-dissociable form. This effect is increased by both high phosphate concentration and low temperature (237). The form present *in vivo* is not known. Despite the affect of ionic strength in dilution experiments, prolonged dialysis against NaCl produces dissociation, and this, coupled with stabilization of dimer from dissociation and of apoenzyme from denaturation, by phosphate, leads to the conclusion that the protein is stabilized by one or more phosphate bridges, which are slowly exchanged for chloride. No spectral (ORD, CD, absorption or fluorescence) differences were observed between the dissociable and non-dissociable forms of the dimeric enzyme, however both prolonged dilution and apoenzyme preparation result in monomeric species with an increased α -helical content (238). Fluorescence intensity reveals an increase in protein fluorescence upon dissociation due to exposure of at least one of the tryptophyl residues per subunit, while energy transfer to flavin is enhanced. Two-stage binding of sodium dodecyl sulfate to lipoamide dehydrogenase is also observed, the first phase being merely hydrophobic in nature and having no spectral or kinetic effect, the second phase being poorly understood, and associated with monomerization (239).

The serious investigation of pyridine nucleotide binding sites began with detection of a (flavin) visible difference

spectrum in the presence of NAD^+ , with a dissociation constant for the complex found from titration to be $K_d = 1.5\text{--}2.0\text{ mM}$ (242). The discrepancy between this value and the competitive NAD^+ inhibition constant, $K_{ia} = 0.2\text{ mM}$ (224), was thought to reflect the difference of the K_d for oxidized and reduced enzyme. Above 5 mM NAD^+ , binding of more than one nucleotide per enzyme subunit was indicated (242). Two pairs of nucleotide binding sites at pH 7.6 and $T = 25^\circ\text{C}$ were tentatively confirmed, but one pair was lost at 5°C (243). A transition temperature of $\sim 15^\circ\text{C}$ was found. Spectral evidence was also obtained that lipoamide forms an abortive ternary complex with the enzyme, having NAD^+ bound at the low affinity site.* A K_{ia} value for NAD^+ in the DHase reaction of 0.2 mM at 25°C is noted, and the two pairs of binding sites were hypothesized to represent duplicate systems of two sites per subunit, with the following distinction (243). One site had high NADH affinity and low NAD^+ affinity, and was termed the catalytic site. The other had low NADH affinity and high NAD^+ affinity, and was termed the regulatory site. This picture was satisfying, remembering the proposal of Massey and Veeger (216) that NADH was bound to the disulfide and NAD^+ to the (regulatory) Y-site. A more complete investigation (245) revealed that the ratio A_{430}/A_{450} of the difference spectrum changed with NAD^+ added (indicative of multiple sites), but disturbingly, linear Scatchard plots were obtained only at the arbitrarily selected wavelength of 430 nm . Utilizing this

* NADH itself was found (kinetically) to form a dead end complex with EH_2 for the rat liver mitochondrial enzyme (244).

wavelength, it was found at 25°C that NAD^+ sites of $K_d = 50-60 \mu\text{M}$ (high affinity) and $K_d = 200-250 \mu\text{M}$ (low affinity) existed at pH 7.2, but at pH 5.6 only the high affinity site remained. In view of the catalytic activity of the enzyme at pH 5.6, the assignment of a catalytic role to the low affinity site exclusively was puzzling, and this situation is further complicated by the facile full reduction to EH_4 by NADH at this pH. It was noted that NAD^+ binding favours the dimer (237), and conditions favouring monomerization favour also full reduction. As the temperature is lowered, a change in FAD environment occurs which favours EH_4 formation, even in the presence of NAD^+ . The presence of NAD^+ drops the pH optimum of the DHase reaction from 6.2 to 5.6, while activating the reaction, and this was shown (245) to be accompanied by release of a proton as binding of NAD^+ to the high affinity site causes the pK_a of an active centre (presumably) residue to drop from 6.2 to ~ 5 .

Following the slight quenching of flavin fluorescence upon NAD^+ binding, Su and Wilson (246) also report the existence of at least two binding sites at room temperature and neutral pH, for both NAD^+ and 3-AP- NAD^+ . TNAD^+ , however, shows monophasic binding. Raising the temperature to 37°C, as well as lowering it to 5°C, caused loss of one site. These authors find a K_d for the high affinity site of 0.2 mM, however, equal to the K_m for NAD^+ in the reverse DHase reaction (243), and consider that the high affinity NAD^+ site is really the catalytic site. An attempt to resolve this dilemma appears in the present work. Kinetic

evidence in support of a regulatory role for NAD^+ appeared in 1973 (247), with demonstration of negative cooperativity by NAD^+ in the reverse DHase reaction.

Scouten *et al.* (248) have immobilized lipoamide dehydrogenase by diazonium coupling to porous silica beads, and observed co-immobilization of NAD^+ near the regulatory site. This was shown by removal of the requirement of NAD^+ for full DHase activity, and a return of that requirement upon NADase treatment. Lowe (249) immobilized the enzyme by thiol-disulfide interchange on sepharose, and found little change in properties due to immobilization except that the sensitivity to Cu^{2+} was greatly decreased. Probably the susceptible sulphydryls were protected by linkage to the matrix. When spacer molecules were introduced (250), the thermal stability of the enzyme decreased, indicating that proximity to the matrix had a stabilizing effect on conformation. When the enzyme was polythiolated by reaction with N-acetylhomocysteine thiolactone prior to immobilization, both stability to heat and organic solvents were enhanced (probably due to disulfide crosslinks), and this stability was further increased by proximity to the matrix (251). This stability reflects a conformational rigidity which apparently accounts for the greatly reduced DHase activity of this species, however.

The first evidence that the two subunits, or at least the two flavin environments, might not be identical, came from fluorescence lifetime studies (252). While free FAD at 20°C had been shown to have a single decay time (from the open con-

formation) of 2.8 ns at 520 nm (253), native enzyme gave two decay times of 0.8 and 3.4 ns. These occurred with equal weighting, indicating that the two flavins per dimer were in different environments. In addition, free FAD displays a strong temperature dependence of its lifetime which the enzyme does not, and it is concluded that changing conformations do not play a part in the biphasicity of the enzyme-bound flavin's decay. Energy transfer experiments according to Forster's theory indicated that one, and only one, of the two tryptophan residues per subunit form a donor-acceptor complex with the flavin, at a separation of 13-16 Å⁰ (254). This result is confirmed by the lifetime studies (252). The hydrophobic nature of flavin binding and tryptophan interaction was further investigated by reconstitution of apoenzyme with the hydrophobic probes 8-anilidonaphthalene-1-sulfonate (ANS) and 4-benzoylamido-4'-amino-stilbene-2,2'-disulfonate (MBAS); (255). Neither probe interacted with the holoenzyme, but two moles of each dye were bound per mole of apoenzyme, and were competitively displaced upon addition of FAD. The fluorescence of both ANS and MBAS was enhanced upon binding, and their absorption spectra were bathochromically shifted, two facts consistent with transfer to a more hydrophobic environment. The protein fluorescence spectrum was perturbed upon removal of flavin, and returned when ANS and MBAS bound, indicating changes in the environment of a tryptophan residue by introduction of a hydrophobic species into the flavin binding site.

Recent evidence for the non-equivalence of flavin binding sites

has come from reconstitution of apoenzyme with 8-chloro-FAD (256). A new method of apoenzyme preparation (short exposure to 5 M guanidine and removal of FAD by gel filtration) allowed quantitative removal of flavin, and reconstitution with the chlorinated analog resulted in formation of a covalent adduct *via* attack of C(8) by a cysteinyl thiolate anion. A monomeric species resulted which, consistent with the work of Veeger (237-239), had a V_{max} 7400 times lower than native dimer in the DHase reaction.* This monomer could dimerize, but only with another monomer containing FAD in a non-covalent linkage, so the resultant dimer necessarily contained one subunit with covalently linked FAD and one with non-covalently linked FAD. Furthermore, the non-bonded 8-chloro-FAD in the dimer could not form a covalent linkage. The indication is that dimerization requires at least one subunit to undergo a conformational change which alters the relative orientations of flavin and a thiol residue.

Modification of a non-metalloenzyme with metal ions is a particularly promising multifunctional probe. A moderately stable cadmium derivative of lipoamide dehydrogenase has been prepared by Stein and Stein (257). One site of chelation appears to be a sulfhydryl formed upon reduction of the active site disulfide and another may be FAD itself. The flavin spectrum is blue shifted when the complex forms. Cd^{2+} does not chelate across the disulfide

*The covalent dimer was only 61 times slower, and both monomeric and dimeric fractions had K_m values comparable to native enzyme, so the low V_{max} of the monomer would not appear to be due to the nature of the FAD linkage (256).

as does arsenite, however, as the complex also forms when one of the nascent thiols is alkylated by iodoacetamide (*vide infra*). The 700 nm charge transfer band which forms on reduction with NADH in the presence of arsenite (223) also forms with the cadmium derivative, but is lost in the alkylated species. The Cd:FAD stoichiometry is 1:1. Most significant in this work are the effects of the modification on the multifunctional activities. DHase activity is inhibited, while THase is decreased to ~20% that of native enzyme. ETase is largely unaffected, while DPase and quinone reductase activities (the latter measured by reduction of aurantiogliocladin) increase 2.5 to 3-fold, results consistent with the conclusions of the present work (see Discussion). It is concluded that the DPase reaction is just a special case of quinone reductase activity.* The importance of looking at all activities is illustrated by this work. It has been seen above that partial denaturation causes loss of DHase activity concomitant with increases in DPase, and in the cadmium work ETase is a valuable "internal-standard". Thus Thorpe and Williams (260) have shown guanidine hydrochloride treatment to cause loss of DHase and THase activities, but increases in DPase.

* Stein and Stein had previously shown (258) the ability of lipoamide dehydrogenase to reduce a wide range of quinones, and the quinoid-like structure of DCIP is obvious. In the present work, the identity of DCIP and quinone reductions is assumed. An interesting though unexplained point is the observation that while non-enzymatic reduction of quinones by NADH requires two unsubstituted ring positions, the rapid enzyme catalyzed reaction requires only one. Fully substituted quinones (such as the coenzyme Q series) are not reduced even enzymatically at significant rates (258). The choice of the fully substituted aurantiogliocladin as quinone substrate for the cadmium derivative is puzzling, and may reflect its 407 nm absorption (259).

The fate of the ETase reaction under such conditions would have been interesting.

Huang and Brady (261) examined a series of divalent metal ions, including Mn(II), Ni(II), Fe(II), Zn(II), and Co(II), and found specific complex formation with lipoamide dehydrogenase with only the last two. Only the cobalt species was studied in detail due to induced spectral changes of the flavin, as well as the colour of cobaltous ion itself. A new band at 650 nm in the 1:1 cobalt:flavin complex was assigned to a d-d transition of cobalt. Optical activity is missing in the 450-nm region of lipoamide dehydrogenase (unusual for a flavoprotein; 262), but is conferred upon the enzyme by Co(II) and Zn(II) complexation (261). The authors suggest tentatively that, from the multifunctional behaviour of the enzyme, cobalt is not chelated by the disulfide thiols, but rather by FAD and an unidentified protein ligand. DHase activity was missing, and both THase and DPase were decreased, but the potentially interesting ETase activity of this artificial metallo-flavoenzyme was again not investigated. The cobalt derivative formed only with the NADH-reduced enzyme, the first chemical evidence that this species is different from that produced by dihydrolipoamide reduction.

The multifunctionality of the Cu^{2+} treated enzyme was sufficiently interesting to stimulate further investigations in which the mechanism of copper inactivation was studied (263), although again only loss of DHase and increase of DPase activities were confirmed. It was shown that two moles of cupric ion bind adjacent to two thiols, probably at histidine binding sites. An

intermediate with the two copper atoms bridged by the two sulfurs breaks down to yield two cuprous ions and a disulfide. The cuprous ions are reoxidized by O_2 , probably prior to release from the enzyme. One of the oxidizable sulfhydryls belongs to an isolable peptide containing two histidine residues. (264).

While the enzyme has not yet been fully sequenced, and has eluded attempts at crystallization, various cysteine-containing peptides have been isolated and sequenced, by Williams' group (264-266), and by Brown and Perham (267,268). This work has been useful in several regards. First, the peptide containing the active site disulfide has been shown to be identical for the PDC (cytosolic) and α -KGDC (mitochondrial) derived enzymes (267), supporting the view of primary structure identity for these species. Bacterial and animal sequences were shown to be similar (268), and the disulfide forms an intrachain loop of six amino acid residues (267,268). This relatively small loop makes it likely that the protein conformation in this region is flat, and the hydrophobicity of the residues involved (-S-Cys-Leu-Asn-Val-Gly-Cys-S-) introduces the possibility that the observed hydrophobicity of the FAD binding site results from stacking of the isoalloxazine ring over this flat portion (265). The identity of this grouping in diverse organisms may reflect the rigid requirements, both catalytic and spatial of this active centre, which must not only harness the activity of the FAD cofactor but also interact in a well defined complex (265). Indeed, of seventeen overlapping residues for this active centre peptide, fourteen are homologous between the *E. coli* and pig heart

enzymes, the longest homology known for analogous peptides from prokaryotes and eukaryotic mitochondria (266).

The enzyme was reviewed a second time by Williams in 1976 (198). The involvement of a lysine residue in both forward and reverse DHase reactions was suggested. This accounted, furthermore, for the difference in pH optimum of the two reactions. In the forward direction the transition state is stabilized by interaction with an -NH_3^+ group (Scheme I(a)), and in the reverse direction, an -NH_2 group acts as a general base catalyst (Scheme I(b)).

Scheme I: Proposed transition states of the forward (a) and reverse (b) DHase reactions; from Williams (198).

Here no structure of EH_2 is intended. Williams states that this "problem seems to rest with the molecular orbital theorist". In subsequent papers, an attempt has been made to probe the active centre further and elucidate the nature of the catalytic base (269-272). Modification of EH_2 with stoichiometric amounts of iodoacetamide (269) resulted in modification of a single, reactive, nascent thiol. This supports the hypothesis implicit in Scheme I

that one of these thiols has a substrate binding role, and that the other interacts with flavin in the reduced species, the two being non-equivalent. It is not clear which nascent thiol is alkylated, as no "EH₂-like" intermediate forms (i.e. reduction is exclusively of FAD, as expected). This could be due to alkylation of the interacting thiolate, or to removal of this thiol from interacting distance by conformational relaxation upon alkylation of the substrate binding thiol (269). Either alkylation should eliminate the NADH-dependent disulfide reduction (DHase reaction), as observed. The THase reaction was found to be slightly enhanced by the modification, accompanied by a slightly blue-shifted flavin spectrum. Addition of NAD⁺ to the mono-alkylated enzyme resulted in an increase in the absorption at 380 nm (270), characteristic of formation of a covalent flavin C(4a) adduct, and it has been tentatively proposed that such an adduct, stable only when the redox partner of the nascent thiolate is alkylated, may be catalytically significant in the native enzyme.

Stopped flow kinetics (271) further implicated a catalytic base, which was thought to be an imidazole ($pK_a \approx 7.9$) and accept a proton in EH₂, leaving a thiolate to form a charge transfer complex with FAD. This base is then catalytic with respect to the reverse DHase reaction (i.e. reduction of enzyme by dihydrolipoamide) according to Scheme II.

Scheme II: Possible catalytic role of an active centre, base; from Mathews *et al.* (271).

Changes in absorption of EH_2 , and in redox potential as functions of pH have indicated a pK_a of ~ 5 for the flavin-interacting nascent thiol (272). This low pK_a is thought reasonable for a base pair of the type $-\text{S}^{\ominus} \cdots \text{H}^{\oplus}-\text{B}-$ (272).

The above studies have all been performed on a mixture of isozymes obtained from one or both of the PDC and α -KGDC systems. In 1965, Stein and Stein (258) isolated three components of the Straub diaphorase on a diethyl aminoethyl (DEAE)-cellulose ion exchange column which were further resolved by analytical electrophoresis into more than thirteen components. Koike and co-workers (273,274) isolated the pig heart enzyme from the PDC and α -KGDC, as well as from an extract devoid of the two complexes. Two enzyme forms were found, by triethyl aminoethyl (TEAE)-cellulose anion exchange chromatography and gel electrophoresis, the one with the higher anodic mobility derived from the PDC. The complex-free extract gave both forms, and likely resulted from *in vivo* or artifactual dissociation of the complexes. Ultra-

centrifugal analysis, peptide mapping, and immunological experiments revealed no difference between the two forms. Either form could be used in reconstitution of either complex. Comparison of thiol content, CD and ORD spectra, and slight differences in electrophoretic patterns after incubation with NADH and arsenite indicated that the two forms differed slightly in active centre conformation involving thiol groups and FAD. Cohn and McManus (275) then found (five or) six forms of the enzyme, three anodic species from the PDC having isoelectric points between a pI of 5.6 and 6.0, and three less anodic forms with pI values of 6.5 to 6.8, by electrofocusing and gel electrophoresis. This indicates differences in net charge between the two groups. Peptide mapping and amino acid analysis revealed no differences in the six forms, and they also concluded that the forms differed only in conformation. Isolation procedures and incubation times caused the relative amounts of the forms to vary, but never less than three could be obtained from one complex. DHase and THase activities and pH optima were similar for isozymes derived from both complexes, and both groups were equally sensitive to Cu^{2+} and p-chloro-mercuribenzoate modification, but the PDC isozymes were more strongly inhibited by the sulfhydryl reagent dithio-bis-(2-nitrobenzoic acid), DTNB, in the presence of NADH (276). Kenney has confirmed the presence of six isozymes (277), and suggests that the previous values of two to thirteen resulted from poor resolution or preparative artifacts. Visser and Strating (278) have separated the isozymes by differences in hydrophobic

interactions with alkylated Sepharose. It may be significant that the isozymes of the PDC are more strongly hydrophobically interacting in this experiment, and that a higher urea concentration is needed to dissociate the pyruvate dehydrogenase complex.

To complete this survey of the work on lipoamide dehydrogenase, we turn to the nature of the enzyme in the intact multienzyme complexes. That the lipoyl moiety must interact with both CoA at a site on the transacetylase molecule to which it is bound, and with FAD on lipoamide dehydrogenase, has been established by the structural and kinetic work of Reed and coworkers (279-281). The lipoyl residue is attached to a lysine residue by an amide linkage (282), leaving the dithiolane ring at the end of a flexible chain of 14 Å, free to rotate to the various catalytic centres (283), the so-called "lysine swinging arm". From mammalian sources, the PDC has a molecular weight of approximately 10^7 daltons and consists of a core of sixty acetyltransferase units, each with one covalently bound lipoate (281). With this core are associated thirty pyruvate decarboxylase molecules and twelve lipoamide dehydrogenase molecules. The pyruvate decarboxylase is tetrameric, of subunit structure $\alpha_2\beta_2$, the α and β subunits having molecular weights of 4.1×10^4 and 3.6×10^4 respectively. The α subunit may catalyze the actual decarboxylation step, while the β subunit may transfer the resultant acetyl group to lipoamide (281, 284). In addition, the complex contains pyruvate dehydrogenase kinase, which catalyzes the ATP-linked phosphorylation of the α subunit of pyruvate dehydrogenase (284, 285) and inhibits the reaction sequence. A fifth enzyme

is also present (284), pyruvate dehydrogenase phosphate phosphatase, which catalyzes removal of the phosphate group and reactivation of the complex. Phosphorylation of the α subunit appears to inhibit formation of the two-carbon adduct of thiamine pyrophosphate (284). The site of phosphorylation has recently been shown (286) to be an active seryl residue, and the regulatory effects of various substrates of the complex upon the kinase have been investigated (287). Much sophisticated structural work which has elucidated the geometry and mechanistic consequences of the simpler complex from *E. coli* is not relevant to the present study (but see 288-292).

MATERIALS AND METHODS

1) Enzymes

Lipoamide dehydrogenase (porcine heart, EC 1.6.4.3) was obtained from Sigma Chemicals Co. (Lot no. 94C-0212-3 used throughout) or Boehringer-Mannheim (Diaphorase, gr. 1; Lot no.'s 1248222 & 1458322). These preparations were suspensions in ammonium sulfate, and were desalted for use by dialysis against appropriate buffers at 4°C, or by gel filtration on Sephadex G-25 or Bio-gel P-2 columns (see below for column preparation). When indicated, further purification was carried out on a calcium phosphate gel cellulose column according to Williams *et al.* (241). The enzyme was run onto the column (2.8 x 15.5 cm, flow rate 200 ml.hr⁻¹) in potassium phosphate buffer (0.10 M, pH 7.00, 4°C) and washed through with 75 ml of the buffer, which caused the enzyme to bind tightly as a yellow band at the top of the column. The enzyme was then eluted with the same buffer containing 4% (w/v) (NH₄)₂SO₄ and 0.3 mM ethylene diamine tetraacetate (disodium salt; EDTA).

Other enzymes were used as obtained. Glutathione reductase (Type III, Yeast; EC 1.6.4.2), alcohol dehydrogenase (horse liver; EC 1.1.1.1), NADase (*Neurospora*, 1.4 u.mg⁻¹; EC 3.2.2.5), and cytochrome c (horse heart, Sigma Type III) were all from Sigma Chemical Co. Catalase (EC 1.11.1.6) was from Worthington

Biochemical Lab.

2) Modification reagents

Iodoacetic acid was from Sigma Chemical Co., iodoacetamide from Pierce Chemical Co. 1- ^{14}C -Iodoacetic acid was a product of New England Nuclear Corp. Methyl acetimidate hydrochloride was from Pierce Chemical Co., 1,2-cyclohexanedione and phenylglyoxal monohydrate from Aldrich Chemical Co. Monopotassium phosphoramidate was synthesized by Paul Inwood from phosphorous oxychloride, following the method of Stokes (293). A nitrogen content of $(10.6 \pm 0.3)\%$ (theoretical 10.37%) and an infrared spectrum matching the literature (294) were found. Bifunctional imidoesters used were dimethylsuberimide dihydrochloride and dimethyladipimide dihydrochloride from Pierce Chemical Co., and dimethylsuccinimide dihydrochloride and dimethylmalonimide dihydrochloride synthesized by Don McRae following McElvain and Schroeder (295). Spin labels were 4-(2-acetamido)-2,2,6,6-tetramethylpiperidinyloxy and 3-[2-[2-(iodoacetamido)ethoxy]ethyl] carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, from Synvar, Palo Alto. All reagents were used as received.

3) Biochemicals

Lipoic acid and lipoamide were products of Sigma Chemical Co. Their reduced forms were synthesized by Dr. C.S. Tsai, following the method of Reed *et al.* (296). β -NAD and β -NADH (Yeast, gr. III),

NADP (Type I), and NADPH (Σ grade) were all from Sigma, as were FMN (Commercial grade) and FAD (gr. III). Riboflavin was from Eastman Organic Chemicals. TNAD and 3-AP-NAD were from Sigma and United States Biochemical Corp., respectively. 1,N⁶-etheno-NAD (eNAD) was a product of P-L Biochemicals, and L-arginine monohydrochloride was obtained from Mann Biochemical Corp.

4) Miscellaneous chemicals

The remainder of the chemicals used are listed here. From Fisher Scientific Co. were obtained EDTA (ACS certified), Na₂S₂O₄ (Lab. Grade), DCIP (ACS certified), H₂O₂ (30%), nitrobenzene, sodium benzoate (reagent), ammonium persulfate (ACS certified), 8-hydroxy-quinoline (reagent), rose bengal (80% pure), CaO (reagent), Br₂ (reagent), CoSO₄·7H₂O (certified), NaAsO₂ (reagent), and CuCl₂·2H₂O (certified). From J.T. Baker Chemical Co. were obtained sucrose (reagent), acrylamide, HCl, and H₃PO₄ (acids analyzed reagent). 2-Chloroethanol and β -mercaptoethanol were from Eastman Organic Chemicals. From BDH chemicals came K₃Fe(CN)₆ (analytical reagent). Urea and NH₂OH·HCl (both reagent) and methylene blue chloride (84% pure) were products of Anachemia Chemical Co. Sigma Chemical Co. supplied N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine, and p-chloromercuribenzoate. Thiophenol was from Aldrich Chemical Co., absolute ethanol from Consolidated Chemicals, and H₂SO₄ (ACS

reagent) from Allied Chemicals. Ammonium sulfate (special enzyme grade) was from Mann Biochemical Corp.

5) Buffers

All chemicals used for buffers were from Anachemia Chemical Co. or Fisher Scientific Co. and were ACS reagent grade. Specific buffers are described below as used. Buffers were prepared by making acid and base components to the final concentration and titrating one against the other. Titrations were done with a Radiometer PHM 62 digital pH meter standardized with Anachemia standard buffers, and read to ± 0.01 pH unit. Due to the sensitivity of lipoamide dehydrogenase to trace metal ions, all buffers were prepared with deionized water obtained from a Millipore Milli-QTM water purification system, at a measured resistivity of $18 \text{ M}\Omega \cdot \text{cm}$ (best experimental value for pure H_2O , $23.3 \text{ M}\Omega \cdot \text{cm}$ (297)). This water is termed ultrapure. In early experiments, 2x glass distilled water was used, with no change in any measurable enzyme parameter. For samples used in kinetic measurements, 0.3 mM EDTA was included in the buffer.

6) Protein determinations

Lipoamide dehydrogenase concentrations were determined spectrophotometrically on a Cary 14 spectrophotometer, assuming a molar extinction coefficient of $11.3 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ at 455 nm (225).

When the protein concentration was too low for significant determination by this method, rough concentrations were found assuming $A_{280}/A_{455} = 6.9$ (see Results). When the flavin content was uncertain, as in monomeric preparations, the micro-biuret assay was used, according to Bailey (298). The biuret reagent was prepared as follows. Sodium citrate (173g) and sodium carbonate (100 g) were dissolved in warm water, to which was added copper sulfate solution (17.3 g in 100 ml water). The solution was diluted to 1 litre. To 4 ml of protein solution (0.1 to 2 mg in 3% NaOH) were added 0.2 ml of this reagent, and the absorbance at 330 nm read after 15 minutes in a Beckman DB Spectrophotometer. Protein concentration is determined from a standard curve prepared with known quantities of bovine serum albumin. Beer's law was obeyed below a concentration of $\sim 1 \text{ mg ml}^{-1}$. A value of $\epsilon_{330} = 0.96 \text{ ml.mg}^{-1}.\text{cm}^{-1}$ was calculated in this range.

7) Determinations of specific amino acids

Free sulfhydryl groups were obtained by mercurial titration following the method of Boyer (299). p-Chloromercuribenzoate (PCMB) was dissolved in 1.0 M NaOH and made up to $5 \text{ } \mu\text{moles.ml}^{-1}$ with water. The concentration was checked assuming an extinction coefficient at 233 nm of $1.7 \times 10^4 \text{ M}^{-1}.\text{cm}^{-1}$. Compartmentalized difference cuvettes were used, the reference and sample cells both containing 1.00 ml of acetate buffer (0.50 M, pH 4.5) and of protein of a known concentration ($1\text{-}2 \text{ mg.ml}^{-1}$) in the same buffer, in separate

compartments. Aliquots (10 μ l) of PCMB reagent were added to the protein of the sample cell and buffer of the reference cell, and in addition 10 μ l of buffer was added to the protein of the reference cell to correct for dilution. By this difference spectroscopic technique, absorption of reagent and protein at 255 nm are automatically subtracted. The absorbance at 255 nm was recorded on a Cary 14 spectrophotometer, and represents only absorption due to the product complex. Titration was continued until no further change in absorbance was seen, and then the mole ratio of PCMB to protein was calculated.

Arginine residues (guanidino groups) were determined by the Sakaguchi procedure, adapted from Litwack (300). Both lyophilized protein and hydrolyzed samples of about 5 mg were used. Dry samples were dissolved in 4 ml of 2x glass distilled water in 17 x 150 test tubes. To each was added 1.0 ml of 10% NaOH and 1.0 ml of 0.02% 8-hydroxyquinoline, the tube shaken, and placed on ice for 10 minutes. NaOBr was prepared fresh by addition of 0.7 ml of Br₂ to 100 ml of 5% NaOH, and 4 drops of this reagent added to each tube by Pasteur pipette to stop the reaction. The tubes were shaken for 15 seconds, whereupon 4 ml of n-butanol (redistilled) were layered on top over the next 10 seconds. Butanol extraction of the red-orange uncharacterized condensation product was carried out following the method of Griotti and Spandrio (301), to avoid destruction of the coloured complex (cf. 302). The tubes were shaken, the butanol layer cleared with 0.2 ml absolute ethanol, and the absorbance read at 512 nm with a Beckman DB Spectrophotometer. A

standard curve of 0.01 to 0.15 μ moles of L-arginine monohydrochloride was prepared, and was linear in this concentration range and extrapolated to zero.

Determination of free amino groups was carried out by titration with trinitrobenzene sulfonic acid (TNBS), a reagent specific for $-\text{NH}_2$ groups of lysine and the N-terminus (303-306). Recrystallization of TNBS from hot 2 M HCl gave the white crystals of the trihydrate, after Fields (304). Samples were diluted in 1.00 ml (final volume) of 0.10 M $\text{Na}_2\text{B}_4\text{O}_7$ in 0.10 M NaOH. Then 0.020 ml of 1.1 M TNBS, stored frozen for up to one week, was added with rapid mixing and let stand at room temperature for 5.0 minutes, at which time the reaction was stopped by addition of 2.0 ml of 0.10 M NaH_2PO_4 containing 1.5 mM Na_2SO_3 (prepared fresh daily). The absorbance was read at 420 nm on a Cary 14 spectrometer, against a blank treated identically but from which protein had been omitted. Duplicate assays of at least three on-scale dilutions of a given value was read from the interpolated lines. Due to the range of extinction coefficients for the complex formed with various lysine-containing peptides (cf. 304), the reaction was allowed to go to completion (45 minutes), and the resultant absorbance attributed to the literature value of 36 lysine residues + 1 N-terminus. This allowed calculation of an average value for ϵ_{420} of $22.7 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in lipoamide dehydrogenase, which is in the expected range (304). Subsequent concentrations were calculated based on this value, and are thus sensible and correct qualitatively.

To determine rates of reaction, the samples were diluted x 2

with the borate buffer. Addition of 0.020 ml of 0.10 M $\text{Na}_2\text{S}_2\text{O}_3$ preceded addition of the same volume of TNBS. The change in absorption (read against a blank devoid of protein) was followed for 45 minutes. A multiple exponential increase (i.e. non-linear semi-log plots *versus* time) was observed, indicating reaction of a number of distinct residues, but the number reacted at a given time could be estimated as a percentage of the total number reacting.

8) Amino acid analysis

Protein samples used for amino acid analysis were initially purified on the calcium phosphate gel-cellulose column, as described above. Following modification, flavin was removed by a modification of the procedure of Veeger and Visser (*vide infra*), the pH being lowered to 1.3 as more extensive denaturation is unimportant here and leads to more efficient removal of flavin. Three repetitions were used. Some samples were analyzed without removal of flavin, and this did not interfere with the determinations. The protein was then desalted by extensive dialysis against ultra-pure water and lyophilized.

Protein (1-3 mg) was dissolved in 1.0 ml of 6N HCl in heavy walled Pyrex tubes with constrictions. The sample was then frozen in a dry ice-acetone or refrigerated methanol bath and evacuated with a mechanical pump (<1 torr for 5 minutes). The tubes were sealed under vacuum and incubated at 110°C for 24 hours. The tubes

were then cooled to $\sim 30^{\circ}\text{C}$, opened, and the contents evaporated to dryness on a water aspirator. The residue was taken up in 1 ml of ultrapure water, frozen, and lyophilized.

Amino acid analyses were performed on a Beckman Model 121-M Amino Acid Analyzer by Mr. Alex Costaigne (NRC) or on a Durrum Model D-500 instrument by Dr. M. Yaguchi (NRC) and on a Beckman 119 BL instrument by Mrs. Cathy Shay (Carleton University). The Beckman instruments were equipped with 9 mm(id) ion exchange columns for loading 100 μl sample volumes, and had detection limits of ~ 10 nmoles, whereas the Durrum instrument used a 1.75 mm column, accepted 10 μl of sample, and had a detection limit down to 0.1 nmoles. All instruments had automatic peak integration and colour correction.

9) Chromatographic techniques

The calcium phosphate gel cellulose column was prepared by the method of Kosicki (307). Sucrose (45.3 g), CaO (7.5 g), and 200 ml of water were stirred overnight. The mixture was filtered, cooled to 4°C , and 6.0 ml of H_3PO_4 (85%) was added dropwise with stirring. The precipitated gel was collected by centrifugation (10 minutes at $4000\times g$) and washed twice by resuspension in 200 ml of water, to a final pH of 7.2. An aliquot of the washed gel was oven dried to determine the concentration, and the suspension then diluted to $30\text{ mg}\cdot\text{ml}^{-1}$ with water. Whatman CF 11 cellulose powder (20 g in 200 ml of water) was dribbled into

100 ml of the calcium phosphate suspension, with stirring, at room temperature. The calcium phosphate cellulose gel was deaerated on an aspirator and poured into a glass column (15.5 x 2.8 cm) at 4°C. Equilibrated with potassium phosphate (0.10 M, pH 7.6), the flow rate was 200 ml.hr⁻¹.

Sephadex G-25 (Pharmacia) cross-linked dextran beads, and Bio-Gel P-2 (Bio-Rad) porous polyacrylamide beads, were used for desalting enzyme in Pharmacia K 9/15 columns (9mm x 15 cm). For molecular weight determinations, Bio-Gel P-100 columns of various sizes were used, as indicated. Void volumes were determined with Blue Dextran 2000 (Pharmacia), and internal volumes with a small amount of methylene blue, or by monitoring the conductivity of the eluent (Radiometer Conductivity Meter, Type CDM 2d) after application of 0.5 ml 1 M NaCl. Standard proteins were used as for electrophoresis.

All gel materials were added to excess buffer at 90°C and hydrated for a minimum of 4 hours, while cooling to room temperature. The suspension was then deaerated on a water aspirator and solvent decanted to leave a thin slurry, which was then poured into a column half full of buffer. When the gel had settled to about 20% of the final bed volume, the column was allowed to flow as the remainder of the gel was added. Columns were run at 4°C. Fractions were collected with an LKB model 7000 Ultrarac Fraction Collector equipped with an 8300 Uvicord II flow system monitoring protein concentration of the eluent by absorption at 280 nm.

10) Kinetic assays

All kinetic studies were carried out in 0.05 M potassium phosphate at 25°C, pH 6.5 or 7.0 as indicated, containing 0.3 mM EDTA. Stock solutions of lipoic acid analogs (10 mM), pyridine nucleotides (2.0 or 5.0 mM), $K_3Fe(CN)_6$ (10 mM) and DCIP (1.0 mM) were prepared fresh daily in 0.10 M buffer and enzyme stock solutions were prepared in the same buffer. Assay mixtures were 0.500 ml ultrapure water, variable amounts of reagents and enzyme as indicated in figure legends and axes, and 0.10 M buffer to 1.000 ml. Reactions were initiated by placing the drop of enzyme solution on the beveled lip of a 1 ml quartz cuvette, inverting twice (Para-film cover), and placing in the sample beam of the spectrophotometer. All measurements were made on a Perkin-Elmer spectrophotometer (Coleman model 124) equipped with a variable output recorder (Coleman model 165) and thermostated circulation maintained at 25°C.

Oxidation (or reduction) of lipoic acid analogs (DHase activity) was followed by the disappearance (or appearance) of NADH at 340 nm ($\epsilon_{340} = 6.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (308)). Transhydrogenase activity (THase) with $TNAD^+$ as acceptor was followed by the appearance of $TNADH$ at 395 nm ($\epsilon_{395} = 11.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (309)). With 3-AP- NAD^+ , the appearance of the reduced form was followed at 365 nm, the value being corrected for the disappearance of NADH at this wavelength ($\epsilon_{365}^{3-AP-NAD^+} = 9.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; $\epsilon_{365}^{NADH} = 3.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; (309)). NADH oxidation (OXase) was followed at 340 nm. Reduction of $K_3Fe(CN)_6$ (ETase) was followed by loss of the yellow colour of this species at 420 nm

($\epsilon_{420} = 1.00 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (308)). Decolourization of DCIP was followed at 600 nm (see Results). Reduction of cytochrome c was followed by the appearance of the reduced form at 550 nm ($\epsilon_{550} = 29.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (308)). Cytochrome c concentrations were based on a molecular weight of 1.24×10^4 daltons (308).

Regression lines for linear regions of the kinetic plots were obtained using a packaged program REGRESS.TSL, available from the Carleton University Computing Centre.

When single point assays were performed, conditions were as above, with the following concentrations unless otherwise specified:

DHase; $[\text{NAD(H)}] = 50 \text{ } \mu\text{M}$, $[\text{lipoic analog}] = 250 \text{ } \mu\text{M}$, enzyme = $2.5 \text{ } \mu\text{g}$

THase; $[\text{NADH}] = 50 \text{ } \mu\text{M}$, $[\text{analog}] = 125 \text{ } \mu\text{M}$, enzyme = $0.25 \text{ } \mu\text{g}$

OXase; $[\text{NADH}] = 125 \text{ } \mu\text{M}$, enzyme = $5 \text{ } \mu\text{g}$

ETase; $[\text{NADH}] = 50 \text{ } \mu\text{M}$, $[\text{K}_3\text{Fe(CN)}_6] = 250 \text{ } \mu\text{M}$, enzyme = $5 \text{ } \mu\text{g}$

DPase; $[\text{NADH}] = 50 \text{ } \mu\text{M}$, $[\text{DCIP}] = 25 \text{ } \mu\text{M}$, enzyme = $5 \text{ } \mu\text{g}$

Cytochrome c Reductase; $[\text{NADH}] = 50 \text{ } \mu\text{M}$, $[\text{cytochrome c}] = 40 \text{ } \mu\text{M}$, enzyme = $5 \text{ } \mu\text{g}$

The terminology used in the expression of the enzyme kinetic results is outlined in Appendix I.

The activity of the *Neurospora* NADase was checked as reported (310,311). One vial (1.7 units) of the enzyme was reconstituted with 1.0 ml of water. To 0.3 ml of KH_2PO_4 (0.10 M) were added 0.1 ml of $4 \text{ mg} \cdot \text{ml}^{-1} \text{ NAD}^+$ and 0.1 ml of the enzyme solution. The mixture was let stand at 37°C for 7.5 minutes, then 3.0 ml of 1.0 M KCN was added. The absorbance of the mixture was read at 340 nm against a

blank to which KCN was added at time zero. The activity was calculated using $\epsilon_{340} = 5.15 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the $\text{NAD}^+ \text{-CN}^-$ complex (310,311).

11) Apoenzyme preparation

Apoenzyme was prepared according to Veeger and Visser (227). Enzyme in potassium phosphate buffer (0.03 M, pH 7.2) with 3 mM EDTA was diluted to 1 ml with cold Tris-acetate buffer (1.0 M, pH 8.1) containing 0.10 M EDTA. One ml of 3.0 M KBr in water was added and the mixture let stand on ice for 20 minutes. A saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was acidified to pH 1.9 with concentrated H_2SO_4 , and 1.0 ml added to the enzyme mixture. The precipitated apoprotein is collected by immediate centrifugation at 4°C (10 minutes at 12,000x g).

12) Anaerobic experiments

Buffer solutions were made anaerobic by bubbling N_2 through them for 10 minutes. Since similar treatment results in foaming of enzyme solutions, such solutions were made anaerobic by six cycles of deaeration on a water aspirator followed by flushing with N_2 . Reduced enzyme forms were stable to oxidation overnight after such a treatment. When spectra were to be recorded, preparations were done in a quartz Thunberg cuvette with reagent added from a side arm bulb by inversion. When multiple reagents were to be

added, this was accomplished from a gas tight Hamilton syringe, through a Para-film seal at the evacuation port of the cell. When spectra were not required, or larger volumes used (> 3 ml), a similar procedure was applied to the enzyme solutions in 25 ml Erlenmeyer flasks with side arm reagent bulbs, sealed by a rubber stopper. When transfer of the reduced enzyme was required, as in the anaerobic dialysis or magnetic circular dichroism experiments, this was done in a glove bag flushed with N_2 . MCD samples were maintained anaerobic by insertion of a Teflon plug and wrapping with Para-film before removal from the glove bag. In all cases, the reduced nature of the MCD sample was confirmed by absorption after the MCD run.

13) Chemical modifications

(a) Reductive alkylations: A general procedure was adopted for all such modifications. Enzyme solutions (2-5 mg in 1-3 ml) were made anaerobic as described above, and then reducing agent was added. Unless otherwise specified, the reductions either involved exposure to β -mercaptoethanol (20 μ l per ml of enzyme solution) for 30 minutes or titration with 10 μ l aliquots of fresh 10 $\text{mg} \cdot \text{ml}^{-1}$ dithionite solution to EH_2 spectrometrically. For carboxymethylation (monomer preparation) and related work, excess solid iodinated alkylating reagent (typically 100 mg) was added by inversion from a side arm. For monoalkylation (including attempted spin labeling) stoichiometric amounts of reagent were

added as 10-20 μ l quantities of freshly prepared stock solutions wrapped in foil to exclude light. All modifications were carried out for 30-90 minutes, in the dark, at room temperature, and stopped by either dialysis against phosphate buffer at 4°C or removal of reagents on the desalting gel columns.

(b) Arginine modification: Lyophilized enzyme (5 mg) was dissolved in 1.0 ml of borate buffer (0.05 M, pH 8.7) in a 10 ml beaker and modifying reagent was added. This mixture was let stand on ice or at room temperature for two hours with continual stirring. Reagent was 1.0 or 10 mg of solid phenylglyoxal, or cyclohexane-dione from a stock solution to 1.0 or 10 mM final concentration. Reaction was stopped by dialysis against phosphate buffer (pH 7.0, 0.10 M, 4°C). Controls consisted of identical preparations from which dione reagent was omitted.

(c) Amidination: Desalted enzyme (2.5 mg) was diluted to 1.25 ml with borate buffer (0.20 M, pH 8.5). A stock solution of 150 $\text{mg} \cdot \text{ml}^{-1}$ methyl acetimidate.HCl was prepared by dissolution in the same buffer and titration to pH 8.5 with 4.0 M NaOH. Addition of 100 μ l of this reagent to the enzyme was followed by continuous stirring for up to 90 minutes at room temperature. Aliquots were removed at timed intervals and either assayed immediately without purification, or purified by gel filtration. Reaction was terminated by gel filtration.

(d) Phosphorylation: Enzyme (2.5 mg) was added to 1.00 ml of 40 $\text{mg} \cdot \text{ml}^{-1}$ phosphoramidate in phosphate (0.10 M) or borate (0.20 M) buffers of varying pH. Stirring was continued for up to 2 hours at

room temperature. Assays and termination of reaction were as for amidine samples.

(e) Cross linking: Protein (2 mg) was dissolved in 1.0 ml N-ethyl morpholine.HCl buffer (pH 8.50), and the pH remained at 8.45-8.50 throughout the course of the reaction. The imidoester cross-linking reagents described in Section 2 were added with stirring at room temperature for 1 to 18 hours at room temperature, to achieve variable degrees of cross linking. Either stoichiometric or 2 mg amounts of reagent were added. Attempts to purify the cross-linked species are discussed under Results. The amount of cross-linking was determined by densitometry of polyacrylamide gels. Samples were dissociated by incubation (2 hours, 37°C) in 8 M urea containing 0.1% SDS and 0.1% β -mercaptoethanol. Aliquots were then subjected to SDS-polyacrylamide gel electrophoresis and stained, as described below. To quantitate the gel bands, the gels were extensively destained and scanned using a Beckman 198402 Gel Scanner 2 accessory on an Acta C III Spectrophotometer at 600 nm. Peak integration of the densitometer tracings was performed with a Keuffel and Esser Compensating Polar Planimeter no. 33624.

(f) Cobalt derivative: Enzyme at pH 10 was obtained by dialysis against 0.05 M borate buffer at 4°C. Prolonged exposure to these conditions at room temperature resulted in denaturation, therefore the solution (2-5 mg protein in 2-3 ml of buffer) was immediately made anaerobic upon warming to room temperature, and reduced to EH_2 by addition of a two-fold excess of NADH. Immediately a two-fold excess of CoSO_4 was added. After 5 minutes the stable cobalt

derivative was obtained by dialysis against cold, neutral phosphate buffer (0.10 M).

(g) Arsenite modification: Enzyme (2 mg.ml^{-1}) was held in phosphate buffer (0.10 M, pH 7.0) in a Thunberg cuvette in the presence of 1.0 mM NaAsO_2 . Upon exclusion of oxygen, reduction with excess NADH (usually two-fold) caused formation of the arsenite derivative, which was confirmed spectroscopically. The sample, if anaerobically maintained, was stable overnight.

(h) Copper treatment: Treatment of the enzyme with copper involved incubation of a solution of enzyme in neutral phosphate buffer (0.01 M) with equimolar NADH and 5.0 mM CuCl_2 at 4°C for up to 48 hours. The modified species was then purified by dialysis against the same buffer with omission of NADH and CuCl_2 .

14) Radioactive incorporation studies

These studies were performed by substituting $1\text{-}[^{14}\text{C}]\text{-}$ iodoacetic acid in the reductive alkylation reaction mixtures. The specific activity of the acid was 13.4 mg.mc^{-1} , and this was diluted 1000-fold with cold iodoacetic acid for use. Aliquots of the reaction mixture were withdrawn at intervals, diluted with water, and centrifuged in Centriflo membrane cones (CF25; Amicon Corp.), washed several times with water, and air dried. The whole dry cones were cut and the radioactivity counted in a Beckman LS150 scintillation counter using Aquasol cocktail (New England Nuclear Corp.). Corrections for counting efficiency were

made using external standard ratio ^{14}C quench curves.

15) Photoreactions

Photoreactions were performed on solutions in Thunberg cuvettes, made anaerobic by the methods described above when applicable. The cuvettes were immersed in an open water bath surrounded by a jacketed vessel connected to a circulating Haake water bath thermostated at $25.0 \pm 0.1^\circ\text{C}$. When O_2 concentrations were measured, the Thunberg cuvette was replaced by the apparatus shown in Figure 7. The capillary tube allows volume changes in the solution to accommodate deviations from atmospheric pressure, yet the rate of O_2 diffusion into the solution *via* the capillary is insignificant and does not effect the reading. The oxygen electrode is described below. When photosensitizers were used, they were at concentrations such as to give an absorption of 0.6 optical density units at the visible absorption maximum (cf. 312). Reactions were carried out by illumination of the sample with a 200 watt photo-flood bulb at a distance of 20 cm from the front surface of the bulb, for varying times as indicated.

16) Dissolved oxygen concentrations

Measurements of dissolved oxygen were carried out using a YSI Model 57 Dissolved Oxygen Meter with output to an Electronik 194 chart recorder (100 mV full scale deflection). Since only

Figure 7: Apparatus for the measurement of dissolved O_2 concentrations in photooxidation experiments. A total solution volume of about 3 ml can be used.

relative O_2 concentrations were desired, the meter was routinely standardized to read 9 ppm in air-saturated ultrapure water.

17) Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in vertical glass tubes as described by Smith (313). Individual sample preparations are described with the results. The buffer was sodium borate (0.10 M, pH 8.5). To 140 ml of buffer was added 10 g of acrylamide and 0.4 g of N,N'-methylene-bis-acrylamide. Just prior to pouring were added 0.3 ml of TEMED and 1.25 ml of fresh 10% ammonium persulfate. During polymerization, a drop of water was carefully added to the top of each gel tube to produce a flat surface. Samples containing several crystals of sucrose and 20 μ l of 0.05% bromophenol blue as tracking dye were introduced into the gels in the assembled circular tray. Electrophoresis was performed at a current of 8 mA per tube using an Ortec 4100 Pulsed Constant Power Supply, at 4°C. When SDS was used, it was included in the buffer to a concentration of 0.1% (w/v). For molecular weight determinations, standards used were lysozyme (monomer, 1.43×10^4), α -chymotrypsin (monomer, 2.51×10^4), bovine albumin (monomer, 6.62×10^4), and LADH (dimer, 8.33×10^4). The molecular weights in parentheses are from reference 308 and suppliers specifications. Under these conditions, the monomer and dimer of LADH were seen, as well as n-mers for the unmodified commercial samples with $n = 4, 3$ and 2 for lysozyme, α -chymotrypsin, and bovine albumin respectively.

These n-mers fit on the standard curve, increasing the number of data points obtained.

Protein was visualized using a Coomassie Brilliant Blue stain (1.25 g in 227 ml methanol + 46 ml glacial acetic acid + water to 500 ml) for 30 minutes followed by destaining in a solution of 75 ml glacial acetic acid and 50 ml of methanol diluted to 1 litre with water (314).

18) Isoelectric focusing

Isoelectric focusing was carried out on a LKB 8100-1 (110 ml) column using LKB 1809 Ampholine carriers (polyamino-polycarboxylic acids) of pH 5-8. All experiments were done at 4°C with ultrapure water. A dense gradient solution (15 g of sucrose + 6 ml 1 M NaOH + 10 ml water) was poured into the column. A glycerol gradient containing 2% Ampholine and 25 mg of protein sample (desalted by dialysis) was then formed as follows. To one upright 50 ml graduated cylinder was added 30 ml of 87% (w/v) glycerol in water + 22 ml of sample in water + 2.0 ml of Ampholine solution (40%). To a second, identical cylinder was added 1.4 ml of Ampholine + 52.6 ml of water. Gentle magnetic stirring was started in the first cylinder. While the contents of the first cylinder were drained by gravity onto the column, a teflon Rotaflo stopcock was opened slightly between the bottoms of the two cylinders. The height of liquid in the two cylinders remained roughly the same, producing a linear gradient. With protein amounts in excess of 25 mg, precipitation occurred upon focusing. A light electrode

solution was then layered on top (1.5 ml 1.0 M H_3PO_4 + 8 ml water). Samples were focussed for 48 - 72 hours at 450 V, DC, using the Ortec power supply, with the anode at the top of the column. Elution was performed by pumping water from the top of the column using a peristaltic pump to maintain a constant flow rate of $0.7 \text{ ml} \cdot \text{min}^{-1}$. The protein absorption was recorded as described above, while 140 fractions (25 drops/fraction) were collected. The pH of the fractions was monitored, then pooled isozyme fractions were extensively dialyzed against phosphate buffer (0.10 M, pH 7.0) to remove reagents.

19) Density gradient centrifugation

Sucrose gradients (5-20% in 0.05 M Tris.HCl, pH 7.5) were formed using a Dekastaltic peristaltic pump, with $\text{Rate}_{\text{in}}/\text{Rate}_{\text{out}} = 0.5$ to form a linear gradient. The gradients were formed in polyallomer tubes ($\frac{1}{2}$ " x 2"). By syringe, 100 μl samples of protein (0.5-1.0 mg) were layered on top, and centrifugation carried out for 16 hours at 40,000 rpm on a Beckman L3-50 ultracentrifuge. The tubes were then punctured at the bottom with a needle and 10 or 20 drop fractions collected through the needle. These samples were diluted to 0.5 ml with water and protein determined at 280 nm. Lysozyme, α -chymotrypsin, and LADH were used as standard proteins, and molecular weights were calculated following Martin and Ames (315). The ratio R of the distances travelled by two species (d_1 and d_2) is inversely proportional to their elution

volume ratio (V_2/V_1), which in turn is inversely proportional to the ratio of sedimentation coefficients (S_1/S_2) which varies as the 2/3 root of the molecular weight ratio, i.e.

$$R = d_1/d_2 \propto V_2/V_1 = S_1/S_2 = \left(\frac{MW_1}{MW_2} \right)^{2/3} \quad (2)$$

20) Ultraviolet/visible spectroscopy

Ultraviolet and visible absorption spectra were obtained on a Cary 14 recording spectrophotometer (Applied Physics Corp.). Changeable slide wires allowed full scale deflections corresponding to 0-1.0, 0-0.5, or 0-0.1 absorbance units. All spectra were obtained in quartz cells (Helma) of 1.000 cm path length. To determine difference spectra, cells with a quartz partition were used, each compartment then having a path length of 0.4375 cm. Two cells were placed in the paths of the Cary 14 double beam spectrophotometer, as in Scheme III. To record a difference spectrum of a molecule M bound to enzyme E, 1.00 ml of buffer is placed in compartments 1 and 3, and 1.00 ml of enzyme solution in compartments 2 and 4. Then a known volume of a solution of M is added by syringe to both compartments 1 and 4 and allowed to equilibrate while an equal volume of buffer is added to compartment 2 to correct for enzyme dilution. Solution

Scheme III: Optical arrangement used for difference spectroscopy. See text for details.

concentrations are adjusted so that additions do not exceed 100 μ l, and titrations usually involve 5 or 10 μ l aliquots.

21) Infrared spectroscopy

Infrared spectra were obtained on a Perkin-Elmer Model 225 Grating Infrared spectrometer. All protein spectra were obtained by the method of attenuated total reflectance (ATR) (315,316). Desalted protein samples (1-2 mg) were layered on polyethylene film (4.6 x 3.6 cm) and dried in an evacuated dessicator over P_2O_5 or Drierite ($CaSO_4$) overnight. The polyethylene squares were then cut to size (4.6 x 1.8 cm) and layered against the ATR crystals. In earlier work, a KRS-5 crystal was used (Tl.BrI, transmission range $>250\text{ cm}^{-1}$,

refractive index 2.37). Later an IRTRAN 4 (Perkin-Elmer Corp.) crystal was employed (ZnSe, transmission range $>513\text{ cm}^{-1}$, refractive index 2.41); (see Results). The assembled sample holder was placed in an RIIC TR-25 ATR mirror assembly (Beckman), to give a 60° incident angle and 25 internal reflections. Spectra were recorded in 12-18 hour scans over the range $4000\text{ to }800\text{ cm}^{-1}$.

22) NMR spectra

The NMR spectra of DCIP were recorded on a Varian T60 spectrometer. Solvent was d_6 -dimethylsulfoxide with tetramethylsilane as internal standard (Silanor).

23) Fluorescence spectra

Fluorescence spectra were obtained on a Perkin-Elmer 204S spectrometer in ratio mode, equipped with a Perkin-Elmer Model 56 chart recorder on 10 mV chart expansion, and were not corrected. All spectra were obtained in 3 ml quartz fluorescence cuvettes of 1.000 cm path length. Optical densities of solutions were kept below 0.10 to minimize internal absorption effects. Quantum yields were determined relative to tryptophan standards (see Results), using optical densities obtained on the Cary 14 spectrometer. Transfer efficiencies were determined from the decrease in fluorescence intensity of the donor chromophore, as described more fully in the Results. Transfer distances were

obtained from the Förster equation (318,319).

$$E = r^{-6} / (r^{-6} + R_0^{-6}) \quad (3)$$

$$\text{where } R_0 = (8.8 \times 10^{-25}) n^{-4} Q_a \kappa^2 J \quad (4)$$

E is the efficiency of transfer, r the distance of transfer, R_0 the Förster distance for 50% transfer efficiency, Q_a the quantum yield of the donor, and n the refractive index of the medium. The orientation factor, κ^2 , between donor and acceptor dipoles, is given by (320-322)

$$\kappa^2 = \cos^2 \theta_{AB} - 3 \cos \theta_A \cos \theta_B \quad (5)$$

where θ_{AB} is the angle between donor and acceptor transition dipole moments and θ_A , θ_B are the angles between these moments and the vector connecting the two molecules. The overlap integral J is given by (317,318)

$$J = \frac{\int_0^\infty F(\bar{\nu}) \cdot \epsilon(\bar{\nu}) (\bar{\nu})^{-4} d\bar{\nu}}{\int_0^\infty F(\bar{\nu}) \cdot d\bar{\nu}} \quad (6)$$

$F(\bar{\nu})$ is the fluorescence intensity of the donor as a function of wavenumber, $\bar{\nu}$, in arbitrary units. The $\epsilon(\bar{\nu})$ is the molar extinction coefficient of the acceptor. These integrals were calculated as follows: F and ϵ values were read from the spectra at 5 nm intervals, multiplied together, and multiplied by the reciprocal fourth power of the corresponding wavenumber. The

donor fluorescence spectrum was also hand digitized to a wave-number scale at 5 nm intervals. Both discrete functions were plotted, interpolated by hand, and integrated using the Keuffel and Esser planimeter. The numerator was computer checked for the native enzyme spectrum. The hand-digitized numerator function was fit to a natural spline function by a Fortran program written by S.K. Gray.* The spline coefficients from this program may be read to a subroutine for integration of the function by Simpson's Rule. Taking into account the sixth-root dependence of R_0 on J , the difference between the two results was insignificant (see Results).

24) Fluorescence decay

Fluorescence decay measurements were performed under the guidance of Dr. D. Rayner in the laboratory of Dr. A.G. Szabo at NRC, Division of Biological Sciences. The equipment was built in that laboratory and has been described in detail (323), as have the deconvolution and curve fitting procedures employed (324,325), and an accuracy of more than 0.1 ns is claimed for the calculated decay times (323). The sample is placed in a 3 ml fluorescence cuvette and illuminated with a nitrogen flash lamp at 400 psi, giving nanosecond pulses at 15-30 kHz. Excitation and emission monochromators are used. Emitted photons are collected at 90°

*Present address: University of California at Berkeley, Chemistry Department.

to the incident pulse, and impinge upon a single-photon counting dynode system. The resultant current passes through a discriminator set to correspond to single-photon energies to minimize random pulses, and is used as the stop signal of a time to amplitude converter. The start signal of the converter is the lamp pulse, detected by a fibre optic. The output of the time to amplitude converter is stored in a 1024 channel multichannel analyzer. This resultant time distribution is a convolution of the true sample decay, the lamp pulse shape and the detection system response. A ratio method for determining the latter two functions has been fully described (323-325) and employs a signal derived from a non-fluorescent scattering sample. To correct for lamp drift over the time of signal accumulation (up to several hours), fluorescent sample and scatterer are placed alternately in the light beam for 30 second intervals. This sample alternation, and its synchronization with signal acquisition is performed with a system of stepping motors. Thus half the channels of the multichannel analyzer are devoted to each sample profile. Signal accumulation is continued until at least 10,000 counts have been stored in the peak channel. The data are then transferred to an IBM 360 computer via a remote terminal. The true fluorescence decay profile is then obtained by numerical deconvolution of the measured profile, taking into account the wavelength dependence of both the lamp and photo-multiplier detection system. The true decay profile is fit to an exponential decay model by least squares, with increasing numbers of exponential terms. The degree

of fit of a given model is assessed statistically, the primary criterion being the demand of a random distribution (determined visually from plots) of the residuals, weighted assuming the reciprocal number of counts in a given channel to be the best estimate of the variance. Exponential terms are added to the best-fit equation until the weighted residual plots appear sufficiently random and the weighted sum of the squares of the residuals fails to decrease further on addition of further terms.

25) MCD spectra

Magnetic circular dichroism spectra were obtained on an instrument built by Dr. B.R. Hollebome of this department. The optical arrangement of a Cary 14 spectrophotometer has been modified to allow placing of the sample in the field of a superconducting magnet (Oxford Instruments). In all experiments, the field was 47 kG at the sample. Spectra are acquired automatically using a stepping motor controlled by a Nova minicomputer. Effectively at least 16 values per datum point are accumulated and averaged. At the next level of processing, the digitized stored spectrum is corrected for baseline. A baseline spectrum in the same cell and of the same solvent is recorded each day. Sample and baseline files are rotated to bring them to the horizontal, minimizing distortion due to a badly curved baseline. Rotation is of a line connecting two points of zero MCD. In some instances this rotation was done by hand. The baseline is then subtracted to produce the final

spectrum, which is then plotted on an X,Y-plotter. All manipulations of data are carried out by the Nova computer using a series of programs written by Dr. Hollebone. The instrument was calibrated by Mr. Ed Jones using CoSO_4 standards, and all MCD values $[\Theta]_M$ are expressed relative to a value of $-0.061 \text{ deg.cm}^2.\text{mol}^{-1}.\text{G}^{-1}$, for hexaquo cobalt (II) at 19,800 wavenumbers (326). We follow the convention that a positive MCD corresponds to $\epsilon_l > \epsilon_r$, where these are the extinction coefficients of left and right hand circularly polarized light respectively. In all cases, concentrations are determined from absorption spectra recorded on a Cary 14 spectrophotometer in the MCD cell after the MCD experiment.

RESULTS

I) Purity

The enzyme obtained commercially has been checked for purity prior to use. Massey (225) and Williams (198) have reported A_{280}/A_{455} ratios of 5.35 and 5.5 respectively for the pure flavoprotein. An increase in this ratio would indicate contamination by non-flavoprotein. Purification on calcium phosphate-cellulose as described should separate the lipoamide dehydrogenase from contaminating protein, as well as from the possibly contaminating and similar flavoproteins glutathione reductase and thioredoxin reductase (241). The presence of divalent metal ions, particularly cupric ion, is undesirable, and may result in catalytic modification of the multifunctional activities.

We have found the Sigma and Boehringer preparations currently available to be sufficiently pure for most purposes, by electrophoresis and isoelectric focussing (*vide infra*). When necessary, the enzyme was purified further by calcium phosphate-cellulose gel chromatography, with no significant change in any of the specific activities. The flavin content was not reduced by overnight dialysis against potassium phosphate (0.1 M, pH 7.0, at 4°C), although prolonged dialysis under these conditions resulted in some denaturation with loss of flavin (cf. Section V). The molar absorption ratios determined on a Cary 14 spectrophotometer before and after calcium phosphate gel chromatography indicate

a low degree of contamination by other protein (Table 5). We have found the A_{355}/A_{455} ratio a good indication of degree of denaturation (discussed below) and these values are included in Table 5 for future comparison. Comparison of the latter ratio for free FAD with that for enzyme indicates that an increase in this value upon denaturation is due to a red shift of the strong UV absorption of the protein component. Massey (225) has reported a value of 0.77 for A_{360}/A_{455} in the pure enzyme. The reasons for our slightly higher A_{280}/A_{455} values (as compared to those of Massey and Williams) are not clear, but do not appear significant.

Samples of 0.25 mg of Sigma enzyme taken directly from the bottle (25 μ l) as well as those diluted 2:1 with phosphate buffer were found by atomic absorption* to contain amounts of copper indistinguishable from the background level of 40 ppb, or less than 0.6 μ M copper atoms for an enzyme concentration of approximately 0.1 mM.

II) Chemical Modifications

i) Reductive alkylations:

Haloacetates have long been exploited by the peptide chemist

* Atomic absorption measurements were kindly performed by Geraldine Blanchette of this department, using a Perkin-Elmer 603 Graphite Furnace Atomic Absorption Spectrometer.

Table 5: Absorption ratios indicative of enzyme purity.
 See text for details. Values are for potassium phosphate
 buffer (0.1 M, pH 7.0).

Species	A_{280}/A_{455}	A_{355}/A_{455}
Sigma enzyme	6.90	0.83
Boehringer enzyme	6.93	0.92
Boehringer after CaPO ₄ gel	6.80	0.77
FAD(a)	1.98	0.61

(a) The ratios at the $\lambda_{\max}$ values are $A_{264}/A_{450} = 3.50$ and
 $A_{375}/A_{450} = 0.78$, in phosphate buffer throughout.

as alkylating reagents, and dependent upon the conditions employed, modification of various nucleophilic amino acid side chains may result from displacement of the halogen (303,327). When an enzyme disulfide is present, its reduction will yield two new nucleophilic sulfhydryl groups, and comparison of oxidized and reduced enzyme may be useful.

-Preparation of reductively carboxymethylated enzyme

Reductively carboxymethylated (RCM) lipoamide dehydrogenase prepared by the β -mercaptoethanol/iodoacetic acid sequence shows an increased DPase activity with DCIP as the acceptor substrate, a reduced ETase activity with $K_3Fe(CN)_6$, and a complete loss of DHase and THase activities (Table 6). As reported by Kalse and Veeger (233) for the DCIP-active monomer obtained in the apoenzyme reconstitution sequence, the degree of enhancement of this diaphorase activity was rather variable, and independent of the absolute magnitude of the changes of activities with respect to other substrates, but definitely real. The appropriate Iodoacetic acid or β -mercaptoethanol treated control enzymes showed no detectable effect of any of the activities to result from β -mercaptoethanol pre-treatment alone, and after 30 minutes in the presence of β -mercaptoethanol under anaerobic conditions, the spectrum of the oxidized enzyme remains unchanged. However, a reduction in all measured activities follows extensive carboxymethylation without the thiol reagent pre-treatment. It should be noted

Table 6: Activities of the preparations are expressed as v_i/E_t . The control enzyme was subjected to identical conditions, with omission of reducing and alkylating reagents from the reaction mixture. ICH_2COOH and $\text{HSCH}_2\text{CH}_2\text{OH}$ controls represent reaction mixtures from which $\text{HSCH}_2\text{CH}_2\text{OH}$ and ICH_2COOH were omitted, respectively. For a discussion of errors on all kinetic parameters, see Appendix I.

Activity	Control ^(a) (Native) (10^3 min^{-1})	Control ^(b) $\text{HS}(\text{CH}_2)_2\text{OH}$ (10^3 min^{-1})	Control ^(b) $\text{ICH}_2\text{CO}_2\text{H}$ (10^3 min^{-1})	Reductive Carboxy- ^(a) methylated Monomer (10^3 min^{-1})
DHase	2.8	2.7 ± 0.3	0.35 ± 0.2	0
THase	1.2	-	-	0
ETase	1.2	1.3 ± 0.1	0.3 ± 0.2	0.25
DPase	0.040	0.040 ± 0.005	0.025 ± 0.01	$0.25-1.3^{(c)}$

(a) Average of >10 experiments. The reported values have standard errors of <10%.

(b) 2 experiments.

(c) This value was quite variable. Minimum and maximum values from many preparations are given.

that variable monomerization (as evidenced by gel filtration chromatography) is seen upon carboxymethylation in the control experiment, and that the assayed enzyme solution contains both monomeric and dimeric species. The RCM preparation will be seen to be exclusively monomeric. Figure 8 shows that an increase in DPase activity, with concomitant loss of DHase activity, is complete by 5 minutes, at which time incorporation of 1-[^{14}C]-iodoacetic acid is also at a maximum.

- Purification of reductive carboxymethylated monomer

On elution from a 35 cm Bio-Gel P-100 gel filtration column, two peaks are seen with absorption at 280 nm resulting from a native enzyme preparation which had been partially denatured by prolonged dialysis (Figure 9a). The major peak (>90% of the total protein) corresponds to a protein with molecular weight above the exclusion limit of the column (>100,000 daltons). The second, minor peak corresponds to a globular protein with a molecular weight of somewhat less than half this value, and is attributable to the lipoamide dehydrogenase monomer. When the reductively carboxymethylated sample was passed through the column, all but a trace of the protein appeared at an elution volume corresponding to this second, minor peak, separated cleanly from the heavier dimer, but poorly resolved from the labilized flavin chromophore observed by its absorption at 450 nm (Figure 9b). Use of a shorter (16 cm) P-100 column gave rise to a similar elution profile with

Figure 8: Progress curves of ^{14}C incorporation into protein from 1- $[^{14}\text{C}]$ -iodoacetate, and changes in activity resulting therefrom. ○—○, moles ^{14}C incorporated per mole of FAD; ●—●, specific activity in DPase reaction; □—□, specific activity ($\times 1/30$) in DHase reaction. Addition of iodoacetic acid to the β -mercaptoethanol pretreated enzyme held anaerobically defines $t = 0$.

Figure 9: Elution patterns from a Bio-Gel P-100 column (37 x 2.5 cm). A) native enzyme; B) RCM-monomer. — absorbance at 280 nm; ---- absorbance at 450 nm.

respect to a clean separation of monomer and dimer, but resulted in an RCM-monomer more heavily contaminated with flavin. Due both to the requirement of FAD for catalytic (DPase and ETase) activity and to its stabilizing effect on the monomer, as discussed below, monomer was routinely purified from the shorter column.

- Characterization of reductively carboxymethylated monomer

The gel filtration chromatography has established a molecular weight for the RCM-monomer corresponding to that of the native lipoamide dehydrogenase monomer. In addition, sucrose gradient centrifugation revealed native enzyme components of 1.01×10^5 and 5.9×10^4 daltons, some monomerization occurring in the sucrose medium, while RCM-enzyme had an apparent molecular weight of 4.6×10^4 daltons. The latter protein thus corresponds to lipoamide dehydrogenase monomer. While flavin is not tightly associated with modified protein, its separation is not complete, and it is eluted with a volume well below that of free FAD (>200 ml; Figure 2b). Complete removal of FAD by dialysis is facile, contrary to the situation with native enzyme dimer.

Under no circumstances was any dimerization of the modified monomer observed, as detectable by either gel filtration, SDS-polyacrylamide gel electrophoresis, sucrose gradient centrifugation, or the return of DHase- or THase-active species.

SDS-polyacrylamide gel electrophoresis shows a single band for RCM enzyme, with a molecular weight of 4.8×10^4 daltons as determined from a standard curve (Figure 10). Unmodified enzyme has a major component with a molecular weight of 8.9×10^4 daltons as determined by the same method. A summary of the molecular weight determinations is given in Table 7. Due to the variation of molecular weights obtained, and to the uncertainty of the dimeric species' Stokes radius (obtained by gel filtration), a deeper analysis of molecular shape does not appear useful.

The isoelectric focussing pattern of this RCM-monomer shows two major bands (Figure 11), in contrast to the six bands of native enzyme (*vide infra*). More bands or shoulders may occur due to denaturation of this relatively unstable, highly modified species, but are present in relatively smaller amounts.

The more extensive separation of flavin from the modified monomer on the longer (35 cm) column leads to decreases in the ETase and DPase activities, and a small contaminating amount of FAD would seem necessary for the activities to be observed. It has been suggested (198) that a rapid turnover of contaminating flavin is responsible for the residual DPase activity of apoenzyme preparations, and we sought further to demonstrate the requirement of FAD for the activities of the RCM-monomer. It was noted that flavin-contaminated samples from the short gel column gained no further activity with a still greater excess of FAD. Some denaturation and precipitation of apoprotein occurs in the

Figure 10: Standard curve for SDS-polyacrylamide gel electrophoresis. Proteins are lysozyme (A), α -chymotrypsin (B), LADH (C), and bovine albumin (D). The m, d, t, and q refer to monomer, dimer, trimer, and tetramer, respectively. On this scale, native lipamide dehydrogenase (Δ) shows an electrophoretic mobility $\mu=0.50$, and RCM-monomer (∇) $\mu=0.69$.

Table 7: Summary of molecular weights determined by various methods for native and RCM enzymes.

Method	Native		RCM enzyme
	Monomer	Dimer	
Gel filtration	5×10^4	$>10^5$	$<5 \times 10^4$
Sucrose gradient ultra-centrifugation	5.9×10^4	1.01×10^5	4.6×10^4
SDS-polyacrylamide gel electrophoresis	4.7×10^4	8.9×10^4	4.8×10^4

Figure 11: Isoelectric focusing pattern of RCM-monomer. Details as for isozyme separations (see text), with ca. 10 mg of protein. 25 drops per fraction collected.

modification procedure, so flavin remaining is indeed present in excess. The protein concentration of such samples was obtained with the micro-biuret assay, and the samples were then dialyzed against buffers containing various molar excesses of FAD. Figure 12 shows that DPase and ETase activities both increase to maximum values at an FAD:monomer ratio of 1:1, and show no further increase upon addition of excess FAD. At relative FAD concentrations of less than 0.3, activity is lost due to visible precipitation of protein during dialysis, before all FAD is removed. In all cases, however, the activities extrapolate to near zero at complete removal of FAD.

Use of 1- $[^{14}\text{C}]$ -iodoacetic acid in the reaction mixture revealed uptake of 21-22 moles of iodoacetate per mole of enzyme-bound flavin, in a reaction essentially complete by 5 minutes (Figure 8). Amino acid analysis showed this to be due to the modification of cysteine, methionine, and tyrosine residues (Table 8). Table 9 shows the protection of one or more mercurial-reactive sulfhydryl groups from carboxymethylation by β -mercaptoethanol pre-treatment, as determined by PCMB titration. The PCMB showed reaction of 11.4 of 14 cysteine residues per subunit (cf. 328) in an LADH control.

- Infrared spectroscopy

If infrared spectroscopy is to be used as a monitor of protein conformational changes, a sensitive spectral feature must be found and must be observable reproducibly for a given

Figure 12: Effect of FAD on the activity of RCM-monomer. $\circ$ — $\circ$ DPase activity $\times 10$; $\bullet$ — $\bullet$ ETase activity.

Table 8: Results of amino acid analyses, showing the number of moles of amino acid per 5×10^4 g of protein, for those residues modified. Obtained on the Beckman 121-M.

Amino Acid	Native	ICH ₂ CO ₂ H control	RCM-monomer
Cysteine + $\frac{1}{2}$ cystine (a)	10	1.8	7.5
Methionine	10	5	0
Tyrosine	7	6.7	4.5

(a) as total of cysteine, cysteic acid, and carboxymethylcysteine peaks, corrected to 10 for native enzyme.

Table 9: PCMB titration showing the number of moles of reactive -SH groups, expressed per mole of enzyme-bound FAD. Values are average of two determinations of a single preparation.

Enzyme Species	Moles -SH/Mole FAD
Native	6.9 ± 0.2
HS(CH ₂) ₂ OH control	5.1 ± 0.5
ICH ₂ CO ₂ H control	3.7 ± 1.9
RCM-monomer ^(a)	5.4 ± 0.5

(a) Dialysed against equimolar FAD.

sample. We have concentrated upon the so-called Amide I ($\nu_{C=O}$) and Amide II (ν_{N-H}) regions of the polypeptide spectra. The absorption maxima of this region have been extensively correlated empirically with peptide conformational structure. A useful tabulation of the results of such correlation has been given by Susi (329) and his data are collected in Table 10 for comparison with our spectra.

Figure 13 shows the reproducibility of the oxidized enzyme spectrum obtained by the ATR method as described above. The two spectra were obtained on the same instrument, three years apart. Curve (a) is of the Boehringer enzyme, and is obtained on an IRTRAN 4 crystal. Curve (b) is the Sigma enzyme spectrum, obtained with the KRS-5 crystal. The IRTRAN 4 crystal is of superior quality, having a higher transmittance (thus improving the signal-to-noise ratio), and a higher refractive index (330). Our spectra offer superior resolution to thin film transmission spectra and H_2O-D_2O subtraction methods (329). The former require more difficult sample preparation (i.e. on IR windows, from a suitable solvent), and, due to the high molecular weights of proteins, require a film thickness difficult to reproduce. The ATR method gives a surface spectrum, any thickness greater than one half the wavelength of the incident light being seen as infinite. Furthermore, the supporting (polyethylene) material is hidden. $H_2O - D_2O$ subtraction requires duplicate spectra, and calculation of difference spectra. In addition, conformational changes between the two solvents can be significant. The ATR method further improves resolution by signal averaging due to multiple contact

Table 10: Characteristic Amide I and Amide II absorptions for various polypeptide conformations, giving maximum of strongest component in cm^{-1} . Data from reference 327.

Conformational feature	Amide I Solid State	Amide I H ₂ O solution	Amide II Solid State
Random	1658	1656	1520
Pleated Sheet	1632	1632	1530
α -Helix	1650	1652	1546
Triple helix	1648	-	1558

Figure 13: ATR-IR spectra of native enzyme, obtained as outlined in methods. (a) Boehringer enzyme on IRTRAN 4 crystal, (b) Sigma enzyme on KRS 5 crystal.

with sample. Our spectra are of comparable or superior quality to those recently obtained by Fourier Transform methods (330), and are superior to those previously obtained.

In Figure 14 are shown spectra of native enzyme (a), apoenzyme (b), RCM-enzyme (c), and control iodoacetic acid treated enzyme (d). The monomeric apoenzyme prepared by the method of Veeger and Visser(227) exhibits a spectrum consistent with the higher α -helical content of that species observed with CD and ORD experiments by those authors (238).

The spectrum of reductively carboxymethylated monomer exhibits an altered absorption pattern as well. The relative intensities of the 1630 cm^{-1} and 1650 cm^{-1} component peaks of the Amide I band are reversed as compared to those of the native enzyme, and the $1500\text{-}1550\text{ cm}^{-1}$ Amide II region is broadened. Both these factors are indicative of a higher α -helical content, with possibly increased randomization of the native conformation as well. The conformation of this monomeric modified species with loosened flavin cofactor resembles that of the monomeric apoenzyme more closely than it does that of dimeric native enzyme. The reductive alkylation sequence is necessary to produce this conformational change, as it is not observed in the control preparations where iodoacetic acid is reacted with oxidized enzyme.

Further evidence supporting this gross conformational change in the RCM monomer comes from the TNBS assay. The colour developed in this assay indicates a $(30 \pm 5)\%$ increase

Figure 14: ATR-IR spectra of (a) native enzyme, (b) apoenzyme, (c) reductively carboxymethylated monomer, and (d) iodoacetic acid treated control. See text.

in the number of exposed ϵ -amino groups accompanying the conformational changes of the modified monomer, over both native enzyme and iodoacetic acid controls.

- Reconstitution and absorption spectroscopy

RCM-monomer can be obtained from longer gel filtration columns lacking visible FAD absorptions ($\lambda_{\text{max}} = 370$ and 450 nm). On incubation with equimolar or excess FAD, at room temperature for 30 minutes, the spectrum of bound FAD is barely discernible by the appearance of a slight shoulder at *ca* 465 nm, lacking in the free FAD spectrum, but present in the enzyme (Figure 15). The extinction coefficient of this shoulder is somewhat less than that of free flavin at this wavelength, as shown by the difference spectrum of monomer + FAD against an equal concentration of free FAD. In the UV, loss of the tryptophan resolution is observed as this peak increases and merges with higher energy transitions. No resolution of the flavin absorption in this region is discernible in any samples due to large protein extinction coefficients.

-Modification with Cu^{2+}

Treatment of native enzyme with cupric ion causes an increase in DPase activity with concomitant loss of DHase (198,217), and for this reason the effect of Cu^{2+} on the monomer prepared here was studied as a further multifunctional probe. Table 11 confirms the earlier reports of enhancement of DPase and loss of DHase

Figure 15: (a) Absorption spectrum of native enzyme (5.3×10^{-6} M in FAD) with inset showing visible region (4.2×10^{-6} M). (b) Reductive carboxymethylated monomer, dialyzed against equimolar FAD (4.4×10^{-6} M; inset 3.4×10^{-6} M). (c) Difference spectrum of the sample of (b), inset, against equimolar FAD. All spectra at 25° in 0.010 M phosphate buffer.

(a)

(c)

activities when native enzyme is incubated with cupric ion. In addition ETase and THase activities are affected differentially, both being decreased but not lost. Similar treatment of the RCM-monomer with cupric ion fails to restore any DHase or THase activity (both absent in this species), and further reduces ETase activity, as expected (cf. Table 11). However, it also results in a large decrease in the DPase activity, in contrast to its effect on native enzyme.

Other reduction/alkylation sequences

In view of the report of mild alkylation of lipoamide dehydrogenase by iodoacetamide following dithionite reduction (269), which specifically modifies the active site disulfide, we investigated further the role of reducing and alkylating reagents in our preparations. With anaerobic reduction by dithionite to the intermediate EH_2 state, followed by mixing with a two-fold excess of iodoacetamide, a species spectrally identical to that of Thorpe and Williams (269) was obtained, as indicated by a λ_{max} of 448 nm. This species has been characterized, and is reported to be dimeric with one sulfhydryl alkylated. This sulfhydryl group arises presumably from the reduction of the active site disulfide residue, which is then prevented from reforming upon reoxidation. Thorpe and Williams reported enhanced THase activity and a total loss of DHase activity for this monoalkylated species, which will subsequently be referred to as E_{ma} . Our results confirm this, and in addition show an increase

Table 11: Effects of incubation of native and RCM-monomeric enzymes with CuCl_2 (5 mM, 48 hrs. at 4°C) in the presence of equimolar NADH. Activities are expressed as a percent of initial activity remaining after incubation, averaged for 2 experiments.

Activity	Native	RCM-monomer
DHase	0	0
THase	15 ± 6	0
ETase	65 ± 23	17 ± 8
DPase	114 ± 30	18 ± 10

Figure 17: Effects of illumination of a solution of lipoamide dehydrogenase. $\circ$ — $\circ$, no sensitizer; $\bullet$ — $\bullet$, with methylene blue added to $A_{600} = 0.6$. Enzyme is 6×10^{-5} M in FAD. Illumination is by a 200 W photoflood bulb at 20 cm, $T = 25.0 \pm 0.1^\circ\text{C}$, in phosphate buffer (0.010 M, pH 7.0)

Table 12: Effect of alkylating and reducing reagents on enzyme activities. See text for explanation. Average of two experiments except where noted. Values are v_i/E_t (10^3 min^{-1}).

	Native (b)	$\text{Na}_2\text{S}_2\text{O}_4$ / $\text{ICH}_2\text{CONH}_2$	$\text{Na}_2\text{S}_2\text{O}_4$ / $\text{ICH}_2\text{CO}_2\text{H}$	$\text{HS}(\text{CH}_2)_2\text{OH}$ / $\text{ICH}_2\text{CONH}_2$	$\text{HS}(\text{CH}_2)_2\text{OH}^{(b)}$ / $\text{ICH}_2\text{CO}_2\text{H}$
Moles-SH(a)	6.9 ± 0.2	$5.3^{(c)}$	$4.1^{(c)}$	$3.9^{(c)}$	5.4 ± 0.5
Activity					
DHase	2.8	0	0.55 ± 0.05	0.31 ± 0.1	0
Thase	1.2	1.6 ± 0.2	0.65 ± 0.4	1.2 ± 0.1	0
ETase	1.2	1.7 ± 0.6	1.3 ± 0.3	0.95 ± 0.3	
DPase	0.040	0.075 ± 0.02	0.065 ± 0.02	0.060 ± 0.005	5-25

(a) Determined by PCMB-titration.

(b) >10 experiments for activities, standard error <10%

(c) Single experiment

or specific DHase, THase, ETase, and DPase activities could be observed, indicating failure to modify the enzyme.

Noting that the biological substrate interacting with this enzyme disulfide is a dithiolane ring, it is reasonable to suggest that the tetramethyl-1-piperidinyloxy moiety is sterically hindering approach, and this moiety was moved further away from the site of nucleophilic attack upon the reagent by attempting the modification with 3-[[2-[2-(iodoacetamido)ethoxy]ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxy. The same negative results accrued, and it becomes clear that steric, electronic, and/or hydrophobicity requirements for attack of the active site disulfide are stringent.

ii) Arginine modifications

In recent years, a number of dehydrogenases and kinases have been found to bind their nicotinamide coenzymes by interaction of a phosphate group on the coenzyme with a protein arginine residue (332-334). Binding of phosphate substrates has also been shown to commonly involve arginyl residues (335-338), and functional arginyl residues are known in the well studied ribonuclease A and lysozyme (339). These studies have been made possible by reaction of the guanidino functionality with α -dicarbonyls (301) (Scheme IV). A problem, of course, is presented by the high pK_a of the guanidino group, but successful results have been obtained at pH 8-9 using such dicarbonyls as phenyl-

Scheme IV : Reaction of arginine with α -dicarbonyls

glyoxal (340,338,341), 1,2-cyclohexanedione (342,339), and 2,3-butanedione (332,334). In an attempt to identify an arginine residue at one of the nucleotide binding sites of lipoamide dehydrogenase we have reacted the enzyme with 1,2-cyclohexanedione and phenylglyoxal, under varying conditions (4°C, room temperature; pH 7.7, 8.1, 8.7, 9.5; 2-18 hrs.) and in no case have found any reproducible alteration in any activity, or any change in the absorption spectrum. The infrared spectrum (Figure 16), however, shows a conformational change resulting from modification of apparently non-essential arginine residues by phenylglyoxal. The Sakaguchi colourimetric method was used to show that 8 of the 26 (single determination) arginine residues were modified by cyclohexanedione independently of the conditions used.

Figure 16: ATR-IR spectrum of enzyme with 8 arginine residues modified by phenylglyoxal. See text.

iii) Photooxidations

To investigate the role of histidine as a possible candidate for a catalytic base, according to the proposed mechanism of Williams (198,271), we illuminated an aerated solution of enzyme in the presence of a sensitizing dye. The dye, such as rose bengal (312) or methylene blue (343), is envisaged as trapping oxygen from an excited state of the dye (344), and the resultant dye- O_2 peroxide species is a mild oxidant of several amino acid residues. However, under the conditions employed, a high degree of specificity for destruction of histidine is observed (312,343). Oster (344) has found both the cationic thiazine dye methylene blue, and the anionic xanthine rose bengal to act within 5 minutes of illumination, yielding aspartic acid and urea (345). We have investigated the effect of both these dyes.

-Flavin illumination, unsensitized

Due to the extensive rapid photolysis of free flavin (346, 347,106,107), it was first necessary to eliminate the possibility of photolysis of enzyme-bound cofactor. In addition, flavin-sensitized photooxidation of histidine has been reported (115), and such an effect must either be ruled out or exploited.

At pH 7.0, illumination of a native enzyme solution for up to 20 minutes had no effect on any of the activities and did not alter the flavin absorption spectrum. There was no oxygen uptake, as monitored by an O_2 -electrode (Figure 17). The enzyme-bound FAD was insensitive to photooxidation. Under

Figure 17: Effects of illumination of a solution of lipoamide dehydrogenase. ○—○, no sensitizer; ●—●, with methylene blue added to $A_{600} \approx 0.6$. Enzyme is 6×10^{-5} M in FAD. Illumination is by a 200 W photoflood bulb at 20 cm, $T = 25.0 \pm 0.1^\circ\text{C}$, in phosphate buffer (0.010 M, pH 7.0)

anaerobic conditions the flavins will photolyze (107), and the experiment was repeated with either free FAD or enzyme in an anaerobic Thunberg cell. After 30 minutes, the FAD solution was 25% bleached (Figure 18), but the enzyme spectrum was unchanged. No enzymic activity was lost. In a final attempt to photodegrade enzyme-bound FAD, it was considered that FAD in the enzyme-bound state was unable to assume the cyclic conformation proposed as the reactive intermediate for free FAD (106, 113). Anaerobic illumination continued for 2 hours at pH 10.0, in order to labilize the FAD (cf. 261). There was still no effect on either activities or the bound flavin spectrum. Finally, the RCM-monomer was used, due to the demonstrated lability of its flavin. At pH 7.0, the rate of bleaching of the flavin portion of E_{RCM} was seen to be comparable to that of free FAD. From Figure 19, the first order rate constants for FAD and E_{RCM} are 0.094 min^{-1} and 0.12 min^{-1} respectively. Thus both flavin and enzyme residues are considered stable to the illumination conditions used, and sensitizers may be employed meaningfully.

- Methylene blue sensitization

Aerobic illumination of enzyme in the presence of methylene blue is seen to result in uptake of O_2 as expected (Figure 17). As methylene blue is not destroyed in these experiments (as evidenced by a constant absorption at 600 nm), it is acting as the catalytic oxygen carrier discussed by Oster *et al.* (344). Because the oxygen concentration drops irreversibly, and the

Figure 18: Anaerobic photolysis; see text for details. $\circ$ — $\circ$, enzyme, 1.9 mg.ml^{-1} ; $\bullet$ — $\bullet$, FAD, $3.5 \times 10^{-5} \text{ M}$. Condition as in Fig. 17.

Figure 19: Anaerobic photolysis, as in Fig. 18. $\circ$ — $\circ$, FAD, $3.5 \times 10^{-5} \text{ M}$; $\bullet$ — $\bullet$, RCM-monomer, 0.95 mg.ml^{-1} .

equilibrium concentration of dye peroxide is expected to be small, the oxygen must be consumed in the oxidation of some enzymic site(s). A decrease in O_2 concentration of ~2ppm represents about 2.6 moles of O_2 consumed per subunit (molecular weight assumed, 5×10^4). Once again, however, no alteration of enzyme activities could be observed, and it may be concluded that no oxidative damage to flavin or active site residues is occurring.

- Rose bengal sensitization

In view of the preference of the enzyme for an anionic species of DCIP to act as substrate (*vide infra*), sensitization was thought to be more likely destructive of an active centre residue with an anionic sensitizer, methylene blue failing this requirement. Aerobic illumination of the enzyme in the presence of rose bengal, an anionic sensitizer, repeatedly gave a 60% reduction in DHase activity, the other reactions being differentially affected (Table 13). The flavin absorption spectrum, obtained after purification from sensitizer by ion exchange, was unaltered, again indicating insensitivity of the FAD cofactor. Amino acid analyses reveal destruction of 5 histidine residues, and no others, in the rose bengal sensitized enzyme. Tables 14 and 15 show independent amino acid analyses of the enzyme. The values in Table 14 are determined by taking the total number of residues output from the analyzer, less the variable Asp, Glu, and NH_3 . The remainder of the residues are totaled, and the

Table 13: Activity alterations in enzyme illuminated 30 min. at 25°C, in the presence of 0.01 mg.ml⁻¹ rose bengal, after purification with CELLEX T ion exchanger. Activities are expressed as percent remaining compared to a control enzyme treated identically, with omission of illumination, wrapped in foil throughout. Values are average of three trials.

Activity	percent remaining
DHase	37 ± 5
THase	68 ± 7
ETase	122 ± 36
DPase	130 ± 25

Table 14: Amino acid analyses of control, methyl acetimidate-treated (E_{amid}) and rose bengal sensitized photooxidized (E_{hv}) enzymes (see text). Analyses of 6N HCl hydrolysates performed on the Durum D-500 system. Values are moles per mole FAD. All values are uncorrected for degradation.

Amino Acid	Control	E_{amid}	E_{hv}
Asp	44.7	44.1	45.4
Thr	24.7	25.0	25.6
Ser	21.4	21.5	22.9
Glu	51.9	51.4	53.0
Pro	19.9	20.4	21.0
Gly	57.4	50.3	52.5
Ala	47.1	46.7	47.5
Cys ^(a)	3.6	2.8	4.1
Val	40.8	40.3	42.0
Met	8.6	7.8	8.9
Ile	32.6	31.7	33.3
Leu	33.8	33.2	33.7
Tyr	8.9	8.8	9.5
Phe	15.0	14.7	15.6
His	12.0	11.9	7.0
Lys	37.8	19.3	37.6
Arg	19.0	19.5	20.2
NH ₃	64.5	57.4	61.6

(a) Subject to extensive degradation upon hydrolysis

Table 15: Amino acid analyses as in Table 14, but performed on the Beckman 119-BL system.

Amino Acid	Control	E_{amid}	E_{hv}
Asp	48.2	52.7	49.8
Thr	25.4	28.0	27.8
Ser	23.1	28.5	25.9
Glu	57.0	58.3	58.3
Pro	20.5	21.9	21.2
Met	9.2	8.9	8.8
Ile	34.3	36.9	34.0
Leu	35.1	38.0	35.1
Tyr	9.0	10.3	8.3
Phe	16.1	17.3	15.1
His	12.0	13.4	7.5
Lys	39.4	20.8	40.2
NH ₃	108	173	88.0

total then corrected to the published value (Table 4), neglecting Asp, Glu, and NH_3 . Individual residue values are then corrected by the ratio of published total to raw data total. The same procedure is adopted in Table 15, except that the residues Gly, Ala, Cys, and Val are also eliminated from the totals due to poor resolution complicating accurate peak integration. When these methods are employed, the results obtained from the two systems are in good agreement with each other and with the literature.

iv) Reaction with methyl acetimidate

Imido esters have been shown to react specifically with amino groups in proteins (348,303) to form imido-amide (so called amidine) derivatives. The reaction may then occur at either the α -amino group of a polypeptide chain N-terminal, or on lysine ϵ -amino groups. The α -amino derivative is unstable to acid protein hydrolysis, but the ϵ -amidino groups are relatively stable (303) and modification may be observed by amino acid analysis. The reaction with methyl acetimidate proceeds according to Scheme V.

To investigate the possible role of lysine residues in lipoamide dehydrogenase catalysis, as well as obtaining further evidence regarding the identity of the active centre base, the enzyme was reacted with neutral methyl acetimidate hydrochloride. Initially reaction was for 100 minutes at pH 8.5 as described

Table 16: Multifunctional activities of E_{amid} after 100 min. reaction time, as percent of control remaining. Conditions as in Materials and Methods. Average of six experiments.

Activity	Percent Remaining
DHase	190 ± 30
THase	100 ± 4
ETase	92 ± 7
DPase	90 ± 12

Table 17: Effects on the activities of lipoamide dehydrogenase of modification with monopotassium phosphoramidate (40 mg.ml^{-1} in 0.01 M phosphate, $\text{pH } 7.0$), purified by gel filtration after 15 min. at room temperature. Assay conditions as in Materials and Methods. Average of seven experiments.

Activity	Percent Remaining
DHase	280 ± 80
THase	104 ± 5
ETase	73 ± 32
DPase	72 ± 30

Figure 20: DHase kinetics of Eamid; see text. (a) Primary double reciprocal plot. Dashed line indicates onset of substrate inhibition. (b) Secondary plot of data from (a). Intercept of regression line is 0.0875 min^{-1} . The ordinate is in units of $(v_i/E_t)^{-1}$ in Cleland's nomenclature, for $E_t = 0.50 \mu\text{g} \cdot \text{ml}^{-1}$, at a molecular weight of 5.0×10^4 , $V = 0.29 \mu\text{mol} \cdot \text{min}^{-1}$, $V/E_t = 2.9 \times 10^4 \text{ min}^{-1}$, $K_{\text{NADH}} = 0.71 \text{ mM}$, $K_{\text{Lip}} = 6.23 \text{ mM}$, $K_{ia} = 33.1 \mu\text{M}$.

Figure 21: ATR-IR spectrum of E_{amid}. See text. Sample purified at 15 min. in the standard reaction (pH 7.0) by dialysis against neutral phosphate buffer, then distilled water for IR

Figure 22: Time dependence of activation of DHase activity by reaction with methyl acetimidate (see text). ●—● methyl acetimidate reaction mixture, ○—○ control. Standard assays are used.

shows that DHase activity is maximal at about 25 minutes after addition of methyl acetimidate, and declines slowly thereafter, possibly due to instability of the conformationally altered enzyme. In several repetitions, similar behaviour was observed, activity peaking at 15-30 minutes.

Lysine determination by TNBS was carried out on dilutions of various samples producing the standard curves of Figure 23. Long reaction times (45 minutes) revealed a total of 20 of the 36 lysine residues to have been modified, while shorter incubation times (5 minutes) showed the absence of 2-8 residues reacting rapidly with TNBS. Amino acid analyses on 15-20 minute samples from the modification reaction mixture are in excellent agreement with the TNBS results (Tables 14 and 15). These results indicate a total of 19 residues modified. In addition, a new peak in the chromatogram follows lysine, which if integrated with the lysine colour factor accounts for 17.6 of these lost residues. The peak is thus attributable to the amidine derivative of lysine. By amino acid analysis, this modification is shown to be highly specific for lysine.

v) Reaction with phosphoramidate

Having implicated both histidine and lysine residues as being involved in the biological DHase activity and conformation, it became relevant to check the effects of the more biologically significant phosphorylation modification. As discussed above,

Figure 23: Standard curves for the TNBS determination of lysine residues (see text).
 ○—○, control; ●—●, △—△, separate E_{amid} preparations.

the PDC has been shown to contain phosphorylating and dephosphorylating enzymes which regulate the complex by inhibitory phosphorylation of pyruvate dehydrogenase, no ATP-linked phosphorylation of lipoamide dehydrogenase itself having been reported. However, this might be due either to a transient incorporation of phosphate at a sensitive site with subsequent transfer or loss of the labile group, or to the inappropriate nature of the conditions (reagents) used.

Figure 24 shows the changes in DHase activity assayed after addition of phosphoramidate to lipoamide dehydrogenase. The activity increases some 5-fold by 10 minutes of exposure, and decreases thereafter, eventually returning to that of a control preparation from which phosphoramidate had been omitted. Table 17 (after purification on Bio-Gel P-2 at 15 minutes) shows this enhancement to be specific for the DHase activity, THase being unaffected while ETase and DPase are slightly decreased. The values in Table 17 fail to reveal the full extent of DHase enhancement (up to 500%) due presumably to the instability of the modified species during gel filtration.

The DHase activation curves for amidination and phosphorylation (Figures 22 & 24) are qualitatively similar, as are the multi-activity effects (Tables 16 & 17). To determine if the same site is responsible for this behaviour, a sequential double modification was performed. Enzyme was reacted first with methyl acetimidate, due to the superior stability of the resultant species, and when timed assays revealed attainment of

Figure 24: Time dependence of activation of DHase activity by reaction with phosphoramidate (see text). ●—● phosphoramidate reaction mixture; ○—○ control. Standard assays are used.

maximal activity, phosphoramidate was added to the usual concentration. Figure 25 shows that at pH 8.0 the activity was further enhanced, but this enhancement proceeded more slowly than usual (cf. Figure 24). This may have been the result of a white precipitate, which also occurred in the absence of enzyme. Although not characterised, the precipitate represented a reaction product of phosphoramidate and the imidoester, which would lower the effective concentration of the phosphorylating reagent.

Figure 25 also shows the results of repeating the experiment at pH 5.7. There is now no effect from the imidoester, the enzyme displaying time-dependent, acid-dependent decreases in activity. The phosphorylating reagent is still effective in enhancing the activity, however, although the increase is less marked due both to the acid instability of the enzyme and possible protonation of a residue involved.

The infrared spectrum of the phosphorylated enzyme purified at 15 minutes by gel filtration (E_{phos}) shows only slight conformational changes to occur (Figure 26(a)) consistent with rapid modification of a few (or a single) residue(s). A spectrum of doubly modified enzyme at 2 hours, however, shows major conformational changes (Figure 26(b)). This may be due either to the cumulative effects of the modifications in $E_{\text{amid/phos}}$ or to a progressive, irreversible conformational change. The latter is the more likely explanation, in view of the small changes in the short term spectra of both E_{amid}

Figure 25: Effect of phosphoramidate on E_{amid} ; see text. Methyl acetimidate is added at 0 min.; phosphoramidate at the arrow. Ordinate represents NADH oxidation in the standard DHase assay. $\circ$ — $\circ$, pH 8.0 (.02 M borate buffer); $\bullet$ — $\bullet$, pH 5.7 (0.01 M phosphate), offset +20 units on the ordinate for clarity.

Figure 26: ATR-IR spectra of (a) E_{phos} , purified by gel filtration at 15 min., (b) enzyme modified by methyl acetimidate followed by phosphoramidate, as in Fig. 25, and purified at 2 hrs., (c) E_{amid} purified at 2 hrs.

(Figure 21) and E_{phos} (Figure 26(a)). The spectrum of E_{amid} (Figure 26(c)) which has been subjected to modification for 2 hours shows no such marked conformational alteration.

The kinetic mechanism of the DHase activity of E_{phos} was investigated. Figure 27 shows the primary and secondary plots, indicating a large increase in the V_{max} of the reaction.

Possible sites of modification by phosphoramidate are the nucleophilic residues cysteine, lysine, tyrosine, histidine, serine, and perhaps threonine and arginine. Phosphoramidate is a potent phosphorylating reagent due to the superiority of ammonia as a leaving group, compared to the -OR group of orthophosphate esters, but it has not been widely used in protein modifications and the reaction products have not been characterized. A primary problem is the unstable nature of the resultant phosphate esters to conventional peptide hydrolysis. Hultquist *et al.* (349) have used the reagent to prepare 1- and 3- phosphohistidines, differing in their rates of formation and reactivity, but both with labile phosphate groups (see Discussion). Schneider (350) has commented that histidine is not a particularly good nucleophile to phosphate esters, and when the amide is used here, the product shares the same reactivity as the reagent. On the other hand, serine or tyrosine phosphates can be expected to display normal phosphate ester stability.

Successful modification of the initially reacting residue(s) at pH 7.0 (Table 17), 8.0 (Figure 24), and 9.0 (not shown), and only a reduction in the degree of DHase enhancement at pH 5.7 (Figure 25), strongly supports modification of an imidazole

Figure 27: DHase kinetics of *E. coli*; see text. (a) Primary double reciprocal plot. Dashed line indicates onset of substrate inhibition. (b) Secondary plot of data from (a). Intercept of regression line slightly negative; V_{max} large. Ordinate as in Fig. 20.

residue ($pK_a \approx 6$) over serine ($pK_a > 10$), cysteine ($pK_a \approx 8.4$), lysine ($pK_a \approx 11$), or other possible residues (pK_a values from 308). The activation profiles also suggest labile reaction products, and to test the hypothesis of modification of a histidine residue we sought further to demonstrate this instability by observing the effect of addition of hydroxylamine to E_{phos} . Addition of hydroxylamine to the reaction mixture at 10 minutes (final [hydroxylamine] = 1.0 mM) causes an immediate decrease (complete within 2 minutes) in DHase activity back to the control level (Figure 28). A similar addition has no effect on control enzyme as measured over 30 minutes.

v) Cross-linking with diimidoesters

Complementary to our preparation of a stable monomeric species, we have attempted to prepare a covalently cross-linked dimer. Because of the ~~number of~~ lysine residues reactive with the imidoester methyl acetimidate (cf. Section (iv)), bifunctional imidoesters seemed a logical choice of cross-linking reagent. The use of these reagents is well documented in cross-linking studies (351-353). The reagents display the same high selectivity for amino groups as do the mono-functional analogs, and the resulting bis-amidines have the same acid stability (351). In addition, since the pK_a of the amidine is higher than that of the ϵ -amino group modified, the protein net charge at neutral pH is left unaltered (351). Cross-linking was carried out as

Figure 28: Effect of hydroxylamine (added to 1 mM at arrow) on E_{phos} (phosphoramidate added at 0 min.), in phosphate buffer, pH 7.0; see text. Ordinate as in Fig. 25. ●—●, with phosphoramidate; ○—○, control.

described in the Methods. The results of using a chain length of varying size are given in Table 18. The proportion of cross-linked dimer to total protein reported in the table was determined by the β -mercaptoethanol/SDS/urea sequence, followed by electrophoresis and densitometry as described. Typical densitometer scans are shown in Figure 29.

To determine the effect of cross-linking on the multifunctionality of the enzyme, it would be desirable to purify cross-linked dimer from naturally occurring dimer under non-denaturing or reversibly denaturing conditions. To this end, several methods were employed without success.

(i) Native enzyme was dialyzed for 24 hours against phosphate buffer (0.10 M, pH 7.0, containing 8 M urea), then passed through a Bio-Gel P-100 column. The majority of enzyme emerges as dimer.

(ii) Following a suggestion of Visser and Veeger (235) a reaction mixture was reduced anaerobically with dithionite in the presence of 8 M urea in the hope that the increased sensitivity of EH_2 to monomerization and irreversible urea denaturation would remove all native enzyme. When the remaining intact dimeric protein was purified by gel filtration, however, it was found to retain a dissociable component under conditions where the amidine linkage is stable.

(iii) Step (i) was repeated in the presence of 1% SDS + 1% β -mercaptoethanol, with similar results.

(iv) In attempts to break the hydrophobic subunit interaction, gel filtration was carried out in phosphate buffer

Table 18: Percentage of cross-linked dimer, from dimer: monomer ratios in integrated densitometer scans. Reaction times are 3 hrs. under conditions described in Methods.

Reagent ^(a)	n ^(b)	Chain Length (Å) ^(c)	% cross-linked
None	-	-	0.4
DMIM	1	2.7	17
DMIS	2	3.9	16
DMA	4	6.5	27
DMS	6	9.0	59
DMS ^(d)	6	9.0	66

(a) See list of Abbreviations

(b) General structure $\text{CH}_3\text{-O-C(=N)-}(\text{CH}_2)_n\text{-C(=N)-O-CH}_3$

(c) Distance between imide carbons, calculated assuming a C-C bond length of 1.54 Å and normal bond angles.

(d) 18 hrs. reaction time

Figure 29: Typical densitometer scans of cross-linked species. Abscissa shows distance from top of gel in arbitrary units. Samples are as in Table 18: (a) control, (b) DMJM, (c) DMIS, (d) DMA, (e) DMS, (f) DMS at 18 hrs.

containing varying amounts of 2-chloroethanol (see 237). At 10%, 2-chloroethanol was insufficient to disrupt the dimer, and 25% caused extensive protein precipitation. Intermediate concentrations gave intermediately unsatisfactory results, 13% being the best. The separation was still not clean enough to remove contaminating amounts of native enzyme, capable, for instance, of dissociating into highly DPase-active monomer, and the enzyme is unstable to repetitive procedures.

Work in this lab is currently underway to solve this problem. For the present, it is circumvented as follows. Samples were cross-linked under varying conditions, to obtain mixtures containing variable amounts of cross-linked enzyme. Aliquots were assayed, and then the samples denatured for gel electrophoresis as above, and the amount of cross-linked dimer quantitated by densitometry. The conditions and monomer:dimer ratios are summarized in Table 19. Specific activities as a function of percentage of cross-linked dimer in the assay mixture are plotted in Figure 30.

The DPase reaction appears the least sensitive to the amount of covalent dimer present, the other reactions varying inversely with that value.

vii) Arsenite modification:

On reduction of the enzyme to EH_2 by NADH, in the presence of NaAsO_2 , and altered absorption spectrum has been reported

Table 19: Monomer:dimer ratios (expressed as % cross-linked dimer) under various conditions, for extrapolation experiments. See text. Ratios are determined for duplicate gels and averaged.

Conditions	% cross-linked dimer
2 mg.ml^{-1} DMIM; 18 hrs.	9 ± 3
Stoichiometric DMA; 18 hrs.	6 ± 3
2 mg.ml^{-1} DMA; 1 hr.	45 ± 10
2 mg.ml^{-1} DMA; 18 hrs.	30 ± 7
2 mg.ml^{-1} DMS; 18 hrs.	17 ± 1

Figure 30: Activity of aliquots of reaction mixtures containing varying amounts of cross-linked dimer (see text). Activities are per ml of reaction mixture, at a constant enzyme concentration of $2 \text{ mg} \cdot \text{ml}^{-1}$, and represent a single experiment. Standard assay conditions (see Methods). $\circ$ — $\circ$, DHase; $\bullet$ — $\bullet$, THase; $\square$ — $\square$, ETase; $\blacksquare$ — $\blacksquare$, DPase.

(223 and see Figure 31), indicative of modification in the neighbourhood of the flavin centre. This has been attributed (223) to chelation of arsenite across the disulfide to give the structure of Scheme VI, and the green colour is explained by

Scheme VI: Proposed arsenite adduct

charge transfer from reduced flavin to NAD^+ . When an attempt is made to repeat complexation with E_{RCM} , reduction in the presence of arsenite causes extensive precipitation of protein, and no such complex can be observed.

Upon exposure to the air, the green complex was stable for several hours, but reverted to the oxidized form upon standing overnight or prolonged dialysis. Thus the samples were stable for immediate rapid assays, the results of which are presented in Table 20.

viii) Cobalt derivative

A relatively stable cobalt derivative has been prepared by Huang and Brady (261), but those authors have not studied the multifunctionality of this potentially interesting species, nor have they characterized it beyond tentative identification

Figure 31: Visible spectra of enzyme held anaerobically in the presence of 1.0 mM arsenite (Conditions described in Methods). (1) oxidized enzyme (5.3×10^{-5} M), (2) addition of NADH to 1.1×10^{-4} M, (3) addition of NADH to 1.1×10^{-4} M.

Table 20: Standard assays of arsenite-modified enzyme (E_{As}), with activities expressed as percent of native enzyme activity. See text. Average of three experiments.

Activity	Percent Remaining
DHase	<4
THase	44 ± 4
ETase	77 ± 7
DPase	160 ± 1

of a flavin and a non-disulfide protein ligand. We initially prepared this species to investigate (a) its ETase activity, and (b) the possibility of flavin fluorescence quenching by cobalt.

The changes in the flavin visible spectrum attendant upon formation of the cobaltous ion derivative (E_{Co}) are seen in Figure 32. They are marked by a loss of resolution of the low energy flavin absorption to produce a spectrum quite similar to that of free FAD (cf. Figure 15) with $\lambda_{max} = 450$ nm. A new, very broad absorption appears centred on *ca* 650 nm.

The ETase kinetics of E_{Co} will be presented in Section VIII below, however the multifunctional assay results are given here in Table 21. DPase enhancements as high as 180% have been observed with this species, but at the expense of ETase enhancement and DHase reduction. Thus this effect may well be due to some dissociative denaturation at the high pH (10) required in the preparation. Great care was necessary in handling the enzyme at pH 10, the series E_{ox} , EH_2 , EH_4 being increasingly unstable at room temperature and ice bath temperatures allowing over-reduction by NADH to EH_4 . This point will be taken up below.

The fluorescence excitation and emission spectra are shown for native enzyme and E_{Co} in Figure 33, with data summarized in Table 22. It is apparent that there is no alteration of the enzyme-bound flavin fluorescence, and the insignificant deviation from unity of the normalized integrated fluorescence intensity ratio (1.008) shows no quenching of FAD fluorescence or energy

Figure 32: Visible spectra of $E. coli$ preparation, as described in Methods. (1) $E. coli$, 6.9×10^{-5} M in FAD, (2) reduced with 1.4×10^{-4} M in NADH, (3) addition of 6.9×10^{-5} M $CoSO_4$, (4) further addition to 1.4×10^{-4} M $CoSO_4$, (5) after 2 hrs. exposure to air, (6) control reduced enzyme (not shown) after 2 hrs. exposure to air.

Table 21: Multifunctional activities of E_{Co} in standard assays. See text. Average of three experiments.

Activity	Percent Remaining
DHase	11 ± 2
THase	70 ± 6
ETase	144 ± 22
DPase	123 ± 40
OXase ^(a)	90

(a) Single experiment

Figure 33: Uncorrected fluorescence emission spectra of (1) native enzyme and (2) E_{Co} . $\lambda_{ex} = 465$ nm. Both samples 6.0×10^{-6} M in FAD, in potassium phosphate pH 7.0, 0.010 M. $T = 25 \pm 1^\circ C$. Native enzyme spectrum offset + 10% F for clarity.

Table 22: Fluorescence data of native enzyme and E_{Co} ; $\lambda \pm 1$ nm.

Species	λ_{max}^{ex} ($\lambda^{em} = 520$ nm)	λ_{max}^{em} ($\lambda^{ex} = 465$ nm)
Native	290, 371, 466	517
E_{Co}	290, 371, 466	516

$$\left(\int F_{E_{Co}}(\nu) d\nu / \int F_{E_{native}}(\nu) d\nu \right) \cdot \left(A_{E_{native}}^{465} / A_{E_{Co}}^{465} \right) = 1.008$$

transfer to the cobalt centre. The reasons for this lack of FAD-Co(II) interaction are not immediately clear in light of the conclusion of Huang and Brady (261) that the cobalt atom has a flavin ligand. The overlap integral of the Forster formula was found to have a value of $J = 2.4 \times 10^{-13} \text{ mol}^{-1} \text{ cm}^6$.

General MCD results are discussed below in Section IV, but the spectrum of the cobalt derivative is given here in Figure 34. The low energy feature in the $12,000 - 18,000 \text{ cm}^{-1}$ region is typical of the transitions of Co(II) in a tetra-coordinate environment, as buried in a hydrophobic region of a substituted metalloenzyme (354-356). The absence of a strong negative dichroism at $19,800 \text{ cm}^{-1}$ indicates complete removal of CoSO_4 reagent by dialysis (Figure 34, inset). The MCD spectrum of the protein region of the E_{Co} derivative is given in Figure 53, below.

Figure 34: MCD spectrum of the cobalt region of E_{Co} (rough solid line) in phosphate buffer (pH 7.0, 0.010 M) at room temperature. The MCD of CoSO_4 is also shown (dashed line), as well as the corresponding E_{Co} absorption spectrum (smooth solid line).

III) Isozymes

- Separation

Despite the several analytical and preparative separations of lipoamide dehydrogenase cited above, no kinetic data have been published on the various forms, and we have preparatively separated six isozymes to allow discussion of their role, if any, in this enzyme's multifunctionality.

Electrophoretically, the Sigma enzyme separates into at least two distinct bands, fuller resolution of which have been reported (277). The more anionic group is associated with the PDC, the more cationic with the α -KGDC. We have improved our resolution by isoelectric focusing.

A typical elution profile following 48 hours focusing time is shown in Figure 35, with resolution into 6 major components. This is the minimum number of discrete components capable of explaining the many elution patterns obtained, and it will be noted that asymmetric peaks are seen even here. These irregularities are thought to result from artifactual, random, subtle conformational changes of a given isozyme. From longer focusing times and/or prolonged pre-dialysis against distilled water, the irregularity of the elution absorption and activity profiles was increased, and in some cases as many as 13 peaks or shoulders could be discerned. This is (perhaps not coincidentally) the maximum number of isozymes which have been suggested to be present (258), but the species numbered in Figure 35 are those which

Figure 35: Isoelectric focusing pattern of Sigma enzyme mixture, with pH gradient (open circles). Conditions as described in methods; 25 drops per fraction.

occur repeatedly at the same isoelectric pH, and are often represented by symmetrical Gaussian peaks. We thus support the view that six major isozymes occur naturally, but may artifactually proliferate due to conformational instability.

Bearing this in mind, the present work concerns mainly isozymes I and VI*, those being (i) the most different with respect to net surface charge, (ii) from different dehydrogenase complexes, and (iii) the least likely to be contaminated by components of opposite complexes.

From a single isolation, the pI, yield, and degree of denaturation (as indicated by the A_{355}/A_{455} ratio) of the individual isozymes are given in Table 23. Also shown are single point assay specific activities for the four reactions. The isoelectric point values are reproducible (to within ± 0.2 pH units); the yields and degree of denaturation are not. From multiple runs, the amounts of individual isozymes present are approximately equal. The denaturation noted here accounts for the relatively low DHase and relatively high DPase activities (see Discussion).

- Kinetics

In Figures 36 and 37 are shown typical kinetic patterns for the DHase, THase, and DPase reactions of isozymes I and VI, at

* The isozymes are numbered from the low pH end, or according to decreasing anodic electrophoretic mobility, in keeping with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN), as published in 1977(357).

Table 23: Data from a single isozyme separation. See text for details. Activities for the reactions are from single point standard assays, pH 6.5, in units of min^{-1} . Focusing was of 25 mg protein.

Isozyme	pI	yield (mg)	A_{355}/A_{455}	Activities (10^2 min^{-1})			
				DHase	THase	ETase	DPase
I	5.82	1.7	>5	0.49	5.9	15	1.1
II	6.05	2.8	2.1	0.43	6.1	12	0.79
III	6.68	4.4	>2	0.45	6.1	10	0.66
IV	7.00	4.6	1.3	0.49	6.5	11	0.65
V	7.26	4.3	1.6	0.60	6.2	10	0.63
VI	7.65	2.4	>4	0.55	5.2	11	0.76

Total recovery = 20.2 mg = 81%

Figure 36: Kinetics of isozyme I. Graphs are double reciprocal plots against both substrates. Values are of three separate experiments from two isolations. Concentrations of second substrate, from the top line down, are:

- (a) [NADH] = 2.0, 6.0, 10.0 μM
- (b) [Lipoamide] = 100, 200, 300, 400 μM
- (c) [NADH] = 20, 40, 60, 100 μM
- (d) [TNAD] = 40, 60, 80, 100, 200 μM
- (e) [NADH] = 40, 60, 100, 200, 300 μM
- (f) [$\text{K}_3\text{Fe}(\text{CN})_6$] = 200, 300, 400, 500 μM
- (g) [NADH] = 20, 60, 100, 200, 300 μM
- (h) [DCIP] = 10, 20, 30, 40, 50 μM

Other conditions are as given in Methods.

Figure 37: Kinetics of isozyme VI. Graphs are double reciprocal plots against both substrates. Values are of two separate experiments from two isolations. Concentrations of second substrate, from the top line down are:

- (a) $[NADH] = 2.0, 6.0, 10.0 \mu M$
- (b) $[Lipoamide] = 100, 200, 300, 400 \mu M$
- (c) $[NADH] = 40, 60, 100, 200 \mu M$
- (d) $[NAD^+] = 20, 40, 80, 100 \mu M$
- (e) $[NADH] = 20, 40, 60, 100, 300 \mu M$
- (f) $[K_3Fe(CN)_6] = 60, 100, 200, 300 \mu M$
- (g) $[NADH] = 20, 60, 100, 200, 300 \mu M$
- (h) $[DCIP] = 10, 20, 30, 40, 50 \mu M$

Other conditions are as outlined in Methods

pH 6.5. In each case, the THase reaction with TNAD^+ as acceptor is seen to proceed *via* a ping pong mechanism at lower NADH concentrations, but at $\text{NADH} > 0.1 \text{ mM}$, the plots converge, indicative of a change to a sequential mechanism. All other reactions are sequential, displaying the same inhibition patterns. Thus DHase reactions are inhibited by both substrates, as are THase and ETase. The DPase reaction in both instances shows inhibition by DCIP, but what is typically considered an activation pattern at higher NADH concentrations. This behaviour is displayed by the RCM-monomer, but is uncharacteristic of native enzyme (cf. Section X where this point is discussed). Regression analysis of all primary plots leads to secondary plots from which the kinetic parameters of Table 24 are derived. Included in the Table, for comparison, are parameters obtained for isozymes II and V, of the same separations, by A.J. Wand of this lab. The same overall kinetic patterns were observed for this pair of isozymes, and there is no notable difference in the kinetic parameters of these four isozymes revealed in Table 24.

- Spectroscopy

Figure 38 shows the flavin absorption spectrum obtained from a single separation. There is a variable degree of denaturation as evidenced by the variable A_{355}/A_{455} ratios in Table 24, but no other differences in flavin structure and/or environment are indicated.

Table 24: Kinetic parameters of the isozymes. When two values are given, they are for complete kinetic determinations on two independent isolations. A refers to NADH, B to the oxidized substrate, the parameters being given in Cleland's nomenclature (see Appendix I). Values for isozymes II & V obtained by A.J. Wand, this lab., included for comparison. In general, the second preparation suffers less denaturation, and reflects the uncertainty of the values due to the isolation procedure, DHase being particularly sensitive.

Activity	Isozyme	$V_{\max}/E_t$ (min^{-1})	K_a ($\times 10^5$; M)	K_b ($\times 10^5$; M)	K_{ia} ($\times 10^5$; M)
THase	I	330,380	1.2,0.5	3.1,21	0.0,0.0
	II	300, -	4.2, -	3.0, -	0.0, -
	V	360,510	2.3,9.5	1.7,3.9	0.0,0.0
	VI	700, -	0.8, -	1.6, -	2.3, -
ETase	I	3500,21000	6.5,6.8	37,105	6.2,23
	II	-	-	-	-
	V	780, -	53, -	10, -	12, -
	VI	4050, -	2.3, -	18, -	15, -
DPase	I	750,3800	13,32	16,99	0.01,4.4
	II	900,2100	12,56	2.0,33	0.0,0.0
	V	1900, -	19, -	-	4.6, -
	VI	2200, -	20, -	15.5, -	1.8, -
DHase	I	- ,1100	-	-	- ,28
	II	2900, -	36, -	60, -	2.0, -
	V	1600, -	11, -	61, -	2.4, -
	VI	-	-	-	-

Figure 38: Absorption spectra of isozymes I-VI, from a single isolation. Isozymes are indicated on the curves. Recorded after dialysis against phosphate buffer (0.010 M, pH 7.0) as described in methods, and concentrations not corrected.

IV Structural Studies

i) Fluorescence decay

The exponential decay of fluorescence determined as described above is fit to equations of the form

$$F_I(t) = \sum_{i=1}^{\infty} A_i e^{-t/\tau_i} \quad (7)$$

where $F_I(t)$ is the fluorescence intensity at time t , τ_i is the lifetime of the i 'th decay component, and the pre-exponential factor A_i is an expression of the amount of the i 'th component present.

All data are initially fit to a single exponential decay model (i.e. $A_2 = A_3 = \dots = 0$ in eq.(7)), then to a double decay ($A_3 = A_4 = \dots = 0$). For higher order decays ($i = 3, 4, \dots$), the number of parameters ($2i = 6, 8, \dots$) becomes too large to meaningfully distinguish the possibilities. We are concerned here only with the improvement of fit on going from single (free FAD) to double (say FAD in a non-equivalent dimeric enzyme) exponential decay models. The statistical criteria for goodness of fit are described in the Methods.

In Table 25 are shown the fitting parameters for the systems studied. FAD and RCM-monomer are fit well by single exponential decay curves, and little improvement is gained by inclusion of a second term. All remaining samples, however, contain dimeric enzyme, and their decay curves are fit with marked improvement upon inclusion of a second exponential term. These conclusions

Table 25: Summary of fluorescence decay fitting parameters. See text. $\lambda_{ex} = 460$, $\lambda_{em} = 520$ for all samples. Data for single and double decay fits are included for comparison.

τ_1, τ_2 in ns.

Species	A_1	τ_1	A_2	τ_2	WSSR(a)	A_1/A_2
Unmodified enzyme	.0338	3.05			261	
	.0217	4.04	.0214	.920	163.5	1.02
Isozyme I	.0340	2.95			388.2	
	.0215	3.92	.0244	.811	37.9	0.88
Isozyme VI	.0372	2.72			143.5	
	.0275	3.48	.0239	.461	19.2	1.15
RCM-monomer	.0333	3.10			35.99	
	.0231	3.74	.0117	1.67	27.77	1.97
Enzyme + NAD ⁺ (c)	.0392	2.57			122.97	
	.0295	3.22	.0268	.383	5.57	1.10
FAD	.0355	2.84			17.23	
	.00393	4.93	.0320	2.60	16.22	.123

(a) Weighted sum of squares of residuals

(b) Sigma enzyme, unpurified

(c) $[\text{enzyme}]:[\text{NAD}] = 1:1$

are born out by the weighted residual plots (not shown). The final accepted decay times, and pre-exponential ratios for the double decay systems, are summarized for clarity in Table 26.

ii) Fluorescence energy transfer

Fluorescence spectra of lipoamide dehydrogenase, FAD, and L-tryptophan are shown in Figure 39. Excitation at 290 nm causes tryptophan emission ($\lambda = 358$ nm in phosphate buffer, pH 7.0; $\lambda = 342$ nm in enzyme) indicative of a non-polar enzyme environment (254). There is a strong FAD emission in the region of 510-520 nm upon excitation of the $\pi^* \leftarrow \pi$ transition at 465 nm. Emission from this band is also seen upon excitation of free FAD at $\lambda = 290$ nm. In the presence of energy transfer from tryptophan to FAD we expect excitation of tryptophan at 290 nm to enhance FAD fluorescence at 520 nm due to the non-zero overlap of tryptophan emission and FAD absorption spectra (Figure 39). Since FAD emission also results from excitation of that chromophore at the tryptophan absorption of 290 nm, the enhancement of the FAD fluorescence cannot be used to obtain the quantum yield enhancement as a result of energy transfer from tryptophan without correction for FAD bound to a hypothetical tryptophan-free enzyme. The requisite enhancement can be obtained directly, however, from the decrease in the tryptophan emission if the loss of intensity is assumed to be due to energy transfer to FAD. The decreased quantum yield is then found from the ratio of tryptophan

Table 26: Summary of fluorescence decay data. See text and Table 25.

Species	τ_1	τ_2	A_1/A_2
Unmodified enzyme	4.04	0.92	1.02
Isozyme I	3.92	0.81	0.88
Isozyme VI	3.48	0.46	1.15
RCM-monomer	3.10	-	-
Enzyme + NAD ⁺	3.22	0.38	1.10
FAD	2.84	-	-

Figure 39: Uncorrected fluorescence emission spectra ($\lambda_{ex} = 290$ nm) of (1) FAD (8.1×10^{-6} M), (2) L-tryptophan (1.5×10^{-5} M), and (3) Lipoamide dehydrogenase (8.4×10^{-6} M in FAD) in phosphate buffer, pH 7.0, 0.010 M, $T = 25 \pm 1^\circ\text{C}$

fluorescence intensity in flavin-free apoenzyme to that in holo-enzyme. The quantum yield of typtophan in the apoenzyme has been carefully determined by Visser *et al.* (254), and is equal to, in the terminology of equation (4), $Q_a = 0.067$. In these experiments, free L-tryptophan is taken as a standard, its quantum yield being accurately known as 0.14 (358,254), and all fluorescent yields are expressed relative to this value. The quantum yield for tryptophan in a given enzyme species, n , is then calculated as

$$Q_n^{\text{Trp}} = (0.14) \frac{\int_0^\infty F_n(\nu) d\nu}{\int_0^\infty F_{\text{Trp}}(\nu) d\nu} \frac{A_{\lambda_{\text{ex}}}^{\text{Trp}}}{A_{\lambda_{\text{ex}}}^n} \quad (8)$$

where $\int_0^\infty F_n(\nu) d\nu$ and $A_{\lambda_{\text{ex}}}^n$ are the relative, unitless integrated fluorescence intensity across the band, and absorbance at the excitation wavelength, respectively, of species n . The sub- or superscript Trp indicates the corresponding values for the L-tryptophan standard solution.

Assuming transfer from a single tryptophan residue to each enzyme-bound flavin molecule (see Discussion, and 254), and with $n = 1.45$ (318,319) and a calculated $J = (1.0 \pm 0.2) \times 10^{-14} \text{ mol}^{-1} \text{ cm}^6$, R_0 values of 21 Å and 20 Å are found for $\kappa^2 = 2/3$ and 0.476 respectively. Using $R_0 = 21$ Å (see Discussion), from equation (3) we find a value in the native enzyme of $r = 14$ Å as the FAD-tryptophan separation.

The experiment was repeated with the mono-alkylated E_{ma} and

the RCM-monomer, the fluorescence spectra of which are shown in Figure 40. The relative r values (i.e. relative to unmodified enzyme) are calculated as follows. For two species a and b ,

$$\frac{r_a^6}{r_b^6} = \frac{R_{o,a}^6}{R_{o,b}^6} \cdot \frac{(1/E_a - 1)}{(1/E_b - 1)} \quad (9)$$

In the series of samples investigated, the FAD spectrum changes little, and changes in $J^{1/6}$ should be negligible. Q_a is constant for tryptophan, and we can assume no better choice of n and κ^2 than above. Thus it can reasonably be assumed that $R_{o,a} = R_{o,b}$, and equation (9) reduces to

$$\frac{r_a^6}{r_b^6} = \frac{E_a^{-1} - 1}{E_b^{-1} - 1} \quad (10)$$

In addition we note that for species n ,

$$1 - E_n = (0.479) \frac{\int_0^\infty F_n(\nu) d\nu}{\int_0^\infty F_{trp}(\nu) d\nu} \frac{A_{lex}^{trp}}{A_{lex}^n} \quad (11)$$

from equation (8). The factor 0.479 converts the value based on tryptophan to that based on apoenzyme, i.e. $Q_a(\text{apoenzyme})/Q_a(\text{tryptophan}) = 0.067/0.140 = 0.479$.

Thus by measuring the fluorescence and absorption spectra

Figure 40: Uncorrected fluorescence emission spectra ($\lambda_{ex} = 295$ nm) of (1) native enzyme (7.3×10^{-6} M), (2) E_{ma} (8.1×10^{-6} M), and (3) E_{RCM} (5.6×10^{-6} M).

of any of a homologous series of enzymes, as well as those of a standard solution, the relative donor-acceptor distances can be found. Table 27 summarizes relevant data, indicating an unchanged tryptophan-FAD distance in E_{ma} , and a much shortened distance in RCM-monomer.

Measurements of quantum yields, defined as

$$Q = \int_0^{\infty} F(\nu) d\nu / A_{\lambda_{ex}} \quad (12)$$

are reported. These are normalized to a tryptophan standard excited at 280 nm with $Q = 0.14$. The values in Table 28 are intended to illustrate the degree of bound flavin character of FAD in the RCM monomer.

iii) FAD binding energy

As relevant to the nature of FAD binding and structure of the binding site, a calculation on the hydrophobic nature of the binding site is included. Janin and Chothia (359) have recently used estimates of protein surface solvation free energies to successfully and quantitatively explain protein - small molecule interactions. In attempts to further discuss the hydrophobic pocket for FAD association with lipoamide dehydrogenase, their calculations have been applied to the present system.

Janin and Chothia (359) find that in general a hydrophobic binding free energy of 25 cal for each square Angstrom of protein

Table 27: Summary of fluorescence energy transfer measurements. See text. Absorption and fluorescence maxima are given in nm with shoulders in parentheses.

Sample	Absorption		Fluorescence ($\lambda_{\text{ex}}=290$)		$\frac{\int_0^{\infty} F^{\text{Trp}}(\nu) d\nu}{\int_0^{\infty} F^{\text{FAD}}(\nu) d\nu}$		$E_{\text{Trp} \rightarrow \text{FAD}}$		$r_{\text{Trp} \rightarrow \text{FAD}}$
	$\lambda_{\text{FAD}}^{\text{max}}$	$\lambda_{\text{Trp}}^{\text{max}}$	$\lambda_{\text{FAD}}^{\text{max}}$	$\lambda_{\text{Trp}}^{\text{max}}$					
L-tryptophan	-	278(272)	-	352	-	-	-	-	-
FAD	450	-	520	-	-	-	-	-	-
Enzyme	455	274	515(585)	334	2.5	92%	14Å		
E_{ma}	448	272	512(586)	333	3.1	92%	14Å		
E_{RCM}	450	no max.	520(596)	334	5.5	99.7%	8Å		

Table 28: Quantum yields, Q , in phosphate buffer (0.01M, pH 7.0), at 25°C, relative to a tryptophan standard (see text). $\lambda_{\text{ex}} = 465$ nm. Averages of two experiments.

Species	Q	Literature value
FAD	$0.05 \pm .01$	0.03 (a)
Enzyme	$0.13 \pm .01$	0.12 (b)
RCM-monomer	$0.11 \pm .01$	-

(a) Reference 81
(b) Reference 185

Table 29: Signs of the MCD, arising from B terms, of various flavin systems. Entries give the signs corresponding to 1L_b and 1L_a transitions, respectively.

Solvent	Riboflavin	FMN	FAD
Water	(+,-)	(+,-)	(+,-)
10 mM phosphate, pH 7.0	(-,-)	(-,-)	(-,-)
Methanol	(-,-)	(-,-)	(-,-)

surface protected accounts for the stability of hydrophobic protein-protein and cofactor-protein interactions. They note that this value is in excellent agreement with that determined for alkanes and amino acids in aqueous systems from solvent transfer studies. We begin by noting that the association constants of FAD and FMN with apo-lipoamide dehydrogenase are $K_a = 2.0 \times 10^5 \text{ M}^{-1}$ (20°C) (233,234) and $0.1 \times 10^5 \text{ M}^{-1}$ (25°C) (236), respectively.* This corresponds to a $\Delta(\Delta G)$ of $-1.76 \text{ kcal.mol}^{-1}$, or noting that solvation is favourable by $\Delta G = -0.025 \text{ kcal.mol}^{-1}$, an additional $70 \text{ \AA}^2$ solvated by FAD binding. Both cofactors are reasonably assumed to bind in the open conformation. Additionally assuming the ribose moiety of the adenosine side chain to have no hydrophobic protecting ability, only the adenine ring must additionally be considered in FAD. A scale drawing reveals that adenine protects a triangular area of roughly $30 \text{ \AA}^2$. A similar FAD-FMN difference is found by simply wrapping aluminum foil to "solvate" scale Dreiding models. Since both protein and a face of adenine are protected, this value should be doubled (cf. 359), yielding $60 \text{ \AA}^2$.

We must, however, account for the unfavourable entropic contribution to the binding free energy. The translational component of the entropic partition coefficient is given by equation (13), (360) where R is the gas constant, N is Avogadro's number, h the Planck's constant, m the mass of the particle, k the Boltzmann's constant, T the absolute temperature, and V the volume

* The K_a of FMN increases with time to that of FAD, indicating induced fit accomodating conformational changes (236).

in which the particle is constrained.

$$S_T = R \ln \left[\frac{e^{5/2}}{Nh^3} (2\pi mkT)^{2/3} V \right] \quad (13)$$

Then at constant temperature, the entropic contribution gives

$$\Delta \left(\frac{\Delta G_{FAD}}{\Delta G_{FMN}} \right) \propto \left[\frac{\ln MW_{FAD}}{\ln MW_{FMN}} \right] \quad (14)$$

To a first approximation, the non-covalent interaction should not reduce the vibrational entropy of the side chain, and any losses in rotational entropy would merely enhance the effect argued here. For molecular weights of 830 and 480 for FAD and FMN respectively, and an average entropic contribution of 20 kcal.mol^{-1} to the total ΔG for small molecule cofactors (359), this represents a loss of $+1.8 \text{ kcal.mol}^{-1}$, eliminating the effect of $-1.5 \text{ kcal.mol}^{-1}$ contributed by $60 \text{ \AA}^2$ of hydrophobic association.

We can then conclude that either (i) the entire side chain is buried in a hydrophobic pocket, or (ii) there are specific (e.g. hydrogen bonding) interactions between adenosine and the protein, which then may also serve in an orientational capacity. Possibility (i) is unlikely due to the polar nature of the phosphate and ribose moieties of the side chain.

iv) MCD spectroscopy

In view of the near absence of published MCD data concerning the flavins (361), we prefaced our investigation of enzyme MCD with a solvent dependence study of the biologically important flavins, riboflavin, FMN, and FAD. Details will be published elsewhere, but a summary of the signs of the MCD of the flavins in various solvents is given in Table 29. Here we shall concern ourselves only with the MCD of FAD in potassium phosphate (0.01 M, pH 7.0) and methanol, as examples of solvents which place the flavin in ionic and less polar environments respectively. In methanol, the resolution of the flavin absorption spectrum approaches that in the enzymes.

Curve resolution of absorption and natural circular dichroism (CD) spectra of the flavins has been carried out by Edmondson and Tollin (362) and we shall follow their peak assignment. The 1L_b transition (ca. 450 nm) is resolved into three bands, designated from the low energy side as I-III, and are taken to be the (0-0) band and is $(0 + 1000 \text{ cm}^{-1})$ and $(0 + 1000 + 1500 \text{ cm}^{-1})$ vibronic components. The 1L_a transition (ca. 370 nm) resolves into two bands, IV (the (0-0) band) and V (the $(0 + 1000 \text{ cm}^{-1})$ vibronic component).

The MCD spectra of FAD in phosphate buffer and methanol are shown in Figures 41 and 42, with the absorption spectra included for comparison. Note that the scales are linear in energy, not in wavelength. Both absorptions display a negative MCD, in both solvents. That corresponding to the 1L_b is very weak in the buffered

Figure 41: MCD spectrum of FAD in 0.010 M phosphate buffer, pH 7.0 (rough line). The inverted absorption spectrum is shown as well (smooth line).

Figure 42: MCD spectrum of FAD in methanol (rough line). The absorption spectrum is shown inverted (smooth line).

solution, and increases somewhat in methanol, although it remains of lower intensity than that of the L_a band. The ordinate shows molar ellipticity per unit field. The increased resolution of the absorption spectrum in methanol is mirrored in the MCD.

The MCD spectrum for the same energy range of FAD in lipamide dehydrogenase is shown in Figure 43. Bands I-III retain the resolution characteristic of FAD in a less polar environment, in both absorption and MCD spectra. They also retain a negative MCD. In contrast to free flavin in any solvent, the second absorption assumes a sign reversal to give a positive MCD in the enzyme. The absorption bands begin to resolve more fully and optical activity results primarily from the $(0-0)$ band IV.

Upon full anaerobic reduction of the enzyme with dithionite, the EH_4 form of the enzyme is produced. The resultant hydroquinone is characterised by an ill-defined absorption beginning at ca. 475 nm and rising to lower wavelength. This feature is present in the MCD spectrum (Figure 44) as a broad negative MCD. A corresponding feature is present when free flavin is reduced by dithionite (Figure 45), but carries a positive sign, characteristic of the pure water solvent as found for oxidized flavins (not shown). An additional feature is present in the reduced enzyme spectrum only; a Faraday A term at 550 nm. When the enzyme is reduced with either NADH or dihydrolipoamide at room temperature, the EH_2 form is obtained exclusively. In both cases (Figures 46 and 47) the intensity of the MCD, associated with bands II and III increases from the value in the

Figure 43: MCD (rough line) and absorption (smooth line) of native lipoamide dehydrogenase in phosphate (0.010 M, pH 7.0).

Figure 44 (a): MCD spectrum of lipoamide dehydrogenase in phosphate (0.010 M, pH 7.0) reduced to EH_4 with a pinch of dithionite.

Figure 44 (b): Expansion of the A term region of part (a).

Figure 45: MCD spectrum of FAD in water, reduced with a pinch of dithionite. Inset shows an expansion of the 17,000 - 20,000 cm^{-1} region with one division equal to $5.8 \times 10^{-6} \text{ deg.cm}^2.\text{mol}^{-1}\text{G}^{-1}$.

Figure 46: MCD spectrum of lipoamide dehydrogenase in phosphate (0.010 M, pH 7.0) reduced with a 5-fold excess of NADH (rough line). Inverted absorption spectrum is also shown (smooth line).

Figure 47 (a): MCD (rough line) and absorption spectrum (smooth line) of lipoamide dehydrogenase in phosphate (0.010 M, pH 7.0) reduced with a 10-fold excess of dihydrolipoamide.

Figure 47 (b): Expansion of the A term region of part (a).

oxidized enzyme. The sign difference characteristic of enzyme-bound oxidized flavin is preserved. In both cases, the A term is present at 550 nm. This feature is also present when the enzyme is titrated to the EH_2 level with dithionite (not shown). In these four cases where the A term appears, it is of nearly constant intensity. When the rose bengal-photosensitized enzyme, E_{hv} , is reduced with NADH (Figure 48), this term is nearly wiped out.

The MCD of the flavin portion of glutathione reductase is shown in Figure 49. The absorption spectrum of this enzyme is similar to that of lipoamide dehydrogenase, except that all bands are red shifted. In the MCD, all features are of positive sign. Upon reduction by NADPH (Figure 50), a broad positive MCD is seen, and an A term appears at 546 nm. This A term is of somewhat lower intensity than those normally found in lipoamide dehydrogenase.

The MCD and absorption spectra of the mono-alkylated species E_{ma} are shown in Figure 51. The MCD spectrum is quite similar to that of native enzyme. In addition, however, a poorly defined A term is discernible at *ca.* 547 nm in the spectrum of the oxidized form of this (inherently reduced) enzyme. Upon reduction, the bleached flavin MCD spectrum is observed, and the A term increases in intensity (Figure 52).

The cobalt-modified enzyme, E_{Co} , shows a new weak, broad absorption centred around *ca.* 650 nm, with an extinction coefficient of $800 \text{ M}^{-1} \text{ cm}^{-1}$ (see 261). The MCD feature centred under this band has been shown above (Figure 33). In the flavin region

Figure 48 (a): MCD spectrum of rose bengal sensitized photooxidized enzyme (E_{hy}) in phosphate (0.010 M, pH 7.0) reduced with a 5-fold excess of NADH.

Figure 48 (b): Expansion of the A term region of part (a).

Figure 49: MCD (rough line) and absorption (smooth line) spectra of glutathione reductase in phosphate (0.010 M, pH 7.0)

Figure 50 (a): MCD spectrum of glutathione reductase in phosphate (0.010 M, pH 7.0) reduced with a 5-fold excess of NADPH. Hand drawn; see Methods.

Figure 50 (b): Expansion of the A term region of part (a).

Figure 51 (a): MCD (rough line) and absorption (smooth line) spectra of the mono-alkylated species E_{ma} , in phosphate (0.010 M, pH 7.0).

Figure 51 (b): Expansion of the A term region of part (a).

Figure 52 (a): MCD spectrum of *E. coli* reduced with a 5-fold excess of NADH, in phosphate (0.010 M, pH 7.0)

Figure 52 (b): Expansion of the A term region of part (a).

(Figure 53), all MCD terms are positive. When reduced to the EH_2 level with NADH, the usual A term centred at 550 nm is seen (not shown).

The MCD spectrum of oxidized lipoamide dehydrogenase in the presence of 2 mM NAD^+ is shown in Figure 54, in view of the binding studies reported below. There is evidence of a weak A term at 491 nm.

Figure 53: MCD (solid line; hand drawn) and absorption (dashed line) spectra of the flavin portion of the cobalt derivative of lipamide dehydrogenase, in phosphate (0.010 M, pH 7.0).

Figure 54 (a): MCD spectrum of lipoamide dehydrogenase in the presence of 2.0×10^{-3} M NAD^+ , in phosphate (0.010 M, pH 7.0).

Figure 54 (b): Expansion of the region of part (a) which lies under the NAD^+ binding site II absorption feature. See text.

V) Properties of Reduced Enzyme Forms, EH_2 and EH_4 .

i) Activities of reduced species

Since the two-equivalent (per FAD) reduced species, EH_2 , is the catalytic intermediate of the DHase reaction (cf. 225), the question arises as to whether the fully reduced EH_4 can be reoxidized at least to the level of EH_2 at a rapid enough rate to constitute catalytic activity with the oxidizing substrate. The only report of experimental evidence concerning this question has been given by Massey and Veeger (216), and their conclusion that EH_4 is inactive has been propagated throughout the literature (see for example 198,225).

Initially EH_4 was produced by reduction with excess dithionite and transferred by gas tight syringe to an anaerobic Thunberg cuvette containing reagents for assay. The spectrum of EH_4 produced by dithionite reduction is shown in Figure 55. The flavin chromophore is bleached. The assay activities are compared in Table 30 with those obtained for the same enzyme solution after reoxidation by exposure to the air until return of the E_{ox} spectrum. This eliminates the need for comparison with fresh enzyme, and accounts for possible damage to the enzyme by either full reduction or exposure to dithionite. When assays and reoxidation were rapid (within 15 minutes), the results of Table 30 were obtained. All activities are reduced in assays where enzyme is added as EH_4 , but significant activity remains (about 80% in the DHase reaction). In all cases, the

Figure 55: Dithionite reduction of lipoamide dehydrogenase. (1) Enzyme ($2.1 \times 10^{-5} \text{ M}$) is held anaerobically in phosphate (pH 7.0, 0.010 M) at 25°C. (2) A pinch of dithionite is added from the side arm of the Thurnberg cell.

Table 30: Specific activities ($\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) of EH_4 and reoxidized enzyme in the standard assays. See text for details. Average values of three experiments.

Activity	EH_4	$\text{E}_{\text{re-ox}}$	$\text{EH}_4/\text{E}_{\text{re-ox}}$
DHase	21.7 ± 7	27.6 ± 4	79%
THase	28.7 ± 4	46.7 ± 4	61%
ETase	33.0 ± 4	38.0 ± 2	87%
DPase	0.51 ± 0.1	0.86 ± 0.2	59%

Table 31: Activities of mono-alkylated enzyme (E_{ma}) or dithionite, as a percentage of those of the reoxidized species, in standard assays. Average values of three trials.

Activity	NADH-reduced	$\text{Na}_2\text{S}_2\text{O}_4$ -reduced
DHase (a)		
THase	56 ± 3	67 ± 8
ETase	99 ± 5	91 ± 4
DPase	102 ± 2	92 ± 3

(a) lacking in this species

activities of reoxidized enzyme are comparable to those of fresh enzyme, indicative of no damage on reduction to EH_4 for short periods of time. On longer reduction times (EH_4 maintained anaerobically for 30 minutes), DHase activity disappears, and does so irreversibly (i.e. is not returned upon reoxidation). This is accompanied by a general, though quite variable, decrease in the remaining activities. If the damage is due to prolonged lability of the flavin, the same effect should be observed to a lesser degree with EH_2 , as discussed below, and a more suitable control would be comparison of EH_2 and EH_4 at the same length of time after reduction. EH_4 as assayed in Table 30 retains 93% of the DHase activity of EH_2 (produced by reduction with a two-fold excess of NADH), supporting the view that the observed decreases are due primarily to damage to the enzyme by artificially enforcing maintenance of a reduced state. This point will be discussed further below.

The above experiment was repeated with E_{ma} , where now of course DHase activity is lacking. Both EH_4 (dithionite) and EH_2 (NADH) reductions were performed, and produce the spectra of Figure 56. Only THase activity (enhanced in this species; cf. Table 12) appears sensitive to reduction (Table 31). Again no real difference is seen between the effect of the two reducing agents, though here the effect is similar (see Figure 56 and later discussion).

The remaining demonstration of the activity of EH_4 is proof that any of the oxidized substrates can reoxidize this species. To produce the reduced form of Massey and Veeger (216), enzyme was reduced to

Figure 56: Reductions of E_{ma} with NADH (a) and dithionite (b). Part (a); Anaerobic enzyme solution (1.9×10^{-5} M in FAD) titrated with NADH stock solution to final concentration ratios of NADH/Enzyme of (1) 0, (2) 0.42, (3) 0.63, (4) 0.83; (5) 1.25, (6) 2.08. Part (b); Reduction with a pinch of dithionite. All samples in phosphate (pH 7.0, 0.010 M) at 25°C.

EH_4 with NADH in the presence of *Neurospora* NADase.* The OXase reaction has not been considered, as of course aerobic reoxidation of EH_4 to E_{ox} demonstrates this activity. Addition of NAD^+ , lipoamide, DCIP, or $\text{K}_3\text{Fe}(\text{CN})_6$ from the side arm of an anaerobic Thunberg cuvette in amounts comparable to those used in the assays, resulted in all cases in an immediate yellowing of the solution and a complete E_{ox} spectrum within the time of recording (5 minutes). Note that enzyme is about 10^3 as concentrated as in the assays, to allow a spectrum to be obtained. The reduction-reoxidation sequence is shown in Figure 57.

ii) NADase/NADH reductions

A vial of Sigma *Neurospora* NADase reported to contain 1.68 units of activity under optimal conditions was found to contain 0.6 units when assayed under conditions identical to those used for the enzyme reductions. Thus in the reductions reported here, 0.3 - 0.6 units of NADase are used, which is sufficient to give total destruction of an amount of NAD^+ equal to the amount of NADH added to its highest concentration (1.0 mM in 2.5 ml) in less than 10 minutes.

Before assays of reduced species were performed, the possibility of complication due to the presence of NADase and its reaction products was eliminated. Adenosine diphosphoribose (ADPR) is a potent inhibitor of some dehydrogenases (363). ADPR was included in standard assay mixtures at low ($[\text{ADPR}]:[\text{enzyme}] = 2:1$, as in reduction

* These reductions are discussed in Section (ii) immediately following.

Figure 57: Reoxidation of EH_4 by lipoamide. (1) Enzyme (2.6×10^{-5} M in FAD) in phosphate (pH 7.0, 0.010 M) at 25°C . (2) Addition of NADH (3.7-fold excess). (3) 10 min. after addition of 0.6 units of NADase. (4) After addition of lipoamide (5-fold excess).

conditions used) and high (0.10 mM) concentrations. As well, a mixture of the usual quantity of NADase and NADH at $[NADH] : [enzyme] = 2:1$ were included. Table 32 shows that neither of these treatments has any significant effect on DHase or DPase activities.

Figure 58 shows the spectral results of addition of NADase anaerobically to an enzyme sample titrated with NADH, followed by a 15 minute incubation. The bleached flavin spectrum indicates full EH_4 reduction, and the strongly sloping baseline is due in part to the crude NADase preparation.

Anaerobic assays show similar activities (Table 33) for this EH_4 species to those of EH_4 produced by dithionite (cf. Table 30); in contradicton to the conclusions of Massey and Veeger. The specific activities are somewhat lower after this treatment, upon reoxidation, in the DHase reaction, and higher in DPase, suggestive of some denaturation (see Discussion).

iii) Comparison of reduced forms

a) Redox potentials: Table 34 is a list of midpoint oxidation-reduction potentials gleaned from the literature, of species relevant to this and subsequent sections.

b) EDTA photoreductions: Massey and coworkers (110,190) have reported that the EDTA-mediated anaerobic photoreduction of flavoproteins, rapid with free flavins, is enhanced by catalytic

Table 32: Effects of possible inhibitors from the NADase reaction on lipoamide dehydrogenase standard assays. Stoichiometric concentrations refer to substance relative to molar quantity of lipoamide dehydrogenase. NADase added is 0.3 units. Activities are expressed as percent of the standard assay value. Values are from a single experiment.

Activity \ Addition	ADPR (2:1)	ADPR (0.10mM)	NADase + NADH(2:1)
DHase	88%	91%	102%
DPase	97%	106%	97%

Table 33: Activities of EH_4 produced by NADH reduction in the presence of NADase. See text. Activities ($v_i/E_t, \text{min}^{-1}$) are in standard assays. Values in parentheses are percent of control activity remaining. Average of two experiments.

Activity	$E_{\text{re-ox}}$	EH_4
DHase	745 ± 15	495 ± 30 (66%)
THase	1400 ± 35	1250 ± 45 (89%)
ETase	1150 ± 35	620 ± 40 (54%)
DPase	84.5 ± 0.5	46.5 ± 2 (55%)

Figure 58: Reduction of enzyme to the EH_4 level by NADH in the presence of NADase. (1) Enzyme (2.6×10^{-5} M in FAD) in phosphate (pH 7.0, 0.010 M) at 25°C. (2) With NADH:Enzyme = 0.45 (3) NADH:Enzyme = 0.91 (4) NADH:Enzyme = 1.82 (5) 10 min. after addition of NADase.

Table 34: Redox potentials of some relevant species, relative to the standard hydrogen electrode, taken from the literature.

Species	$E_{m,pH}$ (mV)	pH	Reference
O_2	+800	7.6	200
$K_3Fe(CN)_6$	+430	7.6	200
	+460	.01fNaOH	364
	+690	.1fH ₂ SO ₄	364
Benzoquinone	+240	7.6	200
DCIP	+160	7.6	200
$NAD^+/NADH$ (a)	-340	7.6	200
	-320	7.0	223
	-298	6.3	198
$TNAD^+/TNADH$	-285	7.0	223
$FAD/FADH\cdot$	-214	7.6	200
	-240	-	78
$FADH\cdot/FADH_2$	-170	-	78
Lipoamide or lipoic acid (b)	-345	8.0	210
	-325	7.6	200
	-286	7.0	210
	-229	6.0	210
Lipoamide dehydrogenase;			
E/EH_2	-280	7.0	272
EH_2/EH_4	-346	7.0	272

(a) varies -30 mV per pH unit at 25°C (210).

(b) varies -60 mV per pH unit at 25°C (210)

amounts of free flavins. In general, the semiquinone form of the flavoprotein is produced in a matter of hours without, and instantly with, the catalyst. We have found no such photoreduction after 3 hours without the catalyst. In the presence of a catalytic amount of riboflavin ($[\text{riboflavin}]:[\text{FAD-enzyme}] = 1:10$), partial reduction was seen after 200 minutes. As revealed in Figure 59, however, the normal EH_2 spectrum was appearing, and not that of either the typical red (anionic) or blue (neutral) semiquinone. Prolonged reduction times resulted in denaturation of the enzyme, possibly due to instability of the EH_2 already formed.

c) Reductions with NADH/NADase: Reductions with NADH give rise to a characteristic spectrum which results from a species which will be referred to as EH_2^{NAD} (e.g. see Figure 58). This spectrum persists despite addition of NADH at up to 100-fold excess, at pH 7.0 and room temperature, as has been reported (216). Addition of NADase to EH_2^{NAD} in the presence of excess NADH, however, causes flavin bleaching and stoichiometric reduction to EH_4 (Figure 58). These spectra are similar to those obtained by Massey and Veeger (216) and are included here for subsequent comparison to other forms.

d) Reduction by $\text{Na}_2\text{S}_2\text{O}_4$: With dithionite as the anaerobic reductant, the enzyme is titrated through EH_2 to EH_4 (Figure 60). It should be noted that here the EH_2 form is distinctly different from EH_2^{NAD} , but that spectra of EH_4 and reoxidized samples are

Figure 59: Illumination of an anaerobic enzyme solution (pH 7.0, 0.10 M) in the presence of 10^{-2} M EDTA and 5×10^{-6} M riboflavin. A 200 watt photoflood bulb at 20 cm is used; $T = 10 \pm 5^\circ\text{C}$. (1) Oxidized enzyme. (2) 80 min. illumination. (3) 140 min. illumination. (4) 200 min. illumination. Insets show typical semiquinone spectra produced by EDTA photo-reduction taken from ref. 198. (a) neutral (blue) semiquinone of thioredoxin reductase; (b) anionic (red) semiquinone of oxynitrilase.

Figure 60: Reductive titration of lipoamide dehydrogenase (5.1×10^{-5} M in phosphate, pH 7.0, 0.010 M) with an anaerobic solution of dithionite. $T = 25 \pm 1^\circ\text{C}$. (1) E_{ox} (2) Addition of 0.50 moles dithionite per mole of FAD. (3) 1.0 moles (4) 1.5 moles. (5) 2.0 moles.

identical for all reductants employed.

e) Attempted reduction with NADPH: Massey has reported without experimental detail (225) that NADPH reduces the enzyme to EH_2 at 10^{-5} to 10^{-6} times the rate of NADH. We have confirmed an insignificant degree of reduction by NADPH, and observed no measurable activity when it is substituted for NADH in any of the multifunctional assays.

f) Reduction with dihydrolipoamide: Since the DHase activity with lipoamide represents a reversible reaction, the reduced disulfide substrate dihydrolipoamide must be a potential reductant of the enzyme. In fact, an anaerobically stable form of EH_2 resulting from reaction with dihydrolipoamide has been well documented (225, 198). In Figure 61, anaerobic titration of E_{ox} with dihydrolipoamide is seen to give a spectrum distinctly different from that of EH_2^{NAD} , and attributable to a species denoted EH_2^{lip} is spectrally identical to the EH_2 species produced by dithionite or EDTA photoreduction. The spectrum persists, however, with no bleaching of flavin even at a 20-fold excess of reductant. The isosbestic points at 500 nm, 385 nm, and 352 nm indicate the presence of only two species, E_{ox} and EH_2^{lip} , with no detectable over-reduction or disproportionation to an EH_4 species. While the rate of the reverse DHase reaction measured with dihydrolipoate is much slower than with the amide derivative, possibly due to electrostatic hindrance suffered by the anionic species (cf. 225 and 210), addition of dihydrolipoate

Figure 61: Reduction of enzyme with lipoate and lipoamide, in phosphate (pH 7.0, 0.10 M) at $25 \pm 1^\circ\text{C}$. (1) E_{ox} , 2.9×10^{-5} M in FAD. (2) Reduced with a 20-fold excess of dihydrolipoamide, (solid line) or dihydrolipoate (dashed line). Performed on a 2.9×10^{-5} M stock solution divided in half.

4

to an anaerobic enzyme solution also causes immediate appearance of the red colour of EH_2 . The resultant spectrum is similar to that obtained with the reduced amide analog (Figure 61).

g) pH Dependence and interconvertibility of EH_2^{lip} and EH_2^{NAD} :

There is ample evidence (for instance the requirement of NADase to allow full reduction by NADH, and the binding studies of Section VI) to suggest that the unique spectral features of EH_2^{NAD} are due to interaction of NAD^+ with EH_2 . Following this line of reasoning, we have attempted to show that addition of NAD^+ to a sample of EH_2^{lip} will cause the appearance of the unique spectral features of EH_2^{NAD} . Figure 48 shows that this is indeed the case. Upon addition of NAD^+ to EH_2^{lip} at pH 7.0, the spectrum becomes virtually superimposable upon that of EH_2^{NAD} . Clearly the added NAD^+ may oxidize the enzyme, producing NADH and E_{ox} , which then react to produce EH_2^{NAD} . However, the catalyzed reaction

has a catalytic efficiency in the forward direction of 9 times that in the reverse, as written (225). Relating the thermodynamic equilibrium constant to the turnover rate *via* transition state theory means that the right hand side of the above equilibrium is favoured over the left by an order of magnitude in the final anaerobic equilibrium mixture. Thus $\text{EH}_2^{\text{lip}} + \text{NAD}^+$ should dominate the spectrum of Figure 62.

An additional check of the interconvertibility of the EH_2 forms has been done using a coupled enzyme reaction. Horse liver alcohol dehydrogenase has NADH-linked aldehyde reductase activity

Figure 62: Interconversion of reduced species (1) E_{ox} (3.2×10^{-5} M in FAD) in phosphate (pH 7.0, 0.50 M) at $25 \pm 1^\circ C$. (2) Reduction with a 10-fold excess of dihydrolipoamide. (3) Reduction of a duplicate sample with a 2-fold excess of NADH (solid line). Addition of a 2-fold excess of NAD $^+$ to the lipoamide-reduced species is also shown (dashed line).

(see Introduction). Thus it will remove NADH from the reaction mixture *via* the reaction

In the presence of a large excess of acetaldehyde, the reaction is essentially irreversible. A solution (2.5 ml) of lipoamide dehydrogenase (8.4 mg), LADH (10 mg) and acetaldehyde (20 μ l) was made anaerobic, and NADH (0.2 μ moles) was added. Failure to alter the oxidized flavin spectrum is proof that LADH was capable of handling all of the NADH (Figure 63). Next, with NAD^+ present from the LADH reaction, addition of dihydrolipoamide causes reduction directly to an EH_2^{NAD} spectrum, which is slightly improved by addition of further NAD^+ . Finally, addition of NADase causes reversion to the spectrum of EH_2^{lip} , completing the proof of complete interconversion of the EH_2^{lip} and EH_2^{NAD} spectral forms by NAD^+ .

At pH 5.7, the same two spectral forms of EH_2^{lip} and EH_2^{NAD} exist (Figure 64), however now a similar excess of NAD^+ to EH_2^{lip} does not cause marked spectral changes. Even at double the excess of that employed in Figure 48, the spectral conversion is only partial.

iv) Stability of reduced forms

To examine the lability of FAD in the reduced state, EH_2

Figure 63: Interconvertibility of spectra in the presence of an LADH-coupled system. (1) Eox (3.1×10^{-5} M in 2.50 ml phosphate, pH 7.0, 0.10 M) with 10 mg LADH. (2) Addition of 2×10^{-7} moles of NADH and 2.0×10^{-5} litres of CH_3CHO . (3) Addition of a 6-fold excess of dihydrolipoamide. (4) Addition of a 1.2-fold excess of NAD^+ . (5) 10 min. after addition of 0.6 units of NADase.

Figure 64: Attempted interconversion of E_{ox} forms at pH 5.7. (1) E_{ox} (2.3×10^{-5} M) in phosphate, pH 5.7, 0.10 M, $T=25 \pm 1^\circ C$. (2) Addition of a 5-fold excess of dihydrolipoamide. (3) Addition of a 2-fold excess of NAD^+ to (2). (4) Reduction of a duplicate E_{ox} sample by a 2-fold excess of NADH.

and EH_4 were produced with NADH and dithionite respectively, and transferred anaerobically (in a glove bag) to dialysis sacs, placed in degassed solutions, and dialysed for 200 minutes anaerobically under nitrogen. A control was merely stored reduced and exposed to the air at 200 minutes. The other samples were reoxidized similarly at this time, and centrifuged to remove some denatured protein existing in all dialysed samples. The results are shown in Table 35. Partial reoxidation by traces of O_2 remaining in the buffer is indicated for EH_2^{NAD} dialysed against buffer. Inclusion of 1 mM NADH in the buffer maintains the EH_2 species and causes more extensive loss of flavin, and denaturation as indicated by the A_{355}/A_{455} ratio. EH_4 proves very unstable in this treatment.

E_{ma} was also found to lose flavin by dialysis, as expected for a reduced species. The irreversibility of this reduction allows investigation by aerobic dialysis. In view of the suggestion (270) that NAD^+ favours covalent linkage of the flavin in this species (and cf. Discussion), it was of interest that, at various times of dialysis, no significant difference in the amount of flavin lost in the presence or absence of 1.0 mM NAD^+ could be observed.

Table 35: Removal of flavin from reduced enzyme forms by anaerobic dialysis, 200 min. at 4°C against 100 ml phosphate buffer (0.01 M, pH 7.0). The samples were subject to uniform slow stirring throughout. Amount of flavin remaining is compared to a control (not dialysed), determined by the A_{455} upon reoxidation. All samples were originally 1.00 ml, and are corrected for volume changes upon dialysis. Values are from a single experiment.

Species	% FAD remaining	A_{355}/A_{455}
Control (EH_2^{NAD} not dialysed)	100	0.94
EH_2^{NAD} (phosphate buffer)	95	1.05
EH_2^{NAD} (buffer with 1.0 mM NADH)	59	1.32
EH_4 (buffer + pinch $\text{Na}_2\text{S}_2\text{O}_4$)	<10	(a)

(a) not determined - too little flavin remaining.

VI) Nucleotide Binding Studies

i) Binding of NAD^+

On binding of NADH to the enzyme, the enzyme is reduced and NAD^+ is the bound nucleotide. If O_2 is present, the enzyme is reoxidized (OXase), and if conditions are anaerobic, only the perturbation of NAD^+ on EH_2 can be observed. Thus the binding of NADH cannot be observed directly.

The binding of NAD^+ does, however, produce changes in the bound flavin spectrum. Figure 65 shows the difference spectrum as lipoamide dehydrogenase is titrated with NAD^+ at pH 7.0, 23°C . Two positive absorption maxima are seen at 507 nm and 385 nm, and two negative minima at 350 nm and 445 nm, the latter having at least two variable shoulders at about 405 nm and 425 nm. The lack of isosbestic points is evidence that we are dealing with more than a simple interconversion of two species, and the variability of the middle region of the spectrum of Figure 65 suggests that there are two or more at least partially independent chromophores resulting from NAD^+ binding. Figure 66 shows a discontinuity in the plot of ΔA_{430} versus NAD^+ , supporting a multiple site situation.

A repetition of the experiment at pH 5.5 supports a two-site model, the minima growing in size while the low energy 507 nm band begins to disappear (Figure 67).

The difference spectrum produced by the binding of NAD^+ to

Figure 65: Difference spectrum of addition of NAD^+ to enzyme ($1.5 \times 10^{-4} \text{ M}$) in phosphate buffer (pH 7.0, 0.10 M) at $23 \pm 1^\circ\text{C}$. NAD^+ added to final concentrations of 9.96×10^{-5} , 3.92×10^{-4} , 1.31×10^{-3} , 1.82×10^{-3} , and $2.14 \times 10^{-3} \text{ M}$.

Figure 66: Titration of enzyme (8.1 mg.ml^{-1}) with NAD^+ . Difference spectrum at 430 nm, in 0.10 M phosphate, pH 7.0, at 23°C.

Figure 67: Difference spectra of enzyme + 2.14×10^{-3} M NAD^+ in phosphate buffer (0.10 M) at $23 \pm 1^\circ\text{C}$. (1) 16.1 mg.ml^{-1} enzyme, pH 7.0. (2) 13.8 mg.ml^{-1} enzyme, pH 5.5.

E_{ma} (Figure 68) provides a rationalization for the difference spectra of Figures 65 and 67. This spectrum is essentially a reflection of the absorption spectrum of this species with $\lambda_{max} = 448$ nm. In addition, a broad, negative band appears at the 530 nm region. A plot of ΔA_{450} versus NAD^+ for E_{ma} at pH 7.0 is shown in Figure 69. Unlike Figure 66, there are no discontinuities here, and a single binding site appears to be responsible for the quenched flavin spectrum of Figure 68(b).

We will now define the binding site-producing flavin quenching in E_{ma} as site I, and suppose (as yet unproven) that the positive absorption feature at 507 nm in the native enzyme is due to binding at a second site, site II. The pH dependence of these hypothetical sites is now investigated.

Figure 70(a) shows the pH dependence of binding to site I, as measured with E_{ma} . In Figure 70(b) the pH dependence of the 507 nm band of native enzyme is plotted. This absorption is identified with site II, and is considered to be well enough separated from other spectral features to be treated cleanly. Figure 70(c) shows the pH variation of ΔA at the wavelength of maximum value (somewhat variable) in the 450 nm region, for native enzyme. This region is clearly not clean, i.e. is at least a composite of the spectral features of sites I and II.

Suppose that in the native enzyme the overall spectrum $f(\lambda)$ is a composite of the sites I and II spectra, that is

$$f(\lambda) = f_I(\lambda) + f_{II}(\lambda) \quad (15)$$

Figure 68: Difference spectrum of NAD⁺ binding (2.14×10^{-3} M) to mono-alkylated E_{ma} ($5.0 \text{ mg} \cdot \text{ml}^{-1}$) at $23 \pm 1^\circ \text{C}$ in phosphate (pH 7.0, 0.10 M). No baseline correction has been applied.

Figure 69: Titration of E_{ma} (5.0 mg.ml^{-1}) with NAD^+ . Difference spectrum at 450 nm, in 0.10 M phosphate, pH 7.0, at 23°C .

Figure 70: pH dependence of NAD⁺ binding at sites I and II. Ordinate in arbitrary units. [See text.]
 (a) Site I, from E_{ma} . (b) Site II from native enzyme at 507 nm. (c) 450 nm region of native enzyme. (d) Site I-Site II, calculated from (a)-(b).

We now consider the values of these two functions at 450 nm as a function of pH, and write

$$f^{450}(\text{pH}) = f_I^{450}(\text{pH}) + f_{II}^{450}(\text{pH}) \quad (16)$$

If we assume that the binding site I is unchanged between E_{ma} and native enzyme, then $f_I^{450}(\text{pH})$ is given by Figure 70(a). We do not know $f_{II}^{450}(\text{pH})$, but will define

$$m = \Delta A_{II}^{450} / \Delta A_{II}^{507} \quad (17)$$

as we do know $f_{II}^{507}(\text{pH})$ from Figure 70(b). This factor m expresses the amount of feature II mixing into the region of measurement of feature I. It must reflect changes in band shape with pH, and so itself is a function of pH. Thus

$$f^{450}(\text{pH}) = f_I^{450}(\text{pH}) + m(\text{pH}) f_{II}^{507}(\text{pH}) \quad (18)$$

Clearly the spectra represent a problem for full curve resolution. Figure 70(c) would in principle allow us to compute $m(\text{pH})$, but the interpretation of this quantity is not straightforward. Let us assume, however, that $m(\text{pH}) = 1$, always. On this basis a calculated $f^{450}(\text{pH})$ is plotted in Figure 70(d). It is in good qualitative agreement with Figure 70(c), which supports the

interpretation thus far.*

We turn now to a fuller analysis of these data in terms of ligand binding equations.¹ Multiple binding sites are semi-rigorously handled by the generalization of the well-known Hill equation (365-368).

$$r = \sum_{i=1}^m \frac{n_i K_i A_f}{1 + K_i A_f} \quad (19)$$

where $r = A_b/E_t$.

Here the subscripts b, f, and t refer to bound, free, and total species present, respectively. E represents enzyme concentration, and A that of a small molecule which binds. The K_i are the intrinsic association constants of the multiple equilibria. In this generalization we assume m distinct classes of binding sites and n sites of each class. A strict application of this theory to the present situation proves difficult. For instance, we know only A_t and E_t , and measure a ΔA according to

$$\Delta A/l = E_b (\epsilon_b - \epsilon_f) \quad (20)$$

where l is the path length of the difference cuvette and the ϵ 's

* It is seen that m is slightly greater than 1 and increases with pH in the range 5-8. That $m > 1$ indicates $\epsilon_I^{M(E_{ma})} > \epsilon_I^{M(E_{Native})}$ or $K_{d,I}(E_{Native}) > K_{d,I}(E_{ma})$, or both.

are the molar extinction coefficients of bound and free enzyme. We assume that the small molecule ligand has no absorption in the spectral region measured, which is justified for NAD^+ at $\lambda > 400 \text{ nm}$. For multiple binding sites, equation (20) should be written as

$$\Delta A/l = \sum_{i=1}^m E_b^{(i)} (\epsilon_b^{(i)} - \epsilon_f) \quad (21)$$

We shall assume $n = 1$. Then if $m = 1$, equation (19) can be put in reciprocal form to yield, after appropriate substitution and manipulation,

$$1/K_1 = E_b^{-2} (E_t A_t) - E_b^{-1} (E_t A_t) + 1 \quad (22)$$

where $E_b = (\Delta A/l) (\epsilon_b - \epsilon_f)^{-1}$ (see Appendix II).

If $m = 2$, the situation is greatly complicated. The difficult curve fitting problem must be side stepped by approximate methods.* A similar problem has been faced by Stockell(369) who has defined a quantity p involving the fraction of sites bound. To compute this, one must assume that a graph of (quantity measured) versus ligand levels off at the saturation value for all sites filled, and that the fraction of sites filled at a given concentration is just the fractional value of the quantity measured. Stockell then shows

* The problem here arises because we have no way of directly measuring ligand binding. It may be argued that we observe enzyme, and may merely reverse the roles of A and E in equation (19). This, however, results in fractional m values which are not handled by the summation.

that

$$\frac{A_t}{p} = n + \frac{1}{K_d} \frac{1}{(E_t - p)} \quad (23)$$

where $p = E_t(\Delta A_{\text{obs}}/\Delta A_{\text{max}})$. A plot of A_t/p versus $(E_t - p)^{-1}$ then gives the number of independent sites n as the intercept and the dissociation constants K_1^{-1} , K_2^{-1} as the slopes of n linear regions.

Figure 71 shows Stockell plots for native enzyme at pH 7.0, plotted for $\lambda = 430$ nm and 507 nm. The two plots are in agreement, and indicate 2 ± 0.4 binding sites with dissociation constants of 0.31 and 1.0 mM. A similar plot at pH 5.5 (Figure 72) does not indicate a second site, rather one with a $K_d = 0.17$ mM.

With the assumption that E_{ma} has a single binding site, we can apply equation (19) directly, with $m = n = 1$. Linearity of a suitable plot will then prove the assumption. The equation is used in the reciprocal form due to Klotz (366,370)

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK_d} \cdot \frac{1}{A_f} \quad (24)$$

If $n = 1$, then

$$E_b = A_b = \Delta A_{450}/(\Delta \epsilon \cdot l) \quad (25)$$

Figure 71: Stockell plot of NAD^+ binding to native enzyme, pH 7.0 in 0.010 M phosphate, 23°C . See text for details. $\text{O} \cdots \text{O}$, measured at 507 nm; $\bullet \cdots \bullet$, measured at 430 nm.

Figure 72: Stockell plot of NAD^+ binding to native enzyme, as in Fig. 57, but pH 5.5 and 430 nm only.

where $\Delta\epsilon = \epsilon_b - \epsilon_f$. Also, the intercept of equation (24) is unity, and we may vary $\Delta\epsilon$ systematically until an intercept of one is obtained, and hence find ϵ_b . The slope will then give the dissociation constant of site I, K_d^I . This has been done by least squares analysis using a Fortran program (Appendix II) with the result that $K_d^I = 0.91$ and 1.4 mM at pH 7.0 and 5.8 respectively. The extinction coefficients of enzyme-bound flavin in the species with NAD^+ bound at site I are found to be 8.8×10^3 and $9.4 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ respectively. The final plots are shown in Figure 73.

Figure 74 shows fluorescence quenching of flavin on NAD^+ binding, and the change in fluorescence intensity with increasing NAD^+ concentration. According to Harbury and Foley (87), equation (26) obtains, and the dissociation constant

$$\frac{1}{F} = \frac{K_d}{(F_E - F_{EA})} \cdot \frac{1}{A_t} + \frac{1}{(F_E - F_{EA})} \quad (26)$$

K_d^I can be obtained as the ratio of slope to intercept of a plot of $1/\Delta F$ versus $1/A_t$. Here F_E and F_{EA} are the fluorescence intensities of free enzyme and bound enzyme respectively, and ΔF is the relative change in fluorescence intensity measured at a given A_t . Figure 75(b) represents such a plot of the data of Figure 75(a). It is linear and gives a K_d^I of 1.9 mM. The various K_d values obtained are summarized in Table 36.

That the decreases in the absorption spectrum parallel fluorescence quenching indicates that we are observing formation of a

Figure 73: Klotz plot of NAD⁺ binding to E_{ma}, 25°C.
 ●—●, pH 7.0; ○—○, pH 5.8. Fit by regression analysis
 to give an intercept of unity (see text).

Figure 74: Fluorescence titration of E_{ma} with NAD^+ . Enzyme is 3.5×10^{-5} M in FAD, at pH 7.0, 0.10 M phosphate at $T = 25 \pm 1^\circ\text{C}$. A stock solution of NAD^+ is made in an aliquot of enzyme sample to ensure no dilution upon addition. Only every second point shown for clarity. Additions of 2.5×10^{-4} M NAD^+ to 2.500 ml of enzyme solution are from the top, in microlitres; 0, 15, 25, 35, 45, 60, 80, 100, 120, 150, 200.

Figure 75: Change in FAD fluorescence upon binding of NAD^+ to E_{pa} ($35\mu\text{M}$) at 7.0 , 25°C . (a) Actual decrease in fluorescence as a percent. (b) Harbury-Foley plot of the data of (a). Regression line $y = (5.15 \times 10^{-3}) \cdot x + 2.74$ with a standard deviation of 0.44 .

Table 36: Summary of dissociation constants for pyridine nucleotide binding to lipoamide dehydrogenase. All values at $24^{\circ}\text{C} \pm 2$.

System	pH	K_d (mM)	Ref. or Method
$E_{\text{native}} + \text{NAD}^+$	7.2	0.05, 0.2	245
	7.0	0.2, 0.9	246
	7.0	0.3, 1.0	Stockell plot
	5.5	0.2	Stockell plot
$E_{\text{native}} + \text{TNAD}^+$	7.0	0.9	246
	7.0	0.02	Stockell plot
$E_{\text{ma}} + \text{NAD}^+$	7.5	1.9	Harbury & Foley plot
	7.0	0.9	Enforced Klotz fit ^(a)
	5:8	1.4	Enforced Klotz fit ^(a)

(a) Fit demanding an intercept of one site.

non-fluorescent ground state complex. We may then apply the Stern-Volmer relation (81,371)

$$I_0/I = 1 + K_a[Q] \quad (27)$$

where I_0/I is the ratio of intensities of incident and emitted light, K_a is the association constant of the complex, and Q is the static quencher. If we assume that the quencher is an enzyme structure (see Discussion), Then $[Q] = [FAD]$ (i.e. a 1:1 complex). The degree of quenching may be taken from references 245 and 246 to be 6 - 10% for native enzyme, at extremes of protein concentration of 8 - 10 mg.ml⁻¹. We then obtain

$$\Delta G = RT \ln\{[Q]/(I_0/I - 1)\} \quad (28)$$

which may be solved at the extremes of the conditions to give $\Delta G = 3.4 - 3.9$ kcal.mol⁻¹, spontaneous in the direction of complex formation. The validity of the Stern-Volmer equation in the present situation will be questioned (see Discussion), and these values are thus very approximate.

In the presence of 2 mM NAD⁺, the active (ETase, DPase) E_{RCM} differed markedly from dimeric enzyme. There was no quenching of flavin fluorescence, and no difference spectrum in the 350 - 600 nm region.

As a model to investigate flavin-flavin interaction upon NAD⁺ binding more fully (see Discussion), similar experiments were

undertaken with glutathione reductase, which binds NADP^+ in its oxidized state (198, 372-374) but has non-interacting paired FAD cofactors by X-ray analysis (375). No difference spectrum could be obtained, however, at pH 7.0, 25°C for 2.5 mg.ml^{-1} enzyme in the presence of 2.0 mM NADP^+ . The dissociation constant of the $\text{E}_{\text{ox}}\text{-NADP}^+$ complex has been estimated kinetically to be $70 \mu\text{M}$ (374). The fluorescence quenching effects of binding were not investigated, due to the non-fluorescent nature of the flavin in glutathione reductase (185).

ii) Binding of NAD^+ analogs

a) TNAD^+ : Titration of native enzyme with TNAD^+ results in a relatively clean reflection of the absorption spectrum (Figure 76), as is obtained from E_{ma} with NAD^+ when only site I is present. In addition, a broad negative absorption in the region 500-600 nm is again seen (cf. Figure 68). A Stockell plot gives a regression line with an intercept of 1.16 suggestive of a single binding site with $K_d = 0.020 \text{ mM}$ (Figure 77).

b) 3-AP-NAD^+ : Figure 78 shows the difference spectrum of binding of 3-AP-NAD^+ to native enzyme. Here the spectral changes even at high nucleotide concentrations are small, indicating a reduced binding constant, and accurate determination of this constant is not possible from the data. It is seen however, that the spectral

Figure 76: Difference spectrum of 1.7×10^{-4} M enzyme in phosphate (pH 7.0, 0.10 M), $T = 25 \pm 1^\circ \text{C}$, containing 1.6×10^{-4} M TNAD $^{+}$.

Figure 77: Stockell plot of TNAD^+ binding to native enzyme (0.17 mM), pH 7.0 in 0.10 M phosphate buffer, 25°C , at 450 nm.

Figure 78: Difference spectrum of 1.5×10^{-4} M enzyme in phosphate (pH 7.0, 0.10 M), $T = 25 \pm 1^\circ\text{C}$, containing 3.3×10^{-3} M 3-AP-NAD⁺.

features are similar to those of two-site NAD^+ binding (cf. Figure 65), containing the positive 507 nm absorption absent with single site binding of TNAD^+ (Figure 76), or of NAD^+ to E_{ma} (Figure 68).

c) $\epsilon\text{-NAD}^+$: The fluorescent analog $\epsilon\text{-NAD}^+$ was investigated as a possible energy transfer probe of the nucleotide-FAD separation distance. The difference spectrum, however, showed no change in the enzyme spectrum in the presence of $\epsilon\text{-NAD}^+$. Figure 79 and Table 37 indicate that there is no fluorescence quenching or enhancement under conditions where NAD^+ does reduce the enzyme FAD fluorescence. It is concluded that there is no (spectroscopically detectable) binding of $\epsilon\text{-NAD}^+$ to the NAD^+ binding sites.

Dehydrogenase activity in the reverse direction was measured with dihydrolipoamide as donor and either NAD^+ or $\epsilon\text{-NAD}^+$ as acceptor. Rates were calculated using $\epsilon_{340}(\epsilon\text{-NAD}^+) = 6.1 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (376,377). In this reaction, $\epsilon\text{-NAD}^+$ was found to have only 2-3% of the activity of NAD^+ , which may well be due to an inability of the enzyme to bind $\epsilon\text{-NAD}^+$. * This activity was not measured due to lack of sufficient spectroscopic distinction between NADH and $\epsilon\text{-NADH}$. The ETase and DPase reactions are irreversible.

*For four other dehydrogenases, Barrio *et al.* (376) find 7,7,19, and 31% of the activity remaining with $\epsilon\text{-NAD}^+$.

Figure 79: Uncorrected fluorescence emission spectra of ϵ -NAD⁺ and enzyme (1.7×10^{-6} M in FAD) in phosphate (0.010 M, pH 7.0), $T = 23 \pm 1^\circ\text{C}$. (1) Enzyme; $\lambda_{\text{ex}} = 288$ nm. (2) Enzyme, $\lambda_{\text{ex}} = 305$ nm (3) Enzyme + 1.0×10^{-5} M ϵ -NAD⁺, $\lambda_{\text{ex}} = 290$ nm. (4) Enzyme + 1.0×10^{-5} M ϵ -NAD⁺, $\lambda_{\text{ex}} = 305$ nm. (5) Spectrum of 1.0×10^{-5} M ϵ -NAD⁺ alone, $\lambda_{\text{ex}} = 305$ nm.

Table 37: Fluorescence data of ϵ -NAD⁺ binding, pH 7.0, 25°C. See text and Fig. 65. Entries are relative peak height intensities, with λ_{max} values in parentheses.

	Enzyme	ϵ -NAD ⁺	Enzyme + ϵ -NAD ⁺
$\lambda_{\text{ex}}=290$	94(335), 53(514)	34(394)	86(335), 47(513)
$\lambda_{\text{ex}}=305$	32(338), 19(512)	37(407)	29(339), 38(401), 20(514)
$\lambda_{\text{em}}=410$	-	37(309)	-
$\lambda_{\text{em}}=515$	-	-	45(291)

VII) The THase Reaction

In view of the above binding studies and isozyme kinetic results, a system can be designed for testing the mechanism of the THase reaction. The kinetic plots of Figures 36 and 37 show this reaction to follow a ping pong mechanism, switching to a sequential mechanism at concentrations of TNAD^+ (Figures 36 and 37) and NADH (occasionally observed before the onset of substrate inhibition; cf. Figure 37) above 0.1 mM. In addition there is evidence for two binding sites for NAD^+ and 3-AP-NAD^+ and one for TNAD^+ . Furthermore, E_{ma} is available as a species with a single pyridine nucleotide binding site. Of course the reaction between NAD^+ and NADH cannot be observed directly, due to the indistinguishability of reactants and products, but the kinetic mechanism of the THase reaction was investigated with TNAD^+ and 3-AP-NAD^+ as acceptors, for both native and monoalkylated enzyme species.

Kinetic plots for both analogs are shown for native enzyme and for E_{ma} in Figures 80 and 81. In Table 38 are collected the kinetic parameters for these plots. It should be noted for subsequent discussion that 3-AP-NAD^+ displays a sequential mechanism (convergent lines) for native enzyme, but goes ping pong with E_{ma} . This statement is based upon the observation that K_{ia} , while non-zero, has decreased from 207 to 7.50 μM . The compounded error in determination of this parameter should be noted (see Appendix I). TNAD^+ is ping pong in both situations, but proceeds ~~via~~ a sequential mechanism at higher (just sub-inhibiting) concentrations of NADH, in native enzyme only.

Figure 80: THase kinetics of native enzyme, 25°C, 0.010 M phosphate, pH 7.0 (a) and E_{ga} under identical conditions (b), with TNAD⁺ as the acceptor substrate. From top, $[TNAD] = 0.1, 0.2, 0.3$, and 0.4 mM.

Figure 81: THase kinetics of native (a) and E_{ma} (b) enzymes, as in Fig. 80, but with 3-AP-NAD⁺ as acceptor. From top, $[\text{NADH}] = 20, 40, 60, \text{ and } 80 \mu\text{M}$.

Table 38: Collected kinetic parameters from Figs. 80 and 81.

	$V(\mu\text{mol}\cdot\text{min}^{-1})$	$V/E_t(\text{min}^{-1})$	$K_{\text{NADH}}(\mu\text{M})$	$K_{\text{NAD}^+}(\mu\text{M})$	$K_{ia}(\mu\text{M})$
Native/TNAD ⁺	0.092	9200	120	240	0
Native/3-AP-NAD ⁺	0.035	3500	29.0	52.0	207
$E_{\text{ma}}/\text{TNAD}^+$	0.13	13000	33.8	146	0
$E_{\text{ma}}/3\text{-AP-NAD}^+$	0.045	450	42	222	7.50

VIII) The ETase Reaction

There is no difference spectrum of the enzyme produced by either potassium ferri- or ferrocyanide. Huang and Brady (261) have found no specific interaction with Fe(II). The reverse ETase reaction, reduction of NAD^+ by $\text{K}_4\text{Fe}(\text{CN})_6$, does not proceed, as predicted from the redox potentials of Table 34 for uncomplexed species. Thus no evidence of an iron binding site has been found, and Fe(III) is reactive with EH_2 and EH_4 only (cf. Section V(i)). Attempts to investigate the ETase activity with dihydrolipoamide or DCIP as the electron donor were unsuccessful, due to rapid reduction of $\text{K}_3\text{Fe}(\text{CN})_6$ by both reductants in the absence of enzyme.

i) pH Dependence

The pH dependence of this reaction is seen in Figure 82 to show a double maximum. The primary maximum is at pH 4.5 and shows a V/E_t value of $5.35 \times 10^4 \text{ min}^{-1}$. A second peak at pH 6.5 represents a lower activity of $2.1 \times 10^4 \text{ min}^{-1}$. It is noted additionally that strong NADH inhibition of this reaction is operative at $\text{pH} < 6.0$. At higher pH, the reaction is insensitive to NADH inhibition up to a final concentration of about 0.2 mM, but below pH 6, inhibition sets in at a concentration an order of magnitude lower, about 0.02 mM.

ii) Stoichiometry

The stoichiometry of the reaction, in the absence of

Figure 82: pH profile of the maximal velocity of the ETase reaction, at 25°C in 0.050 M buffer. At pH $\geq$ 6, buffer is phosphate. At pH $\leq$ 6, buffer is citrate, and values are corrected to the phosphate value from the overlap pH. V/E_t values are consistently ca. 30% higher in citrate. Here they are expressed as $\mu\text{moles} \cdot \text{min}^{-1}$ per mg of protein.

competing reactions, should reflect the overall stoichiometry of the balanced equation, i.e. $[\text{NADH}]$ consumed: $[\text{Fe(III)}]$ consumed should be 1:2. We have investigated the initial rates of NADH and Fe(III) consumption. The equilibrium stoichiometry could well be complicated by reversibility of competing reactions and/or subsequent disproportionation reactions. The enzyme concentrations (typically $0.10 \mu\text{M}$) are roughly three orders of magnitude below those of reagents, and any initial lag between consumption of the two reagents due to build up of an enzyme intermediate cannot be significant.

The rate of Fe(III) consumption can be measured as the change in absorption at 420 nm as usual, and will be called R_{420} . The rate of NADH consumption can be followed as the rate of change of absorption at 340 nm (R_{340}) when this value is corrected for the absorption of iron species at this wavelength.* The initial stoichiometry of consumption is then given by

$$\text{NADH:Fe(III)} = \frac{R_{340} - (0.54 \times R_{420})}{6.22 \times R_{420}} \quad (29)$$

The results show (Figure 83) that while at lower enzyme concentrations the stoichiometry is as expected, relatively higher concentrations show that at least 75% of the reducing equivalents

* We have determined a value $\Delta\epsilon(\text{K}_3\text{Fe(CN)}_6 - \text{K}_4\text{Fe(CN)}_6)$ of $\epsilon_{340}^{\text{Fe}} = 540 \text{ M}^{-1}\text{cm}^{-1}$ at this wavelength. Other extinction coefficients are $\epsilon_{340}^{\text{NAD}} = 6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{420}^{\text{Fe}} = 1.00 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$.

Figure 83: Stoichiometry of the ETase reaction. See text for details. The ordinate gives the ratio of moles of NADH consumed to moles of Fe(III) consumed in the initial stages of the reaction. Open circle; point determined anaerobically. Standard assay conditions are used with varied substrate concentrations.

available from NADH are channeled elsewhere, at a constant $[\text{Fe(III)}]:[\text{enzyme}]$ ratio of 5000:1. When this experiment is repeated anaerobically in a Thunberg cuvette at a relatively high enzyme concentration ($[\text{NADH}]/[\text{enzyme}] = 100$), the stoichiometry remains correct, i.e. $\text{NADH}:\text{Fe(III)} \approx 0.5$. The competing acceptor must be dissolved O_2 , and this finding has led to our subsequent investigation of the oxidase reaction (Section IX).

iii) ETase activity of modified species

The cobaltous ion derivative was shown in Section II to have enhanced ETase activity, and the kinetics of this E_{Co} species were investigated. Regression analysis bears out the visual interpretation of parallel lines in Figure 84; a value of $K_{1a} = 0$ is calculated. Thus the ETase reaction switches to a ping pong mechanism in E_{Co} . The inset of Figure 84 shows that the E_{Co} enzyme is more sensitive to $\text{K}_3\text{Fe(CN)}_6$ inhibition than is the native enzyme. Figure 85 shows a similar trend to a ping pong mechanism in the reductively carboxymethylated enzyme. A kinetic plot of the ETase activity of unmodified enzyme is shown in Figure 86 for comparison (and see also Figures 36 and 37).

iv) Radical scavenging and enhancement

An attempt was made to explore the free radical nature of this obligatory one-electron reaction, using radical trapping of a hypothetical reactive $\text{EH}\cdot$ intermediate. Quinone scavengers were avoided as competing substrates (225,258). Thiophenol or

Figure 84: ETase kinetics of the E_{Co} species, 0.010 M phosphate, pH 7.0, 25°C. Regression line of secondary plot gives $K_{ia} = -5.7 \mu\text{M}$. From top $[K_3Fe(CN)_6]$ is 40, 60, 80, 100 and 300 μM . Inset shows $K_3Fe(CN)_6$ inhibition for control (○—○) and E_{Co} (●—●) at $[NADH] = 0.10 \text{ mM}$. $V = 0.11 \mu\text{moles} \cdot \text{min}^{-1}$, $V/E_t = 1100 \text{ min}^{-1}$, $K_{Fe(III)} = 180 \mu\text{M}$, $K_{NADH} = 93 \mu\text{M}$, $K_{ia} = 0$.

Figure 85: E_t ase kinetics of E_{BCM} ; pH 7.0, 0.10 M phosphate, 25°C. From top, $[NADH] = 100, 150, 200, 250,$ and $500 \mu M$. $V = 0.0043 \mu moles \cdot min^{-1}$, $V/E_t = 43 \cdot min^{-1}$, $K_{Fe(III)} = 150 \mu M$, $K_{NADH} = 270 \mu M$, $K_{ia} = 0$.

Figure 86: Kinetics of the ETase reaction of unmodified enzyme at pH 7.0, 0.050 M phosphate buffer, at 25°C. From top, NADH = 50, 100, 150, and 200 μM . $V = 0.83 \mu moles \cdot min^{-1}$, $V/E_t = 8300 min^{-1}$, $K_{NADH} = 310 \mu M$, $K_{Fe(III)} = 1.0 mM$, $k_{ia} = 120 \mu M$.

nitrobenzene present at 0.10 mM final concentration both caused rapid denaturation of the enzyme, and in addition thiophenol caused rapid non-enzymatic reduction of DCIP. Sodium benzoate has also been shown to be a good radical trapper in biochemical systems (e.g. see 378). The presence of 0.50 mM sodium benzoate in the assay mixtures had no significant effect on any of the activities. It is possible that the $\text{EH}\cdot$ intermediate is not long enough lived to be trapped by external organic species.

Riboflavin was added to the assay mixture in the hope that intermolecular flavin-flavin interactions would compete with an intramolecular mechanism of electron transfer or stabilization of EH_2 . The inclusion of 20 μM riboflavin had no effect on DHase, THase, or DPase reaction rates, but even at 2 μM was an activator of the ETase reaction. Figure 87 shows the effect of increasing concentrations of $\text{K}_3\text{Fe}(\text{CN})_6$ and riboflavin at constant NADH concentration. At concentrations of $\text{K}_3\text{Fe}(\text{CN})_6$ sufficiently high to inhibit the normal assay, an activation is seen upon addition of traces of riboflavin. Note that only the slope of the line is affected. By analogy with competitive inhibition (139), the slope is multiplied by $(1 + [\text{A}]/K_{\text{act}})$ where $[\text{A}]$ is activator and K_{act} is the activation constant. The secondary plot gives a value of $K_{\text{act}} = 1.2 \text{ M}$, noting that this is the negative of an inhibition constant. We have noted a slight biphasicity in the rate of disappearance of $\text{K}_3\text{Fe}(\text{CN})_6$ at higher concentrations. This effect was also present, in the absence of riboflavin, in the partially denatured isozyme preparations. Riboflavin greatly increases this

Figure 87: ETase kinetics at 40 μM NADH, in the presence of varying amounts of riboflavin; phosphate buffer, 0.010 M, pH 7.0, 25°C. From top, [Riboflavin] = 0, 2, 4, 8, 10, and 20 μM . Velocities are from initial phase (see text) which becomes an unmeasurable burst phase at higher riboflavin concentrations (bottom dashed line).

effect, and at 5 μM cause the reaction to proceed in a cleanly biphasic manner, two linear regions being observed for the decrease of absorbance at 420 nm. Figure 87 represents the asymptotic region (first phase) only, which at 20 μM riboflavin has become so rapid as to constitute an initial burst which consumes essentially all the Fe(III) . Absorption spectra taken when the reaction has been allowed to go to completion show that no riboflavin is destroyed in the process, i.e. it is behaving catalytically, and causes no loss of NADH or $\text{K}_3\text{Fe(CN)}_6$ in the absence of enzyme.

The amount of $\text{K}_3\text{Fe(CN)}_6$ consumed in the initial phase was several orders of magnitude greater than the total amount of enzyme present, eliminating interpretations of the biphasicity based upon phenomena such as build up of an enzyme intermediate or half site reactivity. This amount also varied independently of NADH , as in the conditions of Figure 87 where it depends upon both $\text{K}_3\text{Fe(CN)}_6$ and added riboflavin, and under other conditions could be varied independently of both these reagents. This indicates that the biphasicity of the loss of $\text{K}_3\text{Fe(CN)}_6$ may result from a competing reaction, involving either unidentified substrates or products of the initial phase. The most likely candidates including O_2 (the OXase reaction) and H_2O_2 (a product of OXase ; *vide infra*), were subsequently investigated.

The inclusion of 0.10 mg of catalase in a single point ETase assay, in the presence of 10 μM riboflavin, caused a decrease of 26% in the initial phase rate. Thus the effect of added peroxide

(10 μ l of 30% H_2O_2) on all activities was investigated, and bore out an enhancement of ETase activity by this reagent (Table 39). Oxidase and DPase were enhanced, while DHase and THase were adversely affected. Under no conditions was significant reaction observed in the absence of enzyme. The OXase reaction is positively affected by H_2O_2 to a degree unmatched by the other reactions, and since H_2O_2 is a product of this reaction, the OXase reaction is autocatalytic. It is thus reasonable to assume that in the first phase of the ETase assay, the normal ETase reaction dominates, while OXase is minimal. This is in agreement with the stoichiometry studies at usual concentrations (see Figure 83). As H_2O_2 accumulates from low level OXase reaction, however, this reaction proceeds at an increased rate and dominates the second phase. Biphasicity of the isozyme preparations is consistent with this view, as partial denaturation has lowered initial ETase rates to levels where OXase competition can become significant; observable amounts of $K_3Fe(CN)_6$ still remain when H_2O_2 - enhanced OXase becomes predominant. To explain the biphasicity of the riboflavin catalysed reaction, we evoke the opposite situation, enhancement of OXase in the initial phase. This is borne out by Table 39, which shows that an increase in OXase comparable to that in ETase occurs in the presence of riboflavin.

The spectra of Figure 88 show that riboflavin is reduced by enzyme in the presence of NADH, and thus free reduced flavin is transiently present in assay mixtures containing riboflavin. When concentrations of riboflavin and enzyme-bound FAD are

Table 39: Effects of H_2O_2 and riboflavin on the multifunctional activities. The standard single point assays are used (see text). Riboflavin is added to $10 \mu M$, H_2O_2 to 0.3% w/v. Activities are expressed as percent of control (no additions).

Additions	DHase	THase	ETase	OXase	DPase
None	100	100	100	100	100
Riboflavin (a)	100 ± 2	100 ± 4	109 ± 3	108 ± 6	100 ± 6
H_2O_2 (b)	12 ± 4	63 ± 8	142 ± 6	255 ± 12	203 ± 10

(a) Average of three experiments

(b) Average of two experiments

Figure 88: Difference spectra of reduction of enzyme by NADH in the presence of riboflavin, against equimolar enzyme (3.8×10^{-8} moles in 2.50 ml phosphate, pH 7.0, 0.010 M) (1) Absorption of E_{ox} (2) Difference spectrum on addition of 3.7×10^{-8} moles of riboflavin. (3) After reduction of both sample and reference solutions with 12.5×10^{-8} moles of NADH. (4) Addition of a further 12.5×10^{-8} moles of NADH. (5) Absorption spectrum of reduced enzyme.

comparable, Figure 88 shows that initial additions of NADH preferentially reduce riboflavin, and excess NADH is needed to reduce enzyme.

v) Cytochrome c reductase activity

Massey (207-209) has established that lipoamide dehydrogenase is devoid of cytochrome c reductase activity, presumably due to steric factors. (see Discussion). We therefore investigated the possibility that the reaction could be catalyzed by a riboflavin "carrier". For the assay conditions described, we found an intrinsic activity of 6.5 min^{-1} of NADH-linked cytochrome c reductase in the unpurified Boehringer sample. This is 2-3 orders of magnitude lower than the lipoamide DHase activity under similar conditions and is a further test of the purity of this sample (i.e., indicating substantial freedom from contaminating cytochrome c reductase). No increase in this rate was found in the presence of $4 \text{ } \mu\text{M}$ riboflavin, possibly due to the much more rapid reaction of the free flavin (reduced) with O_2 than with cytochrome c (cf. 200).

E_{RCM} , a species with a naturally labile flavin and no OXase activity was checked, using the same rational, for cytochrome c reductase activity. With E_{RCM} , $V/E_t = 38 \text{ min}^{-1}$ was found, a six-fold increase over background, and a value comparable to native enzyme DPase activity under similar conditions.

IX) The OXase Reaction

The OXase reaction (catalytic oxidation of NADH in aerobic solution) must proceed *via* an ordered B_iB_i mechanism, as it represents reoxidation of EH_2^{NAD} . However here it is treated as a one-substrate reaction in NADH, as systematic alteration of the dissolved O_2 concentration is difficult.* The V_{max} value is then taken as the reciprocal of the intercept of the one-substrate double reciprocal plot.

The pH profile of unmodified enzyme shows an optimum pH of 4.4 for this reaction, and similar behaviour is observed with E_{ma} (Figure 89). Despite the decreased OXase activity of E_{ma} at pH 7, the V_{max} at pH 4.4 is double that of unmodified enzyme. E_{RCM} preparations which showed significant ETase activity and several-fold increases in DPase, could not be made to show OXase activity, a result which remains quite puzzling.

The native enzyme has been found to have increased sensitivity to NADH inhibition below pH 6 in other reactions.[†] An identical inhibitory effect was also noted for OXase. It is observed at all pH's for E_{ma} OXase, however, as it was for ETase. NADH inhibition begins at about 50 μM for native enzyme OXase (and ETase and DPase) below pH 6, and for E_{ma} this concentration causes inhibition

* At all times assays are done in O_2 saturated solutions, which when corrected for ambient temperature, pressure, and salt concentrations represent about 9 ppm O_2 .

† ETase - this work, Section VIII
 DPase - this work, Section X
 Quinone reductase - reference 258.

Figure 89: pH profiles of the OXase reaction, standardized to phosphate buffer. pH $\leq$ 6, 0.020 M citrate; pH $\geq$ 6, 0.020 M phosphate. ●-●, unmodified enzyme; ○-○, $E_{ma} \cdot V_{max}/E_t$ in $\mu\text{moles} \cdot \text{min}^{-1}$, per mg. of protein. $T = 25 \pm 1^\circ\text{C}$

throughout the range (Figure 90).

The enzyme also displays a dihydrolipoamide-linked oxidase activity, but due to the slow rate of this reaction and low extinction coefficient of substrate (ϵ_{233} of lipoamide = $151.4 \text{ M}^{-1} \text{ cm}^{-1}$; 364), the reaction was monitored with an O_2 -selective electrode. Relative rates in $\Delta(\text{ppm } \text{O}_2) \text{ min}^{-1}$ are converted to specific activities by comparison with the spectrophotometrically measured NADH reaction and assuming the same stoichiometries for the NADH and dihydrolipoamide oxidase reactions. The rate comparisons for reduced substrates at pH 5.0 and pH 7.0 are given in Table 40. The NADH activity is higher in both cases, but it increases at the lower pH, whereas the opposite situation is observed for dihydrolipoamide. Since both substrates reduce the enzyme rapidly, and O_2 is the anomalously slow oxidant, substrate oxidation is not expected to be rate determining, and the observed rates should not be governed by the oxidation potentials of NADH and dihydrolipoamide. A calculation based on the oxidation potentials of Table 33 bears this out. It predicts rate ratios $k_{\text{NADH}}/k_{\text{H}_2\text{-lipoamide}}$ of 14 and 1200 at pH 7.0 and 5.0 respectively, and also indicates that both rates should decrease with pH.

To test for the formation of peroxide in the OXase reaction, the O_2 concentration was monitored with the O_2 electrode. At various times after addition of lipoamide dehydrogenase to a solution of NADH as above, catalase was added to a final concentration of 0.06 mg.ml^{-1} , sufficient to cause rapid destruction of any peroxide present. According to equation (30), if O_2 is

Figure 90: OXase kinetics of E_{ox} in 0.050 M phosphate buffer, showing NADH^{ma} inhibition.
 ●—●, pH 5.0; ○—○, pH 7.0, $T = 25 \pm 1^\circ\text{C}$.

Table 40: Specific activities in the OXase reactions, measured by the O_2 electrode, in 0.05 M phosphate buffer, 26°C. Values in v_i/E_t (min^{-1}). Average of two experiments.

Reaction	substrate (mM)	pH 5.0	pH 7.0
NADH OXase	.06	75 ± 5	31 ± 2
Lipoamide- H_2 OXase	.50	4.3 ± 0.8	14 ± 2

converted solely to peroxide by the OXase reaction,

50% of it should be regenerated by catalase. In several determinations, $40 \pm 6\%$ of the O_2 was regenerated, while addition of catalase in the absence of lipoamide dehydrogenase had no effect on the oxygen concentration.

X) The DPase Reaction

i) Characteristics of 2,6-dichloroindophenol

In the oxidized state, 2,6-dichloroindophenol has a pK_a of 5.90 for protonation of the phenoxide form (379), and of 0.52 for protonation of the imine (380).^{*} Two resonance structures can be drawn for the phenoxide species, which become tautomers upon protonation (Scheme VII)

Scheme VII: Equilibria of DCIP (from references 379,380).

* Reference 380 states a pK_a of 5.57 for protonation of the phenoxide. Analysis of the additivity of the spectra of mixtures of the two species at pH values in the range of 5.8-6.2 leads us to confirm the higher value of reference 379.

The question arises as to whether the protonated phenol or deprotonated phenoxide form is the most reactive species for the DPase reaction, and which tautomer or resonance contributor is of major importance. The visible absorption spectra of the two species are shown in Figure 91, the acid form being obtained at pH 4.0, where only 3% of the basic form is present, and the basic form at pH 10.0, where essentially none of the acid exists. On superposition of spectra at intermediate pH's, the isosbestic point at 522 nm reported by Armstrong (379) is confirmed. Armstrong also reports the results of careful determinations of the extinction coefficients of the two forms. In the present work these values have been determined on the Coleman 124 spectrophotometer used in the kinetic studies. Due to inferior response characteristics of this instrument, the values used are somewhat lower than Armstrong's, but are in the correct ratio. For consistency with the kinetic data of Section (ii), our values are used throughout, and are as follows:

$$\epsilon_{522} = 5.36 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}; \epsilon_{600}(\text{DCIP}) = 1.68 \times 10^3 \text{ M}^{-1}\text{cm}^{-1};$$

$$\epsilon_{600}(\text{DCIP}^-) = 1.37 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}.$$

In determination of these values, the dry formula weight was corrected for 11.3% moisture as reported by the supplier. The value was confirmed by overnight drying at 110°C to be $12 \pm 1\%$.

The NMR spectrum in d_6 -DMSO is shown in Figure 92. In this solvent, the bright blue spectrum of the phenoxide form (inset) is obtained, though solvent red-shifted by 50 nm. In addition, no phenolic proton is seen up to 10 ppm offset in the 60 MHz spectrum.

Figure 91: Absorption spectra of 5×10^{-5} M DCIP (1) in phosphate-NaOH buffer at pH 10.0, (2) in phosphate-HCl buffer at pH 4.0, and (3) in phosphate (0.10 M, pH 7.0).

Figure 92: 60 MHz NMR spectrum of oven-dried DCIP in d_6 -DMSO with TMS standard. Inset shows the absorption spectrum of DCIP in DMSO at about 1×10^{-5} M.

The aromatic protons integrate 4:2 to quinoid protons.* This indicates that the dichloro-quinone, *para*-substituted phenoxide structure of Scheme VII is the major resonance contributor in DMSO.

When the reaction is assayed spectrophotometrically by DCIP absorption at a pH range of 6 ± 2 , the amount of both species present becomes significant, and rates must be corrected. When an absorption band is composed of two overlapping bands, A and B, the changes in absorbance at two wavelengths X and Y in this band are given by

$$\begin{aligned}\Delta A_X &= \Delta A_X^A + \Delta A_X^B = \epsilon_X^A \Delta[A] + \epsilon_X^B \Delta[B] \\ \Delta A_Y &= \Delta A_Y^A + \Delta A_Y^B = \epsilon_Y^A \Delta[A] + \epsilon_Y^B \Delta[B]\end{aligned}\quad (31)$$

where the superscripts refer to the species causing features A, and B and the subscripts refer to measured wavelengths. Thus two simultaneous equations must be solved for the concentration changes of species A and B. Here the $\Delta A_{X,Y}$ are measured as functions of time for the DCIP reaction, so that the concentration changes represent rates of disappearance of DCIP and DCIP⁻. By choosing one of X,Y to be 522 nm, the isosbestic point, only three extinction coefficients must be determined. The rate ratio for the two species, corrected for amounts present, obtained from the pK_a value and

* The protons of benzoquinone are a singlet at $\delta 6.8$ (381).

assuming first order in $[\text{DCIP}]$,* is then given by

$$\frac{R_{\text{DCIP}}}{R_{\text{DCIP}} - \Delta[\text{DCIP}]/t} = \frac{[\text{DCIP}]}{[\text{DCIP}] - \Delta[\text{DCIP}]/t} \quad (32)$$

where t represents time.

This point has been belaboured because it has been tacitly assumed in the diaphorase literature that one need merely measure the total change in absorption at a given wavelength, ΔA_{λ}^T , to get the total change in concentration, ΔC^T , using a single extinction coefficient ϵ_{λ}^T , that is

* The concentration dependence of the rate is actually of the form (see Appendix I),

$$v_i = \frac{V_{AB}}{K_{ia}K_b + K_aB + K_bA + AB}$$

If at constant A (NADH), B is increased n -fold, a first order dependence on $[\text{DCIP}]$ necessitates that $v_{i,2}/v_{i,1} = n$. The ratio is

$$\frac{n \cdot VAB}{VAB} \cdot \frac{K_{ia}K_b + K_aB + K_bA + AB}{K_{ia}K_b + nK_aB + K_bA + nAB}$$

which equals n only if $(K_aB + AB) = n(K_aB + AB)$, i.e. if A and K_a are small so that $(K_aB + AB)$ is small compared to $(K_{ia}K_b + K_bA)$.

This is approximately justified by the low concentrations of NADH used and the K_a for NADH of an order of magnitude lower than that for DCIP in this reaction at 25°C (Dr. C. S. Tsai, unpublished). The correction, however, is approximate. Note also the same K_b is assumed for DCIP and DCIP⁻.

$$\Delta A_X^T = \epsilon_X^T \Delta C^T \quad (33)$$

Clearly this is incorrect. In fact

$$\begin{aligned} \Delta A_X^T &= \Delta A_X^A + \Delta A_X^B \\ &= \epsilon_X^A \Delta[A] + \epsilon_X^B \Delta[B] \end{aligned} \quad (34)$$

and so

$$\begin{aligned} \epsilon_X^T \Delta C^T &= (\epsilon_X^A + \epsilon_X^B) (\Delta[A] + \Delta[B]) \\ &= \Delta A_X^T + \epsilon_X^A \Delta[B] + \epsilon_X^B \Delta[A] \end{aligned} \quad (35)$$

Thus equation (33) is not valid in an enzyme assay even if A and B are not distinguished by the enzyme.

ii) Species dependence of DPase

Using equation (31) and the above extinction coefficients, and the rates of DPase measured at 522 and 600 nm, the rates of disappearance of both species are then found by solving

$$\begin{bmatrix} 5.36 \times 10^3 & 5.36 \times 10^3 \\ 1.68 \times 10^3 & 1.37 \times 10^4 \end{bmatrix} \cdot \begin{bmatrix} \Delta[\text{DCIP}] \text{ min}^{-1} \\ \Delta[\text{DCIP}^-] \text{ min}^{-1} \end{bmatrix} = \begin{bmatrix} v_{\text{app}}^{522} \\ v_{\text{app}}^{600} \end{bmatrix} \quad (36)$$

having calculated the apparent maximal velocity, v_{app} , at each wavelength, in units of $\Delta A \cdot \text{cm}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$. The corrected rate ratio is then found from equation (32). This value is plotted

as a function of pH in Figure 93. The sharpness of the inflection is indicative that protonation of an enzyme group at pH 6.0 is responsible for favoring reaction of DCIP^- over DCIP. This argument is electrostatically sound. The rate ratio is calculated assuming a pK_a of 5.90 for DCIP. This value does not effect the inflection point of the relative rate *versus* pH curve (which depends only on the first term of the right hand side of equation (32)), but rather determines the difference between the ratios at extreme pH values.

An attempt has been made to show that, above pH 6.0, the rate ratio can be accounted for by a difference in redox potential of the two species. Assuming that for deprotonated enzyme

$$\frac{R_{\text{DCIP}}}{R_{\text{DCIP}^-}} = 2 = \exp \left[\frac{(\Delta G_{\text{DCIP}^-} - \Delta G_{\text{DCIP}})/kT}{1} \right] \quad (37)$$

and noting that 2 equivalents of charge transfer in the reduction, then a difference of 9.0 mV in redox potential accounts for the observed favoring of the phenol form.

Data are available for the 2,6-dibromo-analog (382), which has a similar redox potential and pK_a to DCIP. While the redox potential of the dibromo analog increases with decreasing pH, there is a discontinuity at the pK_a which corresponds to an excess of +40 mV in the phenol species (Figure 94). This is more than enough to account for the difference in reduction rates of the DCIP species in the absence of electrostatic factors.

Figure 93: Rate ratio of deprotonated to protonated DCIP forms as a function of pH. Calculated from V_{apparent} values at 522 and 600 nm, and corrected for relative concentrations assuming a first order dependence of rate on concentration (see text).

Figure 94: pH Dependence of the reduction potential of 2,6-dibromoindophenol, showing a discontinuity at the pK_a . Copied from Clark (382).

iii) DPase kinetics of denatured species

It has been noted that in the isozyme preparations the K_{ia} value decreases for the DPase reaction, and it was considered that this tendency to a Ping Pong mechanism might have resulted from destabilization of the ternary complex (or Theorell-Chance equivalent) by partial denaturation. Thus a sample of lipoamide dehydrogenase was partially denatured by exposure to ten cycles of freezing and thawing. The sample retained only 10% of control DHase activity and had an A_{355}/A_{455} ratio of 1.24.* A plot of the DPase activity of this species in Figure 95 shows nearly parallel lines, and regression analysis gives a negative K_{ia} value, indicating a shift to the Ping Pong mechanism upon denaturation. Note the low NADH concentrations. This species is extremely sensitive to NADH inhibition.

iv) DPase kinetics of E_{RCM}

Figure 96 shows that, whereas native enzyme experiences NADH inhibition of DPase activity, similar concentrations with E_{RCM} cause an activation. The K_i for NADH is increased about 9-fold, from 40 μ M to 360 μ M. Native enzyme is shown in Figure 97.

* Denaturation was also seen by rapidly bubbling nitrogen through enzyme solutions to cause foaming. This produced A_{355}/A_{455} ratios as high as 1.6, with occasional complete loss of DHase activity, but was not sufficiently reproducible to warrant further investigation. Sonication at cavitation threshold levels for 30 minutes had no detectable effect on the enzyme.

Figure 95: DPase kinetics of enzyme denatured by a freeze-thaw cycle. See text. Phosphate buffer; 0.010 M, pH 7.0, 25°C. From top, [DCIP] = 15, 20, 25, and 30 μM . $V = 0.012 \mu\text{moles} \cdot \text{min}^{-1}$, $V/E_t = 2.4 \text{ min}^{-1}$, $K_{\text{NADH}} = 22 \mu\text{M}$, $K_{\text{DCIP}} = 33 \mu\text{M}$, $K_{ia} = 0$.

Figure 96: Kinetics of the DPase reaction catalysed by E_t ; phosphate buffer, pH 7.0, 0.050 M, 25°C. From top $[DCIP]_{RCM}$: 20, 30, and 50 μM . $V = 3.6 \times 10^{-3} \mu\text{moles} \cdot \text{min}^{-1}$, $V/E_t = 36 \text{ min}^{-1}$, $K_{NADH} = 1.4 \mu\text{M}$, $K_{DCIP} = 28 \mu\text{M}$, $K_{ia} = 36 \mu\text{M}$.

Figure 97: DPase kinetics of native enzyme; phosphate buffer pH 7.0, 0.050 M, 25°C. From top, $[DCIP] = 20, 30, \text{ and } 50 \mu M$. $V = 4.3 \times 10^{-3} \mu moles.min^{-1}$, $V/E_t = 43 min^{-1}$, $K_{NADH} = 16 \mu M$, $K_{DCIP} = 29 \mu M$, $K_{ia} = 14 \mu M$.

v) pH Dependence of DPase

In determining the pH dependence of the DPase reaction, we must correct the observed value of the rate for the amounts of the two DCIP species present, as well as for their inherently different rates above and below pH 6.0. This has not been considered in previous studies. When it is taken into account, the pH profiles of Figure 98 are obtained. These values are calculated by determining $V_{\max}$ at 522 nm and assuming the overall rate to be due to the sum of rates of both species. At the pH extremes, the values are quite certain, due to the low concentration of one species present. A pH optimum of about 7.6 is indicated for both species.

Figure 98: pH profile of DCIP measured at 522 nm (●—●), and corrected profiles of DCIP^- (○—○) and DCIP (Δ — Δ); see text.

DISCUSSION

We have noted and investigated the isozyme composition of native lipoamide dehydrogenase, and have in most cases worked with the native mixture. We have probed the structure-function relationships of the enzyme by way of its type C2 multifunctionality, and have exploited specific chemical modifications, techniques of kinetic analysis, and sensitive spectroscopic probes of the flavin. If ambiguity is to be avoided, then, similarity of the isozymes in terms of primary structure, kinetic mechanisms, and active centre arrangement must be demonstrated. Previous work reviewed above has indicated such similarities, and we have supplemented these observations. In addition, we have shown the kinetic properties of the native enzyme mixture to be shared by all of its constituent isozymes. Properties of the isozymes are summarized in Table 41, and are discussed further below. Detailed investigation of the native enzyme mixture appears justified.

Previously, alkylation of lipoamide dehydrogenase has been carried out with iodoacetamide in slight excess, reacting with the EH_2 form of the dithionite-reduced enzyme (269). One nascent thiol is modified, and the enzyme is trapped with its disulfide broken. We have carried out a fuller investigation of the dependence of modification upon reducing reagent and alkylating reagent, in the hope of further altering the multifunctional properties of the enzyme.

Alkylation of the β -mercaptoethanol reduced enzyme with

Table 41: Properties of the isozymes of lipamide dehydrogenase.

Isozyme	I-III	IV-IV	Reference
Origin	PDC	α -KGDC	274, 275
Molecular weight	10 ⁵		274
Sedimentation ($S_{w,20}$)	5.4, -5.5 S		275
Amino Acid Composition	5 fractions identical in each a.a. to $\pm 10\%$		274
N-terminal	Ala		This work, 274
Absorption Spectra	Identical resolved FAD spectra $\lambda_{max} = 356, 455$		
ORD: $[\alpha]_{233}^b$	-4241	-4140	
CD: $[\theta]_{370}$	-99	-103	274
pI	+31,900	+50,900	
FAD Fluorescence Decay	Broad dichroism 450 nm 5.6-6.0 5.8-6.7	none 6.5-6.8 7.0-7.7	276 This work
Kinetic patterns (I, II, V, VI)	(I) $\tau = 3.92, 0.81$ ns biphasic, $A_1/A_2 = 0.88$	(VI) $\tau = 3.48, 0.46$ ns biphasic, $A_1/A_2 = 1.15$	"
DRase	Similar inhibition/activation patterns in all rxns.		"
THase	Ping Pong, becoming BiBi at high nucleotide		
ETase	BiBi		
DPase	BiBi, approaching Ping Pong with denaturation		

iodoacetate has been demonstrated to produce a monomeric species of a molecular weight of about 4.8×10^3 daltons. This species has no tendency to reassociate to dimer, and will be seen to be not only useful in discussing the functional attributes of monomeric and dimeric species, but, due to the drastic changes in FAD environment attendant upon its production, useful as a model in understanding the effects of the protein environment on flavin chemistry as well. The flavin spectrum in the visible region strongly resembles that of free FAD under the same conditions. The difference spectrum obtained against equimolar free FAD is slight. This difference spectrum shows tailing in the intermediate UV region which results from enhanced protein absorption. It is argued that this is due to exposure of a second tryptophan residue per subunit on dissociation. The flavin is readily removed by dialysis and prolonged gel filtration chromatography. Six lines of evidence argue for an association of flavin with the reductively carboxymethylated monomer, however.

i) The enzyme is still reduced by NADH, resulting in a rapid bleaching of flavin anaerobically, as observed with flavoproteins and immobilized flavins, but not with free flavins.

ii) Reconstitution experiments show that one mole of flavin per mole of apoenzyme monomer is necessary (and sufficient) for maximum catalytic activity. This argues for a specific, single site interaction, rather than exclusively a rapid turnover of flavin, as has been suggested to account for residual activity of the apoenzyme monomer of the unmodified enzyme(198).

iii) By gel filtration, the flavin has a much higher molecular weight than a species below the fractionation ranges of the columns. This phenomenon can be explained by an association with protein which shifts the partition equilibrium of flavin between mobile phase and gel particle interior in favour of the protein-containing mobile phase.

iv) There is a slight difference spectrum for the visible region of reductive carboxymethylated monomer and free FAD.

v) Rapid denaturation of the enzyme occurs upon complete removal of FAD, indicating a stabilizing role of flavin association.

vi) The quantum yield of flavin fluorescence in monomer is found to be 0.11, comparable to that of enzyme-bound flavin (0.13), and enhanced 4-5-fold over free flavin. The slightly lower Q value for the monomer, compared to the native enzyme, may well be accounted for by the excess free flavin which results upon precipitation of some apoprotein in the acidic carboxymethylation step.

Extensive conformational changes are seen to accompany modification. The α -helical content of E_{RCM} is increased, much as observed by us and others (238) for unmodified apoenzyme monomer. There is some evidence for slight increases in the random coil content of the protein also, which would necessitate the conclusion that conformational changes are occurring at the expense of β -sheet structure. This is consistent with the substantial decrease in the intensity of the large 1630 cm^{-1} component of the native enzyme upon modification. This absorption

is uniquely associated with β -structure (cf. Table 10).

Here monomerization may also be the result of conformational changes arising from modification of some residues, or from the concomitant removal of flavin, or from the acid treatment itself. In early methods of apoenzyme preparation (227), a low pH was used and resulted in a monomeric species. Addition of the excess iodoacetate causes a drop in pH of approximately 4 units, and this effect is necessary for extensive modification. The reassociation may be blocked by the extensive conformational alterations, but since apoenzyme monomer will reassociate in the presence of flavin (233), the current modification must render the dissociative conformational changes irreversible. The introduction of about 21 carboxymethyl groups per monomer may also sterically hinder the hydrophobic reassociation process (cf. 233).

The reductive carboxymethylation modifies 21-22 residues, by amino acid analysis, including all methionine residues. This is at first sight puzzling in view of the report by Crestfield *et al.* (383) that β -mercaptoethanol protects methionine in carboxy methylation studies. However, their work was carried out at pH 8.6. It is likely that at the low pH attained in the present study, methionine modification is subject to general acid catalysis. Deprotection at low pH could also result in formation of the carboxymethylsulfonium derivative by $\text{ICH}_2\text{CO}_2^-$, which decomposes

to several products upon hydrolysis with 6N HCl* (303).

Modification of sulfhydryl residues is decreased by the β -mercaptoethanol pretreatment, which must therefore protect some residues from modification[†]. This protection could take two forms; (i) direct protection through mixed disulfide formation in excess β -mercaptoethanol (cf. 385), or (ii) conformational changes in the presence of sulfhydryl reagents (cf. 231) could mask some reactive-SH groups. It is clear, however, that pretreatment with β -mercaptoethanol is necessary to produce the monomeric species observed. Conformation changes are induced which alter the reactivity patterns of cysteine, methionine, and tyrosine towards carboxymethylation, and result in a stable monomer, extensively modified but retaining ETase and DPase activity, which has a limitingly labile flavin. The sulfhydryl

* We have recently checked this proposal by modification of horse liver alcohol dehydrogenase in the same manner. With the exception of cysteine (not determined), amino acid analysis (Beckman 119 BL system) showed exclusive modification of methionine, with still some modification at pH 8.6 but more extensive modification at pH 3.0. Numbers of methionine residues were: control, 8.9 (published, 9 (384)); pH 8.6 modification, 6.3 residues; pH 3.0 modification, 4.7 residues.

[†] Because the flavin chromophore itself has a non-negligible absorption at 250 nm, the wavelength monitored in the PCMB titration, it must be assumed that its value remains constant throughout, and this may not be the case if dissociation from the protein occurs (225). This dissociation appears not to occur upon treatment of unmodified enzyme with PCMB (comparing the number of residues detected with that from amino acid analysis, and cf. 217). In the E_{RCM} species the flavin is, for practical purposes, fully dissociated to begin with. Dissociation is a problem in mercurial determinations of reduced native enzyme, however (217).

reagent may also be viewed as favoring monomerization. However, the identity of the flavin visible spectra before and after β -mercaptoethanol treatment indicates that the active centre disulfide is not reduced.

When dithionite is used as the reductant, a slight excess of iodoacetamide gives specific modification of one active site nascent thiol (269). This is accompanied by a blue shifted flavin spectrum ($\lambda_{\max}$ going from 455 to 448 nm), indicating loosening up of the hydrophobic flavin binding site. We have observed that the flavin is removable from this species by dialysis against neutral phosphate buffer, in marked contrast to the native enzyme, indicating further the partial loosening of the cofactor. The E_{ma} remains dimeric. Iodoacetic acid substituted for the amide in this procedure does not have the same effect, but gives a modification pattern more characteristic of that obtained with the $\text{HSCH}_2\text{CH}_2\text{OH}/\text{ICH}_2\text{CO}_2\text{H}$ sequence. It would appear that the anionic reagent cannot approach the active centre, and more conclusive evidence for this interpretation will be discussed below. There is a distinct possibility that the two cysteine residues which appear to remain in the reductively carboxymethylated monomer originate from the active site disulfide. Both $\text{Na}_2\text{S}_2\text{O}_4/\text{ICH}_2\text{CO}_2\text{H}$ and $\text{HSCH}_2\text{CH}_2\text{OH}/\text{ICH}_2\text{CONH}_2$ combinations have less drastic effects than the other two sequences, allowing some DHase activity to remain. This indicates that the active centre disulfide is left intact in these modifications. Furthermore, it is evident that both the charge of the alkylating reagent and the conformational dependence

upon sulfhydryl reagents must be considered.

That the steric requirements for entry into the active centre are also rather strict is shown by the attempts at spin labelling. While EH_2 reacts readily with stoichiometric amounts of $\text{ICH}_2\text{CONH}_2$, elaboration of the amide with hydrophobic side chains effectively prevents any reaction.

As shown in the Introduction, fluorescence work has been used to argue for FAD binding in the open conformation, and the hydrophobicity of the binding site is well established. The evidence presented here is consistent with binding of the flavin in an extended conformation, with the isoalloxazine and possibly adenine moieties in a hydrophobic pocket. The high quantum yield of the enzyme-bound flavin (89) and strong fluorescence (185) rule out the usual intramolecular quenching of free FAD. Thus the cofactor is generally accepted to be bound in the open conformation. Speculation upon the relative orientations of the disulfide and NAD(H) binding sites to the flavin is offered below. Product analyses of flavin photodegradation reactions outlined above (78,106-108) have shed light on the photochemical pathways open to these systems, and the reluctance of the FAD molecule of lipoamide dehydrogenase to react photochemically supports rigid binding in an open conformation. Attack by $\text{N}(1)$ of the $2'$ -position of the naturally occurring flavins may result in Norrish type II hydrogen abstraction in a 6-membered transition state (Scheme VIII(a)), or in abstraction of the hydrogen from the $2'$ -hydroxyl group in a 7-membered transition state (Scheme VIII (b)). Both reactions occur

Scheme VIII: Transition states for abstraction of H $\cdot$ from the 2'-carbon (a) or 2'-OH group (b) of the ribitol side chain of a flavin. See text.

(a)

(b)

in the singlet as well as triplet state, and the increased electron density on N(1) found in the triplet (97) is not necessary for photodegradation. Thus failure to form a triplet, or a redistribution of spin density, in the enzyme can be ruled out as a source of the photochemical stability of bound FAD.

Examination of models and Scheme VIII shows that if the side chain is held rigid with a staggered conformation at all carbon centres, or is fully extended, these intermediates cannot form. Rigid binding of FAD in the open conformation to lipoamide dehydrogenase is sufficient reason, therefore, for the unusual photochemical stability of this flavoprotein.

Demanding an open binding conformation ensures that the adenine ring will also be available for binding. Following the method of Janin and Chothia (359), we have calculated the expected hydrophobic binding energy of adenine alone. This binding energy is insufficient to account for the reported (236) differences in the association constants of FMN and FAD with lipoamide dehydrogenase. Rather, the anchoring of the side chain by more specific interactions is implied, and will have an orientational effect on the bound cofactor. The association constant for binding of FMN to apoenzyme rises with time to that of FAD (236). This suggests an induced fit mechanism which can accommodate cofactor binding through isoalloxazine-protein interaction. This tight interaction accompanies loss of activity, however, and it may be suggested that the adenosine moiety of FAD promotes strong binding while allowing sufficient exposure of the isoalloxazine nucleus for chemical

reactivity. The adenine moiety may serve to bind the flavin while at the same time maintaining a sufficiently open active centre for substrate approach. It may also serve in an orientational capacity, maintaining a correct geometry of the flavin with respect to the disulfide redox couple and/or substrate binding sites. Thus the picture emerges of the isoalloxazine ring hydrophobically stabilized, possibly over a planar disulfide ring structure containing six amino acid residues (cf. 265, and a similar argument for the *E. coli* enzyme (386,387)), and in a rigidly specific orientation with respect to catalytic and binding sites. The side chain is peeled back and itself helps in binding and orientation, and possibly has a role in flavin determination of protein conformation, while also allowing enhanced fluorescence and exposure of the isoalloxazine ring for chemical reaction with a variety of substrates.

The proximity of a tryptophan residue to the flavin binding site has been confirmed by fluorescence energy transfer measurements.

Earlier work had found a distance of Trp $\rightarrow$ FAD of 13-16 Å (254), consistent with our value of 14 Å, based on the assumption that only one tryptophan residue per flavin is transferring energy. This assumption has been justified by the work of Visser *et al.* (254). Polarization spectra showed both tryptophan and flavin to be rigidly attached to the enzyme with an angle of about 50° between the tryptophan L_a absorption transition vector and the flavin L_b emission vector. The theoretical efficiency of transfer in such a situation from a single donor to acceptor matched the experimental efficiency. In the absence of better structural data, no accurate

value of the orientation factor κ^2 can be obtained, but we have used both the values 2/3 and 0.476 for rapidly reorienting and rigid randomly oriented molecules respectively (254,318,321, 322), with the result that the calculated Förster distance varies by less than 5%. We report Trp $\rightarrow$ FAD distances for $\kappa^2 = 2/3$. Although a value of 0.476 has been widely used (318,321), it has been pointed out that this value arises from a description of concentration quenching of donor by randomly distributed acceptor molecules in rigid media, and may not be generally applicable (322). The calculated distances are seen to be relatively independent of the κ^2 value due to the sixth root dependence of R_0 upon it, and the same situation applies to J , the overlap integral. Our calculated value of R_0 is in good agreement with that of Visser *et al.* (254) of 22-24 Å, and is typical of indole $\rightarrow$ aromatic acceptor R_0 values (318).

The insensitivity of the transfer distance calculations to the various parameters has been widely reported (318,319). It has recently been pointed out (320), however, that as κ^2 tends to zero, the results become uncertain. This occurs when the angle between donor and acceptor transition dipoles approaches 90° (see equation (5)), although under these circumstances a low probability of energy transfer should appear. Thus, while the absolute values of the tryptophan-flavin distances reported here are thought to be good estimates of the true values, we are primarily concerned with the relative values for the various species examined.

A single tryptophan residue is in a non-polar environment of

the native protein at about 14 Å distance from the flavin. It is thus probably too far removed from the flavin to have a large stabilizing effect on flavin binding. The insensitivity of the tryptophan emission to flavin environment, and preservation of the flavin-tryptophan distance when the active centre is opened up by alkylation of the disulfide (in E_{ma} , to produce a dialyzable flavin), argue that the appearance of the tryptophan residue in this hydrophobic region of the protein is coincidental. We furthermore present the preserved distance in E_{ma} as good evidence that gross conformational changes have not occurred in this species, but rather are localized to the region of the flavin environment and active centre. While opening of the disulfide ring destabilizes FAD binding, the distance between the active site FAD and a distant protein structure is unchanged.

The situation is quite different in the ERCM. Here gross conformational changes have been seen by infrared spectroscopy to occur, as well as by titration of various exposed residues. The observed A_{280} value of this species, with greatly enhanced flavin fluorescence upon excitation of tryptophan, may reflect attainment of a more effective alignment of donor and acceptor dipoles by a mobilized flavin. More likely it indicates exposure of the second tryptophan residue to the proximity of the flavin environment. The ultraviolet spectrum of this species shows loss of resolution of the tryptophan absorption maximum at 272 nm, as it increases in intensity and merges with higher energy transitions. Previously CD and ORD measurements (238) had indicated that the

second tryptophan residue was exposed upon formation of the unmodified apoenzyme monomer. It thus appears that such exposure may be characteristic of monomeric species, and the residue may exist as part of the hydrophobic interface of the two subunits. The proximity to FAD seen in the energy transfer experiments would then suggest that the flavins exist near the interface of the subunits, although this becomes highly speculative. More will be said about possible flavin interactions later.

The decay of fluorescence in free FAD and in native lipoamide dehydrogenase has been previously investigated (89,252,253). Wahl *et al.* (253) have found a value of 2.82 ns for the decay time of free FAD fluorescence in aqueous solution at 20°C ($\lambda_{\text{ex}} = 370$ nm, $\lambda_{\text{em}} = 520$ nm), while for the enzyme a double decay with $\tau_1 = 0.8$ ns and $\tau_2 = 3.4$ ns was found under similar conditions, with equal amplitudes for the two components (252). Multiple exponential decay can arise from competition with newly induced radiationless decay processes in the enzyme environment (388), including possible interflavin energy transfer in systems with interacting flavins. Our observation that the two decay components contribute equally in all dimeric species ($A_1/A_2 = 1.0 \pm 0.1$), despite independent variations of the actual decay times, argues strongly that we are observing two fluorophores in different environments, present in equal amounts. The appealing interpretation is that the two flavins on separate subunits of the dimeric enzyme are present in slightly different environments, not detectable by steady state fluorescence or absorption spectroscopy. The existence of quenched fluorescence

complexes cannot explain a variation in fluorescence lifetime, since static quenching is independent of the lifetime and depends only on the concentration of the quencher, i.e. on the equilibrium constant of the non-fluorescent complex (79,371). Thus the difference between the two lifetimes observed in the dimeric enzyme must represent more than a variable degree of non-fluorescent complexation of the flavin with some enzyme residue. Two different environments are indicated. One flavin resembles more the free fluorophore in aqueous solution, with perhaps a slight increase in fluorescent lifetime due to immobilization and protection from collisional quenching by buffer ions. A second, more rapid decay ($\tau < 1$ ns) is unique to the enzyme. The reasons for this rapid decay are unclear, but may indicate an increased dynamic interaction (i.e. of an excited state flavin) with a protein residue. Such a residue, if charged, would also be expected to destabilize the hydrophobic enzyme-flavin interaction, and might well result in one FAD molecule being more mobile (and hence more efficiently quenched by a collisional process) than the other. In early studies, de Kok *et al.* (79) were unable to observe the rapid, second decay component, but did conclude that quenching was almost entirely dynamic for the enzyme-bound flavin.

The slightly faster decay of both components of native enzyme fluorescence observed by Wahl *et al.* (252) compared to our work may well reflect the slightly different experimental conditions used. Collisional quenching of tyrosine by phosphate decreases the lifetime from 3.4 ns in water to 2.6 ns in neutral phosphate

buffer of 0.10 M (371). Our values were obtained at 1.0 mM phosphate concentration for all measurements, while those of Wahl *et al.* were recorded in the presence of 30 mM phosphate.

With reference to the isozymes, it is noted that τ_1 and τ_2 are slightly lower for both isozymes I and VI than for the native enzyme mixture. This may well reflect a variation amongst the six isozymes, the composite τ_1 and τ_2 values being the average of the slow and fast decays of the individual components. The partial denaturation of the isozymes, as evidenced by the increased A_{355}/A_{455} ratios and low specific DHase activities, may also allow slightly more efficient relaxation by loosening of the flavin. There is, however, no consistent pattern of denaturation among the isozymes, and their flavin components are still firmly bound, as indicated by absorption spectroscopy and equilibrium dialysis. Consistent with the spectroscopic results of Sakurai *et al.* (274), these lifetime studies reveal a difference in the flavin environment between the isozymes derived from the PDC (I) and α -KGDC (VI), and furthermore indicate a more intimate interaction of flavin and protein in the α -KGDC isozyme. In any case, an isozymic origin of the double decay process is eliminated.

Consistent with the loose flavin interaction of the RCM-monomer, a single decay component is observed for this species, of lifetime comparable to that of free FAD. As shown above, however, several lines of evidence argue for a specific stoichiometric interaction of the flavin with the active centre of this monomeric species, and it is attractive to postulate that the

single decay time observed for this species is not due merely to the existence of essentially unbound FAD. Rather it would appear that FAD finds itself in a single (protein) environment in this species, which then suggests that the non-equivalence of sites in the dimer results from the dimerization process. It is noted also that the quantum yield of this species is comparable to that of FAD unfolded by binding to the enzyme. This could be due to asymmetric dimerization of identical subunits, or to a conformational asymmetry induced by association. The resolution of this problem may await X-ray structural analysis. Evidence that the monomeric subunits have not merely been so extensively modified as to mask subtle isozymic differences comes from two lines of reasoning which corroborate the asymmetric dimerization proposal.

Firstly, we have observed the isoelectric focusing pattern of the monomer to give rise to two major bands only, containing variable but unequal amounts of protein. For non-equivalent subunits, a minimum of four must occur to explain the native enzyme pattern, that is, two for each complex. While the results of prolonged isoelectric focusing of the relatively unstable monomer are admittedly not conclusive, they favour, when taken

* Thus for instance the mixture might be described as $\alpha\alpha, \beta\beta, \alpha\beta$ and $\alpha'\alpha', \beta'\beta', \alpha'\beta'$ if no subunit exchange between the complexes occurs during isolation. This would necessitate, however, predominance of two subunits, say α and α' , in the monomeric sample, which does not explain the relatively higher proportion of dimers of other subunits in the native mixture.

with other corroborative evidence, the interpretation of asymmetric association of identical subunits within each complex. Secondly, recent work by Massey (256) has shown that while monomeric enzyme species covalently bind 8-Cl-FAD, the dimer may only reassociate when one of the subunits retains the FAD analog in an unbound state. The dimer must then have undergone a conformational change which prohibits approach of the C(8) position of the flavin analog to the residue which forms the site of covalent attachment in the other subunit.

This conclusion does not seem unreasonable in view of the fact that complex conformational changes occur upon dimerization of reconstituted apoenzyme monomer (233,236), and leads logically to the questions (i) what enzyme structural features are responsible for the asymmetric conformational changes?, (ii) how are such changes induced?, and (iii) what is the mechanistic significance and functional advantage of such changes, if any? The second question appears to be beyond the scope of present knowledge. Speculation on the remaining questions focuses attention upon the flavin moiety. Monomerization loosens the flavin, and removal of flavin destabilizes the protein. Reduction and various chemical modifications, as well as pH extremes, were seen to concomitantly destroy flavin binding and protein integrity. It can be concluded that presence of the oxidized flavin molecule is crucial for maintenance of the native enzyme conformation. Thus the asymmetry of the flavin environments represents an asymmetry of regions fundamentally involved in protein structure-function relationships. The possibility of flavin

interaction clearly merits investigation. Since the conventional spectroscopic measurements prove at best ambiguous, the MCD investigation was therefore undertaken. The work of Tollin (361) and Edmondson and Tollin (362) has established natural CD as a sensitive probe of flavin environment and conformational changes, although correlation with spectral features remains largely empirical. We extend this work to include the MCD.

Tollin (361) has discussed preliminary work on the MCD of flavin analogs.

The isoalloxazine chromophore of the flavins is optically inactive, and any natural optical activity must therefore arise from interaction with the chiral ribityl side chain. The ribityl group is non-chromophoric in the visible and near UV regions. Very little natural optical activity has been found to be associated with the 450 nm L_b transition.

An MCD spectrum may consist of three types of features (389, 390), known as the Faraday A, B and C terms. The A term is observed when a degenerate excited state has its degeneracy removed in the magnetic field so that two transitions now occur, one allowed for left and one for right circularly polarized light. These will give MCD's of opposite sign and produce a feature similar in appearance to the derivative of a Gaussian absorption band. The C term arises from Zeeman splitting of a degenerate ground state, and can be detected by its temperature dependence, the non-degenerate states being populated according to the Boltzmann distribution. The B term arises from mixing of states in the magnetic field. As the

isoalloxazine chromophore is of low (C_s) symmetry, its MCD spectrum must be composed exclusively of Faraday B terms arising from magnetic field-induced mixing of excited electronic states. The spectra reported thus consist of a series of B terms corresponding to the vibronically resolved bands I-V of the 1L_b and 1L_a transitions.

Michl (391-393), using a free perimeter electron model, has predicted that in general the 1L_b transition of a cyclic π system will have a contribution to $[\theta]_m$ of zero from the B term. From the same model, a negative $[\theta]_m$ is predicted for a 1L_a transition. The flavin transition at 450 nm is long-axis polarized and 1L_b in character, while the next transition is 1L_a and has a significant short-axis component (Figure 5 and reference 100). Thus our observations of weak and negative MCD's for the 1L_b and 1L_a transitions respectively are in agreement with the theoretical predictions. However, the large number of ring heteroatoms and substituents in the flavin systems makes the extension of Michl's work uncertain, and more model compound studies are needed to establish the predictability of the MCD signs of the flavin chromophore.

The B term occurs when electronic states of the molecule can mix under the magnetic dipole operator and such mixing arises in the magnetic field. The expression for the B term thus represents an expansion of the basis functions to include mixing of the ground state g or the excited state j with other molecular excited states k .

In general, only excited states are sufficiently close in energy to

significantly perturb one another, and the major contribution comes from the term representing the mixing of the k into j (391).

This dominating term is given by

$$B_{g \rightarrow j} = \sum_k I_m \{ \langle j | \mu | k \rangle \cdot \langle g | \vec{m} | j \rangle \cdot \langle g | \vec{m} | k \rangle \} / \Delta E_{j,k} \quad (38)$$

where g and j are the wavefunctions representing the ground and excited states, respectively, of the transition considered. The summation is over all molecular electronic states k which mix with j , and $\Delta E_{j,k}$ is the energy difference between j and a particular k . The μ and $\vec{m}$ are the magnetic and electric dipole operators respectively, and I_m denotes the imaginary part of that which follows.

Two possibilities exist to explain the sign reversal of the B term corresponding to the 1L_a transition in the enzyme environment. If the flavins interact in a dimer complex, the two 1L_a states could interact to give rise to an exciton splitting about the absorption maximum. In the coparallel or collinear limits, the intensity of one transition goes to zero, and the B terms become of opposite sign (394). Thus we would expect sign reversal to be accompanied by a shift of the band centre of the B term from the position in the free flavin. Such an exciton splitting is not indicated by the spectra since no appreciable band shift is observed. In addition, the 1L_a sign reversal is observed in the MCD spectrum of glutathione reductase, where the X-ray structure (375) reveals an absence of flavin-flavin interaction.

Secondly, the sign reversal could result through alterations to the higher energy perturbing k states in the enzyme environment. Such states are masked by the intense protein dichroism and are unobservable in the enzyme systems studied here. The 1L_a transition has been shown both experimentally and theoretically to be polarized at about 40° from the long axis 1L_b (102) as shown in Figure 5, although more recent estimates (105) indicate that this angle may be as low as $20 \pm 5^\circ$. Thus the 1L_a state is perturbed effectively by in-plane, short-axis polarized energy states, k . The 1L_b transition has effectively no short axis component, and the lack of such a component is consistent with retention of the negative MCD sign in native lipoamide dehydrogenase. The perturbation of the short axis component of the 1L_a transition is likely produced, therefore, by higher energy, short-axis polarized flavin transitions. This necessitates a change in polarization of such transitions to account for the sign reversal in the enzyme.

Michl (395) has examined the B term behaviour of anthracene and its aza analogs acridine and phenazene. These systems are classified as soft chromophores by Michl, meaning that their B term signs are sensitive to substituent effects. These structures are analogous to the alloxazine system, which is thus also a soft chromophore. The perimeter model implies that the L transitions of these compounds have MCD signs sensitive to mesomeric substituent effects, dominated by substitution at the 9, 10, 1 and 3 positions of the ring. The flavin system is substituted at both positions 3 and 10. Corresponding to N(9)

55

OF / DE

of phenazene, N(5) is unsubstituted in the flavins, but there is good evidence that in both lipoamide dehydrogenase and glutathione reductase a disulfide is within interacting distance of N(5). The presence of this electropositive group is therefore expected to affect the polarization of short axis transitions. To predict the nature of such changes it will be convenient to use a molecular orbital description. In the unperturbed system, low lying π^* orbitals on the N(5) are available as acceptor orbitals for an excited electron. In free flavins, the transition dipole of the lowest energy short-axis polarized transition is most likely directed towards N(5), and away from the more electropositive alkane nitrogen atom at position N(10). This picture is in agreement with the higher spin density on N(5) found by Fox *et al.* (96) for addition of an electron to the isoalloxazine system, and by Song (97) for the lowest triplet state of flavin. However, back donation from the filled p orbitals of sulfur close to N(5) will increase the electron density of the N(5) π^* orbitals, since N(5) may act as a π -acid. If this π charge transfer is sufficiently strong, the π^* orbital at N(5) can become electropositive with respect to N(10), effectively reversing the direction for the short axis dipole and destabilizing the antibonding state. This reversal of sign of the short axis component of the perturbing transition dipole will result in a reversal of sign of the MCD B term of the 1L_a state (cf. equation (38)). Furthermore, the destabilization of the perturbing state increases the value of $E_{j,k}$, and is the most likely cause of the two- to three-fold decrease in molar ellipticity of

the observed MCD in the enzyme. This argument is in qualitative agreement with the substituent effects deduced by Michl (395).

Filling of the low-lying π^* orbital by interaction with an aromatic amino acid residue in the enzyme suggests an alternative explanation. However, only one tryptophan residue is observable per FAD in lipoamide dehydrogenase, and is at a distance of 14 Å as measured by fluorescence energy transfer. Tyrosine is not implicated in the hydrophobic flavin binding site of this enzyme (see 265,198). The imidazole residue present does not appear to interact directly with FAD in glutathione reductase (375).

The appearance of an A term at about 550 nm in reduced enzyme species is of particular interest since it is absent in the reduced free flavin. It is well established that both lipoamide dehydrogenase and glutathione reductase contain active centre disulfides which are reduced in addition to FAD in the fully reduced EH_4 enzyme forms (198). Furthermore, both show similar EH_2 spectra. The early assumption that the red EH_2 forms of the enzymes represented flavin semiquinones were abandoned in favour of strongly interacting biradicals and then thiolate-to-flavin charge transfer complexes, although no conclusive physical evidence has been presented and all structures are still in contention (198). The absence of any new bands attributable to C terms in the MCD of all EH_2 forms rules out any unpairing of electron density in the ground state of this species. In addition, an A term centred under the 550 nm shoulder of the absorption spectrum indicates unpairing of electron density in the excited state of this species and is

strongly supportive of triplet character in a charge transfer complex. The singlet transition associated with this charge transfer gives rise to a shoulder to the low energy side of band I, but is devoid of magnetic field-induced optical activity. The remaining features of the oxidized enzyme spectrum remain but are somewhat blue-shifted.

That the A term remains at full intensity when FAD is also reduced in EH_4 produced by dithionite, is at first puzzling. Reduced flavins are charge transfer donors (80), and the degree of charge transfer would be expected to decrease upon reduction of the flavins. Furthermore, the A term remains at 550 nm. The energy of a charge transfer transition is dependent upon the electron affinity of the acceptor, which must clearly decrease when the flavin is reduced. The possibility that the thiolate anion is stabilized by charge transfer complexation with a protein residue has not been previously considered. This possibility merits re-evaluation. A base at the active centre has been proposed to be involved in the catalytic mechanism of the DHase activity (198, 271). Evidence that such a base exists and is an imidazole will be discussed below. Stabilization of the thiolate by charge transfer to the imidazole (actually an imidazolium cation, probably forming an ion pair with the thiolate (271,272)) rather than to FAD must be considered.

Glutathione reductase is shown also to have a thiolate EH_2 structure by MCD. The disulfide is viewed as being positioned for reduction by N(5) of the flavin ring, and this has been confirmed

by X-ray (375). However, the X-ray structure has also revealed the presence of what appears to be a histidine residue (His 450) near the proximal nascent thiol. It has been proposed by those authors that a thiolate anion occurring in the catalytic process is stabilized by an imidazolium cation of His 450.

The rose bengal sensitized photooxidation of lipoamide dehydrogenase destroys five histidine residues and produces a species E_{hv} with multifunctional behaviour consistent with loss of a catalytic base in the DHase reaction. The A term intensity in the 550 nm region of the EH_2 form of this species is greatly reduced, consistent with a thiolate-to-imidazole charge transfer as the origin of this term in the unmodified enzyme. The remaining A term intensity may result from unreacted enzyme, as no attempt has been made to purify E_{hv} .

The site of alkylation in E_{ma} has not been established, beyond its identification as one of the nascent thiols arising from reduction of the disulfide (269). These thiols appear to act in catalytic and binding capacities respectively in the DHase reaction (271). The unequivocal identification of catalytic and binding residues is of interest. The major problem in this regard has been the failure of E_{ma} to display the characteristic EH_2 spectrum. This could arise from alkylation of the catalytic thiol, or merely from loss of interaction between this thiolate and a stabilizing residue as the active centre changes to a more open conformation on splitting of the disulfide. The MCD spectra show the usual A term to be present, though weak, in the oxidized form of the monoalkylated

enzyme, and to increase to a normal intensity upon reduction of the flavin. A conformational change must occur when the flavin is reduced, (as both absorption and MCD spectra show it to be upon reaction of E_{ma} anaerobically with NADH) which allows restoration of the strong charge transfer. This is strong evidence in support of the view that the binding thiol is alkylated, and explains the loss of DHase activity in this species as resulting from a failure to bind the disulfide substrate. It also argues strongly against the proposal of Thorpe and Williams (270) that addition of NAD^+ to E_{ma} induces covalent binding of the flavin at C(4a) to the catalytic thiol. Our reduction was done with NADH, which generates bound NAD^+ as the enzyme reduces. The thiolate remains free and gives rise to the A term.

The cobalt derivative, E_{Co} , has been shown by MCD to contain cobalt in a tetracoordinate environment. This is consistent with the conclusions of Huang and Brady (261), and with the multifunctionality of E_{Co} as discussed below, that the single cobalt atom per subunit has at least one flavin ligand and is not chelated by the active centre disulfide. Tetracoordination is consistent with sequestering of the Co(II) in the hydrophobic region of the flavin, as exposure to water in neutral aqueous media favours a higher coordination number for Co(II) (396). Sign reversal of both the 1L_a and 1L_b B terms as compared to free flavin indicates strong interaction of the flavin and cobalt atom, which is consistent with conferral of natural circular dichroism upon the low energy flavin absorptions as noted by Huang and Brady (261).

The common factor in the five multifunctional enzymic activities discussed is the usage of the NAD^+/NADH couple as the electron donor or acceptor. In addition, reducibility by NADH is the property conferred upon the flavin by its immobilization in the enzyme system. An understanding of pyridine nucleotide binding phenomena is clearly crucial to understanding of the mechanistic basis of this flavoprotein's activity, and we have attempted to improve upon and extend previous work in this regard, to the point where nucleotide analogs, environmental factors, and enzyme species can be varied rationally as a mechanistic probe.

In native enzyme at pH 7.0, 25°C, two binding sites are found for oxidized NAD^+ . The interesting situation with NADH cannot be observed directly, as aerobically the nucleotide is catalytically transformed (OXase reaction), and anaerobically the enzyme is quantitatively reduced at the expense of the nucleotide. Subsequently it will be argued that the reduced and oxidized nucleotides differ only in their affinity for the same sites, however. Our dissociation constants are in close agreement with those determined by Su and Wilson (246), and somewhat larger than the values reported by Veeger and coworkers (243,245). It would appear, however, that the results of the former authors are fortuitously correct, as they were obtained by measuring quenching of fluorescence with excitation of the flavin at 463 nm. We have shown that the absorption spectrum of the enzyme- NAD^+ system changes in this region in a non-linear fashion with increasing NAD^+ concentration. Therefore fluorescence intensity must be corrected for the decreased absorption at the

excitation wavelength, even if a constant quantum yield of the fluorescent species (presumably unbound protein) is assumed. The Stern-Volmer equation (equation 27) is only applicable to determination of the association constant, K_a , if a) the complex is known to be non-fluorescent, and b) the quencher Q does not alter the absorption of the fluorophore in the region of excitation and emission (80). The Stern-Volmer relationship is implicit in the method of Harbury and Foley (87) as used by Su and Wilson (246). The left hand side of equation (26) varies with changing absorption at the excitation wavelength as well as with reductions in quantum yield due to complexation (see Appendix II).

The work of Veeger *et al.* (243,245) also suffers from a fundamental problem. Across the region of the difference spectrum, at least two spectral features are changing, with at least partial independence. This results in non-systematic changes in the absorption differences at a given wavelength, as we have observed. The observation of a regular Scatchard plot by Visser and Veeger at a single wavelength (430 nm) in the difference spectrum would again appear fortuitous, and does not justify the method.

Five general questions remaining after the work of previous authors will form the basis of the present discussion.

(i) Identification of the catalytic site(s)

There were problems in determining which of the high and low affinity sites constituted the catalytic binding site, based upon the assumption that one site was catalytic in function and the other regulatory. Su and Wilson (246) found the K_d of the high affinity site to be the same as the K_i of NAD^+ acting as a competitive

inhibitor in the DHase reaction (200 μ M). Veeger and coworkers (243,245) make the same observation regarding their low affinity site, the discrepancy reflecting the different K_d values determined by the two groups. In addition Su and Wilson note a K_i of 190 μ M for NAD^+ as a non-competitive inhibitor in the DPase reaction. Thus Su and Wilson assign the high affinity NAD^+ site a catalytic role, while Veeger's group argues that the low affinity site is acting in this regard. It was argued from the regulatory effect of NAD^+ , which is seen as binding to Massey's Y-site (216), that it must represent the high affinity site (see 243). It was also shown that it was binding of NAD^+ to the high affinity site which favored the dimeric form of the enzyme (237,245). However, the high affinity site was the only one present at pH 5.6, where reaction still occurs. Either this site must be viewed as having catalytic capacity as well, or the association constant has been altered. We will argue that the THase mechanism indicates a catalytic role for each site, with the low affinity site II having in addition a regulatory role. A previously encountered difficulty was that conditions for isolating a single site (e.g. low temperature or pH) could not be found which did not drastically alter the observed dissociation constants and the relevant kinetic parameters. We have circumvented this problem by identification of the spectral features associated with each site.

In the present work, monoalkylation of the enzyme at a single nascent thiol residue has been found to quantitatively remove one site, cleanly leaving one NAD^+ binding site per subunit. The kinetic

parameters of the resultant enzyme species are only slightly altered when the multifunctionality of the enzyme is investigated, although the biological activity is exclusively removed, presumably due to loss of the lipoamide binding site. The remaining site has the affinity constant of the low affinity site at neutral pH, and furthermore displays the spectral features of that site. Since full catalytic activity remains in all but the DHase reaction, we favor the interpretation that this low affinity NAD^+ binding site is the true catalytic site. We denote it site I. The second, high affinity site we denote site II, and will tentatively consider it to be a regulatory site. Discussion of both regulatory and catalytic roles for site II will be offered.

Assignment of a catalytic role to a low affinity site may appear catalytically inefficient, and requires comment. If NAD^+ is to serve in a regulatory manner, it is desirable to have it binding in this capacity initially. This ensures maintenance of a proper active dimeric conformation (237), and also protects the enzyme from full reduction as we shall see. Subsequent NADH binding at site I may then proceed with productive consequences. We shall also suggest below that site I has a higher affinity for NADH than site II, contrary to the situation with NAD^+ . In neither case does catalytic efficiency suffer, as Massey *et al.* (214) have determined that in both directions reaction with the lipoate substrate is rate determining for the DHase reaction. Also, an increase in the affinity of site I for NAD^+ in the presence of lipoamide has been reported (245). This may be catalytically significant.

(ii) Binding properties of TNAD^+ and 3-AP-NAD^+

The number of TNAD^+ binding sites was investigated by Su and Wilson (246), who determined a single association constant. This binding was observed indirectly, however, by the quenching of flavin fluorescence in the presence of the pyridine nucleotide analog, and it was not possible to state unequivocally that the situation did not represent two or more sites with the same affinity. The precise number of binding sites cannot be found by the method of Harbury and Foley used by those authors. We have found Stockell plots of data obtainable from the difference spectrum to give linear fits with a single binding site indicated. In addition, a single spectral feature (the hypochromic shift of the flavin spectrum) as identified with site I above is observed. It is concluded that TNAD^+ binds to the catalytic site (site I) only of the native enzyme. We have in addition observed the two-site binding of 3-AP-NAD^+ as observed by the previous authors.

(iii) Spectral features of binding to Sites I and II

The spectral features resulting from binding at both sites have not been discussed in the literature, although Thorpe and Williams (270) have suggested that the decrease in extinction coefficient of the flavin of E_{ma} in the presence of NAD^+ results from covalent binding of flavin to protein through C(4a). We shall consider the characteristic spectral features of sites I and II in turn.

Binding of NAD^+ occurs exclusively at site I in E_{ma} , as does binding of TNAD^+ in native enzyme. In these situations it is

associated with the catalytic activity of the enzyme, and is characterized by a decrease (without further perturbation) of the visible spectrum of bound FAD. Assumption of similar consequences accounts nicely for the difference spectrum obtained when NAD^+ binds to native enzyme, and we conclude that a similar site I phenomenon is occurring. The binding is also associated with quenched fluorescence of FAD.

Five possible explanations exist for the observed spectrum

- (a) Vibronic interaction of flavin with the pyridinium system could appear.
- (b) Vibronic interaction of flavin with an aromatic enzyme residue could be induced or enhanced by conformational changes attendant upon binding.
- (c) Flavin-flavin interaction could be induced or enhanced, resulting in self quenching.
- (d) A covalent flavin adduct could occur, as suggested by Thorpe and Williams (270).
- (e) Quenching could be induced due to non-specific changes in the flavin environment, resulting from protein conformational changes upon NAD^+ binding.

The second possibility above does not appear likely. McCormick (397) has recently shown that interaction of flavin with the three aromatic amino acid residues causes unique spectral perturbations which are not seen when binding occurs at site I of E_{ma} . Charge transfer complexes between flavin and indoles or phenol form, which have characteristic spectra (80) unobserved for site I binding.

Both native and monoalkylated enzyme have a single tryptophan residue transferring energy to the flavin, but at a distance of 14 Å. Furthermore, this Trp to FAD distance is the same in both species, despite a greatly reduced extinction coefficient for E_{ma} upon binding of NAD^+ at site I. Also, one calculates a free energy for formation for the non-fluorescent complex of E_{ma} - NAD^+ of about 1.5-2.0 kcal.mol⁻¹, which does not appear sufficient to account for significant conformational changes in the protein structure. The uniquely high fluorescent quantum yield of this flavoprotein points strongly to the absence of any protein residue quencher in the neighbourhood of the flavin.

The best evidence against flavin-pyridine interaction comes from the RCM-monomer. Nucleotide binding in this species must occur, as shown by the maintenance of two catalytic activities, *via* sequential mechanisms. The binding is almost certainly at site I. We would expect the conformationally sensitive site II to be missing in this species (cf. Section (iv) below). However, no difference spectrum or fluorescence quenching are seen in this species, in the presence of saturating amounts of NAD^+ , indicating that the dimeric structure is necessary for the interaction producing these results. Since this interaction does not appear to be of a flavin-aromatic amino acid nature, we must seek another explanation.

Thorpe and Williams (270) have proposed that the decrease in flavin extinction coefficient of E_{ma} in the presence of NAD^+ is due to a favored formation of a thiol-C(4a) adduct. They have shown this quenching to be rapid, and suggest such an adduct

may be catalytically significant. Other workers (398,399) have shown that dithiols may be oxidized by flavins with such adducts as intermediates, and this possibility must certainly be considered. However, the MCD spectrum of E_{ma} shows the active site thiolate to be intact, and to remain in the reduced state. NAD^+ is present in the mixture, as the reduced enzyme was produced with NADH, but is equimolar with enzyme and well below site I saturating concentrations. NADH is present in excess, and should be turning over in a transhydrogenation reaction, so if a thiolate adduct forms during catalysis we might expect a reduced A term. This is not observed. Of course the same argument applies to reduced native enzyme. We note also that flavin is removed from E_{ma} at approximately equal rates by dialysis against neutral phosphate buffer in the presence or absence of 2 mM NAD^+ . This seems inconsistent with the hypothesis that NAD^+ induces covalent binding of the flavin in this species. This result is obtained despite the fact that NAD^+ shifts the monomer $\rightleftharpoons$ dimer equilibrium in favor of dimer in the native enzyme, and flavin is readily dissociable from monomer (245). The conclusion of van Muiswinkel-Voetberg and Veeger with respect to binding of NAD^+ at the regulatory site (i.e. site II, missing in E_{ma}) appears correct, although our E_{ma} preparation may not be sufficiently dissociable to make this effect significant.

By gel filtration on a column equilibrated with NAD^+ Thorpe and Williams (270) have found a stoichiometry of less than 1 mole NAD^+ bound per mole of FAD. The reasons for this are unclear,

and the actual stoichiometry may not be accurately determined by this method. However those authors extrapolate their results to completely stoichiometric binding and produce a spectrum having a bleached L_b transition and an increased L_a extinction coefficient, which may be consistent with the covalent adduct. This is not observed by us, as both transitions are quenched in the difference spectrum. Additionally we observe similar spectral features when TNAD^+ binds at site I of unmodified enzyme. In this case a covalent adduct is not expected, in this fully oxidized system. A fuller investigation of the stoichiometry of binding of NAD^+ to E_{ma} is in progress in Williams' group (270), and we cannot unequivocally rule out the covalent adduct in this species at this time.

We favor the explanation suggested by van Muiswinkel-Voetberg and Veeger (245) that the partial quenching of flavin absorption and fluorescence on binding of NAD^+ to native enzyme at least, result from a change in flavin environment. Subtle conformational changes are indicated, which may result in new flavin-flavin or flavin-protein interactions. The MCD spectra give no indication of flavin π - π interaction, but interactions of other types cannot be ruled out. Binding studies with glutathione reductase were undertaken since binding of both NADP^+ and NADPH to both the E_{ox} and EH_2 forms of that enzyme is established (198,372-374). Although the reaction catalyzed by this enzyme (reduction of glutathione by NADPH) is essentially irreversible (198), NADP^+ was shown to bind to EH_2 produced by NADPH (372), as found in the corresponding

lipoamide dehydrogenase system. Kinetic evidence showed that NADP^+ is a competitive inhibitor of NADPH, forming a dead end complex with E_{ox} (373,374,398), apparently at the catalytic nucleotide binding site. Since no perturbation of the E_{ox} spectrum by NADP^+ has been reported, all evidence for binding has been kinetic. We hoped to observe this binding spectrophotometrically, in analogy to the experiments with lipoamide dehydrogenase. That no difference spectrum was detected in the presence of saturating levels of NADP^+ is consistent with the view that the difference spectrum upon binding of NAD^+ to the catalytic site of lipoamide dehydrogenase is due to flavin-flavin interaction, the latter being impossible in glutathione reductase. The enzymes are thought to function by similar catalytic mechanisms (198) and this result is also inconsistent with formation of a covalent adduct in both enzymes. More work on the possible flavin-flavin interaction in lipoamide dehydrogenase is suggested. The absence of an NAD^+ difference spectrum in monomeric E_{RCM} is also noted in this regard.

The presence of site II in native lipoamide dehydrogenase is characterized by a sharp positive absorption at 507 nm in the difference spectrum, and a relatively complex negative feature at higher energy. A positive feature below 400 nm is also associated with binding to this site. This spectrum bears a striking qualitative resemblance to the charge transfer spectra of aromatic donors to flavin (e.g. see Figure 99 taken from Slifkin, (80)). The energy of the transition, furthermore, is well below anything occurring in the uncomplexed, oxidized flavin.

Figure 99: Difference spectra of flavin charge transfer complexes, reproduced from Slifkin (80). Curve 1, FMN-adenosine; curve 2, FMN-tryptophan.

Both flavin and the pyridinium ring of NAD^+ are fully oxidized and tend to act as charge transfer acceptors, although their reduced forms are good donors. Donor-acceptor complexes of the types $\text{NADH-Fl}_{\text{ox}}$ and $\text{Fl}_{\text{red}}\text{-NAD}^+$ have been documented (80). It appears likely that if the site II feature is charge transfer in nature, the adenosine portion of the nucleotide may donate to the flavin (and cf. Figure 99). Additional criteria for a charge transfer interaction, namely reversible temperature dependence of the intensity of the transition and linear dependence upon ionization potential and electron affinity of donor and acceptor respectively, are difficult to demonstrate in the present situation. Binding site II is lost by a change in the temperature outside a narrow range. Reconstitution of the enzyme with FAD analogs is difficult, and is accompanied by extensive denaturation. The binding to site II is very sensitive to minor changes in the structure of the pyridine nucleotide, as shown by the properties of TNAD^+ , 3-AP-NAD^+ , ϵNAD^+ and NADP^+ . The MCD evidence supports the charge transfer interpretation of this site, a weak A term appearing at 490 nm for native enzyme in the presence of 2 mM NAD^+ . The unpairing of spin density giving rise to this term is thought to result from the transfer of an electron to adenosine.

iv) Chemical nature of the two sites

The catalytic site I shows a steep sigmoidal pH profile upon approach of the maximum from low pH. The deprotonated species is favored. The pK_a value for a single site involved is estimated to be 7.5. Site II, however, shows a gradual decline of binding capacity

on lowering the pH over several units. This indicates that a single residue is not involved, and is suggestive of a dependence upon a gradually changing protein conformation. At high pH, both sites show a sharp decrease in binding capacity, possibly due to deprotonation of a second residue, or to a rapid denaturation of the enzyme. The apparent pK_a of the second deprotonating residue is 7.8 ± 0.1 , and in view of Massey's report (210) asserting a pH optimum of 7.9 for the reverse DHase reaction, this value may be significant.

In keeping with the interpretation of a conformationally sensitive site II is the observation of facile loss of this binding site upon temperature and pH changes and chemical modifications of the enzyme, as well as partial denaturation by freeze-thaw cycles, under conditions which allow site I to remain. Ionic strength dependence of binding of NAD^+ at site II has also been reported (245). A single site has been found to remain below $\sim 15^\circ C$ (243), an observation which may be related to the discontinuity in the Arrhenius plot of the enzyme at $22^\circ C$ (400). Koster and Veeger (401) have explained this latter effect as arising from a reversible transition between two conformations of equal activation energies but different probabilities of transition, necessitating conformational changes around the active centre. In addition, binding to site II is sensitive to changes in all moieties of the nucleotide structure, the nicotinamide ring (NAD^+ and 3-AP- NAD^+), the adenosine ring (ϵNAD^+), and the intervening chain ($NADP^+$). Site I is relatively insensitive to modification of the nicotinamide ring, again

implicating perhaps a single binding residue acting distal to the redox site. This residue does not appear to be the commonly utilized guanidino structure of arginine, however, argued both from the inability of extensive arginine modification to affect the catalytic activity of the enzyme, and from the observed pH dependence of the binding phenomenon.

v) Relative geometry of the two sites

We are now in a position to speculate on the proximity and relative orientations of the two sites. It is clear that nucleotides bound at both sites are in close proximity to the flavin and hence to each other. NADH at site I reduces the flavin directly. NAD^+ at site II forms a charge transfer complex with the flavin, and may also be catalytically active. The two fluorescent lifetimes in native enzyme are both reduced by the presence of NAD^+ , presumably by dynamic quenching, indicating the binding phenomena are similar at each subunit. This is consistent with the net stoichiometry of reduction of the dimeric enzyme.

The following picture emerges. NADH binds at the catalytic site I in an orientation favourable for reduction of the flavin at one of several possible positions, including N(1). The isoalloxazine ring is in a hydrophobic enzyme environment, probably in a shallow pocket and protected on one face by hydrophobic residues. Reduction by NADH would involve edge-on approach by the nicotinamide moiety, as would subsequent reduction by flavin of the protein disulfide. Reduction of flavin at N(1) followed by oxidation by disulfide at N(5) or C(4a), is chemically

feasible, and explains facile nucleotide binding and flavin reduction even when the disulfide acceptor is blocked, as by alkylation or metal ion chelation. A similar mechanism is supported by recent X-ray structural work on glutathione reductase in the presence of nucleotide (375), an enzyme very similar to lipoamide dehydrogenase in the reduced state (198). Furthermore, this edge-on approach of nucleotide explains the absence of any spectral perturbation at site I by the pyridine nucleotide since the π -clouds may remain orthogonal. A portion of the second face of the flavin is left open and is available for formation of a charge transfer complex with the adenosine portion of the second NAD^+ molecule bound at site II. The situation is pictured in Figure 100.

An interesting sidelight to this picture comes from consideration of the stereospecificity of dehydrogenases for the A or B form of NADH (402). Isotopic labeling of one of the two hydrogens at the 4-position of the pyridine ring produces the R (A side) or S (B side) configurations. Weber *et al.* (403) observed a scrambling of deuterium label at this site in the THase reaction with 3-AP- NAD^+ . The reaction was investigated in both directions to rule out an isotope effect, and the enzyme showed only a 60% preference for the B form. This situation appears unique amongst dehydrogenases (404,14). It has been suggested (14) that scrambling of the label may occur if the cofactor binds in the open conformation. If site II is a site of charge transfer complexation with adenosine, this cofactor will be held open, and the A and B sides may become

Figure 100: Proposed active centre structure of lipoamide dehydrogenase with NADH bound at site I. Dashed line is the adenine portion of NAD⁺ bound at site II, solid line is the protein backbone. The position of the amino acid ring and the geometry of the adenine-flavin stacking are somewhat arbitrary (but cf. Slifkin (80) for the stacking geometry).

indistinguishable. The label would then scramble when site II is involved in the THase reaction. Isotopically reduced NAD^+ and analogs may prove useful in further elucidating these nucleotide-enzyme interactions.

This heuristic description does not account for the relative affinities of the two sites. Actually, the NAD^+ at site II is bound first, as we have assigned this site the high affinity constant for NAD^+ . The relative affinities of the two sites for NADH remains unknown, and need not necessarily parallel those of NAD^+ . We have assumed that in E_{ma} site II is destroyed, yet it retains three NADH-linked activities (THase, ETase, DPase) with normal activity. This indicates that in the unmodified enzyme the activities operate through this site, which is then the preferential NADH binding site. We postulate that while site II has the higher affinity for NAD^+ , site I has the higher affinity for NADH. This postulate will allow us to treat the THase mechanism consistently below, and there is additional circumstantial evidence for its correctness. The early view of binding of NAD^+ at the Y-site to protect the enzyme from over reduction appears essentially correct. Thus this site may have been developed to allow early, rapid binding of NAD^+ . NADH will still require access to site I for catalysis, and further should not block site II. It has also been reported (245) that the affinity of site I for NAD^+ increases in the presence of lipoamide. This may be of catalytic significance in the reverse DHase reaction. The sensitivity of site II to modification of the nicotinamide ring should be noted. 3-AP- NAD^+ binds with lowered

affinity and TNAD^+ not at all. In NADH this ring is reduced. If the enzyme requires the nucleotide at this site to remain in an open conformation, this may be achieved by a specific interaction with the nicotinamide moiety.

The implications of the above model are supported well by kinetic studies of the THase mechanism with analogs. The native enzyme allows binding of NAD^+ at sites I and II, of 3-AP- NAD^+ at sites I and II, and of TNAD^+ at site I only. Thus we observe that at low NADH concentrations the THase reaction with TNAD^+ as acceptor proceeds *via* a ping pong mechanism. Binding of NADH at site I forms the reduced EH_2 species, and the product NAD^+ must dissociate to free the obligatory site I for subsequent TNAD^+ binding and completion of the catalytic cycle. Only at high NADH concentrations ($> 0.1 \text{ mM}$) does the reaction become sequential, and this necessitates ternary complex formation. This would indicate that the remaining site, site II, is binding NADH. The high concentration of NADH required suggests a low affinity at this site, consistent with our postulate that site I is the high affinity NADH site. A similar effect is seen when the concentration of TNAD^+ rises to 0.2 mM . This may reflect a very low (spectroscopically undetectable) affinity of this analog for site II. More likely, the high concentration of TNAD^+ is saturating site I and NADH binds at site II by default. A concentration of NADH high enough to compete for site I in this region of the kinetic plots cannot be used due to the onset of substrate inhibition. Note the very high association constant we observe for TNAD^+ at site I of native enzyme (50 mM). Further,

the K_d of site II for NADH, with TNAD^+ bound at site I, is unknown.

These observations suggest a catalytic role for site II as well. When NADH binds to this site it is capable of reducing an oxidized cofactor at site I. That this reduction is flavin-mediated has not been established, but appears likely. The model is summarised in Scheme IX.

With 3-AP-NAD^+ replacing TNAD^+ as the acceptor, both sites are now open to both nucleotide species, and ternary complexes may form. Thus a sequential mechanism is observed. This situation is further evidence for a catalytic capacity of site II.

The species E_{ma} is considered to have site II destroyed, and thus, as observed, it is expected that the TNAD^+ THase reaction will remain ping pong even at high NADH concentrations. The ternary complex of Scheme IX cannot form. Interestingly, the reduction of 3-AP-NAD^+ still occurs, but now also goes ping pong, as the model necessitates.

The role of site II in prevention of full reduction to EH_4 by NADH merits further discussion. The native enzyme at room temperature and neutral pH displays the onset of substrate inhibition by NADH at a concentration of about 0.1 mM in all reactions. Under some circumstances, the sensitivity to NADH inhibition is enhanced, occurring at 0.01-0.02 mM. We will refer to this as sensitive NADH inhibition. Below pH 5.5, the ETase and OXase reactions are the only ones with high activity, and there display high sensitivity to NADH inhibition. Stein and Stein (258) have reported strong NADH inhibition of the

(a) Low nucleotides :

(b) High NADH or High TNAD^+ :

- Scheme IX: Two-site model of the Thase reaction with TNAD^+ .
 (a) At low concentration of both nucleotides, no ternary complex forms: (b) At high $[\text{NADH}]$, significant binding at site II occurs, and a ternary complex can form. At high $[\text{TNAD}]$, site I is saturated and NADH binding at site II is obligatory.
 $\text{T} = \text{TNAD}^+$; $\text{N} = \text{NADH}$.

quinone reductase activity below pH 5.8, which we have confirmed for DCIP reduction. The partially denatured isozymes displayed the phenomenon to a degree which made kinetic investigation difficult in view of their already decreased activity. The E_{ma} species also displayed sensitive NADH inhibition under all conditions. Thus the appearance of sensitive NADH inhibition correlates well with conformational alterations leading to loss of site II. It should also be noted that in the circumstances indicated, reduction by NADH was seen to produce a fully reduced flavin.* Thus we conclude that NADH inhibition results when site II is lost, because NAD^+ bound at that site protects the enzyme from full reduction by NADH. Over-reduction is the cause of the observed inhibition. This statement should not be confused with the conclusion of Massey and Veeger (216) that the EH_4 form is inactive (in the DHase reaction at least). We disagree with this conclusion. Enhanced NADH inhibition with increasing tendency to form EH_4 is observed in the multifunctional reactions of this enzyme, not the biological activity alone. Thus we have shown EH_4 to be rapidly reoxidized by O_2 , DCIP, $K_3Fe(CN)_6$, NAD^+ , and lipoamide. We view the first three of these substrates as potent, non-specific oxidants of reduced flavin (as established for free flavin (200)), which may reoxidize EH_4 stepwise via the flavin. NAD^+ is viewed also as reoxidizing the EH_4 molecule through bound flavin; lipoamide may well pull the electrons in the opposite sense, working through the disulfide couple. Thus catalytic activity appears to remain in all reactions with the EH_4 species.

The discrepancy between our results and those of Massey and

* Only the isozymes were not investigated in this regard, due to the relatively small amounts obtained.

Veeger appears explicable. We were unable to repeat experimental conditions exactly due to the very high quantity of NADase used by the earlier authors (75 units). However, we found production of EH_4 to be efficient in the presence of approximately 1 unit of the enzyme. The assays of the previous workers were conducted at pH 5.9 in the presence of 0.20 mM NADH. Under these conditions, we have found almost total inhibition of the DHase reaction by NADH, especially when, as in the work of Massey and Veeger, NAD^+ is excluded and EH_4 formation allowed. At neutral pH and low NADH concentration, however, comparable to cellular conditions, loss of DHase activity is not an inherent property of EH_4 , produced either by dithionite or the NADH/NADase combination.

Before suggesting a likely explanation for the phenomenon of inhibition by overreduction, three additional facts should be noted.

- (i) When assayed immediately after its production, EH_4 has nearly normal DHase activity, which is all but lost after standing 30 minutes at room temperature.
- (ii) Flavin is removable by dialysis from all reduced species EH_2 , EH_4 , and E_{ma} , but not from native enzyme. Furthermore, this removal accompanies denaturation of the protein, apoenzyme being relatively unstable.
- (iii) The return to the oxidized enzyme spectrum upon treatment of EH_4 with the various oxidized substrates represents a single catalytic cycle. The observed kinetic inhibition represents a decrease in catalytic efficiency corresponding to several thousands of cycles per

second.

The conclusion is that reduction of the disulfide causes a conformational change at the active centre which favours release of flavin from the enzyme. This in turn causes further conformational changes which lead ultimately to denaturation. When the flavin itself is reduced, it must adopt the butterfly conformation due to sp^3 hybridization at N(5). This both disrupts the hydrophobic interaction stabilizing the planar aromatic oxidized flavin, and reduces the degree of fit at the already altered flavin binding site. Binding of NAD^+ at site II forms a charge transfer complex with the isoalloxazine ring, which may both electrostatically stabilize the aromatic oxidized state of the flavin and disfavor its reduction by sterically disfavoring the butterfly flavin conformation. When site II is missing, a certain concentration of NADH is necessary to maintain EH_4 due to rapid reoxidation of EH_2 by oxidizing substrate. When site II is present this sufficient concentration is at the normal level. We suggest that when conditions favour formation of EH_4 , decreases in activity are observed due to loss of effective orientation of flavin for nucleotide oxidation and disulfide reduction in subsequent cycles, and with time, due to loss of flavin-protein interaction entirely. The effects may be enhanced by "wagging" of the flavin in and out of the butterfly conformation if EH_4^* is cycled during catalysis. To press the analogy, the

* More accurately, any species in which the flavin is fully reduced. In native enzyme, this represents EH_4 , but the same argument applies when flavin is reduced preferentially to the disulfide, due to blockage or reorientation of the latter.

butterfly may fly from its protein site. An inherent inability of substrate to oxidize EH_4 , however, is not supported.

We turn now to consideration of the multifunctionality of lipoamide dehydrogenase. At the outset, it is concluded that the isozymes do not contribute to this multifunctionality, but within experimental error all share the kinetic mechanisms and activity of the native enzyme mixture. Any physical differences in the isozymes detected by us and others do not appear to be reflected in a different spectrum of reactivities. Thus the observed multifunctionality of the native enzyme mixture is a true molecular property, and results from the active centre environment of this flavoprotein.

Throughout our studies, it has become apparent that when a perturbation to the native enzyme is applied, the various activities are affected in a common order, that being DHase, THase, ETase and DPase. Table 42 gives a summary of the multifunctional variation gleaned from the Results, for all systems where selective effects are observed, and forms the basis of the statement above. The OXase reaction has not been fully studied due to the relatively low activity displayed by the enzyme in this regard, and to the experimental complications of varying dissolved O_2 concentration as a second substrate. OXase appears to fit into the sequence somewhere around ETase and DPase, however.

The general applicability of this sequence has lead to development of the reactivity scale (Figure 101) which will form the basis of subsequent discussion. An understanding of the basis of Figure

Table 42: Summary of multifunctional variation. Activity expressed as percent of a control; see text.

Species ^(a)	DHase	THase	ETase	DPase
E _{As}	<4	44	77	160
E _{Cd} ^(b)	2	16	110	273
E _{Cu}	0	15	65	114
E _{Co}	11	70	144	123
E _{RCM}	0	0	22	600-3000
E _{RCM/Cu}	0	0	17	18
E _{ma}	0	139	148	188
E _{amid}	190	100	92	90
E _{phos}	280	104	73	72
E _{hv}	37	68	122	130
+20 μ MRib	95	98	>250	95
+0.03% H_2O_2	12	63	142	203

(a) All abbreviations are given in the List of Abbreviations

(b) From reference 257

Figure 101: Activity scale of lipoamide dehydrogenase. See text.
Arrow shows increasing activity/decreasing vulnerability.

100 begins from systematic investigation of the multifunctionality as reported here, and leads to a still deeper understanding of the mechanisms involved. In Table 42, it is seen that one of ~~the~~ criteria for multifunctionality, namely the independent increase and/or decrease of the various activities, is extensively met. The DHase reaction is selectively eliminated by treatment with cupric ion, or reductive alkylation of the active site disulfide, or blocking the disulfide, as with arsenite. More extensive alkylation, to produce the monomeric species, causes loss of THase activity as well, although the mono-alkylation actually enhances this activity. Photooxidation decreases both these activities while increasing the remainder, but the DHase is the most severely affected. Both amidination and phosphorylation, however, selectively increase the DHase activity. The ETase activity is selectively enhanced by formation of the cobalt derivative, as well as by inclusion of trace amounts of free flavin in the reaction mixture. It is also by far the most significant activity (excluding OXase) occurring at its pH optimum of 4.8. The DPase activity of the enzyme appears relatively indestructable, and no means of severely reducing it were found, short of denaturation of the other activities. It can, however, be increased by a number of treatments, including chelation or alkylation at the reduced active site disulfide. The most profound increase in this activity comes from extensive carboxymethylation leading to monomerization. Based on these analyses, we will list the factors affecting the multifunctionality of this enzyme; the position of the monomer $\rightleftharpoons$ dimer equilibrium, the environment of

the flavin prosthetic group, the status of the disulfide, the enzyme conformation, and the dual site phenomenon.

(i) The monomer $\rightleftharpoons$ dimer equilibrium

It has been shown that reappearance of DHase activity follows dimerization in reconstitution of monomeric apoenzyme (233). This supports complete loss of this activity resulting from the monomeric nature of the E_{RCM} species, although this species is also extensively modified and conformationally altered. It is clear, however, that the DPase and ETase activities of this enzyme do not require the dimer, and that the flavin is still rapidly reducible by NADH in the monomer. Furthermore, the dimer most certainly appears to be required for biological activity. Whether this requirement is due to the necessity for paired active centres, or due to the favourability of the conformational changes occurring upon dimerization, or both, remains unclear. It is clear, however, that the dimeric species retains all activities, so that the observed multifunctionality is not due to a mixture of monomer and dimer in the assay mixture. The cross-linking experiments show that as the percentage of cross-linked dimer increases, all activities decrease. None extrapolate to close to zero at 100% cross-linking, however. The reason for this decrease in activity compared to control enzymes treated with similar functionality under non-cross-linking conditions (shorter chain length or monofunctional reagents) is not clear. It may be suggested that enforcing the dimeric conformation imposes a rigidity which interferes with flexible control normally exercised through the flavin-active centre interaction, and that, by the reverse

argument, the latter is disrupted. This hypothesis would be difficult to test, but a precedent for it has been set by the work of Lowe (249-251). It was found that spacer molecules could be used to alter the rigidity of lipoamide dehydrogenase immobilized in a porous matrix, and that increasing rigidity (thermal stability) correlated with decreasing DHase activity.

(ii) The environment of the flavin

The environment of the flavin in lipoamide dehydrogenase is readily characterized by two types of observation. The polarity of the environment is observable in the visible spectrum of the flavin. A red-shifted spectrum with increased resolution of shoulders in the L_b transition, and a blue-shifted next-lowest energy L_a absorption with resolution into a double peak, are characteristic of FAD in a hydrophobic environment. These characteristics are observable in the spectrum of the native enzyme, but when the flavin becomes less buried it is exposed to a more polar environment and spectral changes in the direction of free flavin are seen. In the limiting case, the very exposed flavin of the E_{RCM} species has a spectrum virtually indistinguishable from that of free FAD. The degree of binding of the flavin can be followed by dialysis at neutral pH. As the active centre is perturbed, the flavin may loosen up and become readily dialyzable. In the present system, the results of these two methods of observation correlate well.

We have been unable to bring about loosening of the flavin without adversely affecting the DHase activity. The species that display full or enhanced activity in this reaction (native enzyme,

E_{amid} , E_{phos}) all have a non-dialyzable flavin and show the fully resolved hydrophobic spectrum. On the other hand, loosening of the flavin, even at the expense of partial denaturation, benefits the DPase reaction. In the extreme situation we observe many-fold enhancement of DPase activity with E_{RCM} . It is concluded that the biological activity is most effectively catalyzed by a tightly bound flavin with proper orientation with respect to nucleotide(s) and the disulfide active site. Reduction of the artificial acceptor DCIP, however, appears to be governed primarily by the accessibility of the flavin. The other activities are intermediate in this regard, not requiring direct participation of the disulfide, but also not facing the same steric requirements for approach of substrate to flavin as does DCIP. Thus we suppose that they may more effectively take advantage of a partially oriented flavin.

(iii) The status of the active centre disulfide

We have shown that in the unperturbed enzyme, substrates fail to reduce the flavin, but rather result in an EH_2 species which has the disulfide broken, one nascent thiol actually being deprotonated at pH 7.0 and participating in a charge transfer complex with an active centre residue, possibly an imidazole. The other nascent thiol appears to be a binding site for the dithiolane substrate. We will discuss this point below when considering the DHase mechanism. Thus we expect an intact disulfide to be obligatory for DHase activity, and furthermore a correct orientation for interaction with pyridine nucleotide *via* flavin to be desirable. This is born out by Table 42. Both cadmium and arsenic derivatives have been characterised and seen

to have the disulfide blocked by bidentate chelation of the metal ion. E_{ma} has also a blocked disulfide due to irreversible alkylation of one of the sulfur atoms. All three species show negligible DHase activity. Instability of the arsenite derivative, subject to hydrolysis (223), may account for its DHase activity not being reportable as zero. These same species, however, display marked enhancements of DPase activity, which again appears to relate to loosening of FAD upon disruption of the active centre disulfide. Again the other two reactions are intermediately affected, perhaps being positively influenced by exposure of the flavin but negatively affected by the attendant conformational changes. The E_{ma} species, which still has a partially resolved, though blue-shifted, low energy flavin absorption, and a slowly dialyzable flavin, has thus suffered only subtle denaturation and has enhanced THase and ETase activities.

(iv) Conformational sensitivity

The conformational sensitivity of the reactions is not as easy to characterize objectively as the above factors. Clearly the biological reaction is sensitive, and this reflects the severe orientational requirements of interaction of the biological substrate with pyridine nucleotides through two intervening prosthetic groups acting as redox couples. It may also reflect the regulatory nature of the enzyme displayed necessarily in this reaction, as well as the conformational dependence of other factors such as the position of the monomer $\rightleftharpoons$ dimer equilibrium and the association constant of nucleotide binding at site II. At the other extreme we have a DPase activity enhanced many-fold by the near-denaturing conditions employed in the

preparation of E_{RCM} . The evidence for the placing of the other two activities in the sequence, with respect to the property of conformational sensitivity, is more circumstantial. It rests mainly on the observation that these two reactions show a strong tendency to deviate from their native activities by amounts which are intermediate between DHase and DPase, and are ordered in keeping with the sequence. Furthermore, as argued in the above paragraphs, the relatively moderate changes of these two activities are best understood to be dominated by conformational factors, and thus the order of the activity changes is thought to reflect conformational sensitivity.

(v) Dual site phenomenon

This property has been dealt with above. All DHase-active species retain site II. The THase reaction can take advantage of the dual sites at high nucleotide concentrations. The other activities appear to require only site I as a means of providing reduced flavin by reaction with NADH. DCIP, $K_3Fe(CN)_6$, and O_2 all react slowly or not at all with NADH in the absence of enzyme, but rapidly when an enzyme species with site I intact is present.

To gain a better understanding of lipoamide dehydrogenase multifunctionality, we must turn to the molecular mechanisms of the reactions. That discussion will be preceded, however, by consideration of a hypothesis put forward by Dixon in 1971 (199-201) which appears applicable to the present model system and to flavoprotein multifunctionality in general. We will deal with the role of the flavin and the nature of the active centre before

considering specific reactions.

Dixon found very rapid (half-time less than 5 seconds) oxidation of reduced flavin (FMN or FAD) by O_2 , $K_3Fe(CN)_6$, benzoquinone, DCIP, and methylene blue. In fact, with ferricyanide, the reaction is almost complete in less than 3 ms (405), and the same occurs with quinone (406). Cytochrome c had a half-time of 10-20 seconds while H_2O_2 was only moderately reactive, with a half-time of 0.5-4 minutes. NAD^+ was found to be totally incapable of reaction with reduced flavin. Dixon (201) then looked at twenty flavoproteins with respect to their acceptor specificities. With few exceptions, no enhancement of the natural reduced flavin activity was found. Rather, in all cases some of the reactions were quenched, and generally those remaining were found to have rates slower than or equal to those of the free flavin. The apoprotein is thus viewed as selectively harnessing the natural activity of the flavin. In addition, enzyme bound flavin becomes reactive with the pyridine nucleotides in both directions. As discussed above, this has now been shown to be due not to the apoprotein *per se*, but rather is a property of flavins immobilized in many synthetic polymer matrices. The pattern of reaction quenching was not found by Dixon to be systematic. Thus each apoenzyme confers its own distinctive pattern of inhibition. None of the acceptors listed above was reactive with all twenty flavoproteins surveyed. Moreover the reactivity of a given flavoprotein with one acceptor did not parallel the effects with another. The acceptor pattern does not correlate with flavin content, quaternary

structure, metal content, mechanism of catalysis of the biological reaction, fluorescence of the bound flavin, or its redox potential. The apoprotein appears to have tight control over what activity the flavin is allowed to display. According to Dixon, lipoamide dehydrogenase displays no reactivity with cytochrome c or benzoquinone, greatly reduced reactivity with O_2 , and reduced activity with ferricyanide, methylene blue, and DCIP. From this viewpoint, an understanding of the multifunctionality of this enzyme represents an understanding of the observed inhibition pattern of flavin-acceptor reactivity. An additional acceptor, the biological substrate, appears to be a special case. While dihydrolipoic acid reacts only slowly with free flavins (220,221), the reaction with enzyme is rapid. This appears not to result from direct interaction with the flavin at all, but rather is due to interaction directly with the enzyme disulfide.

The biological role of the flavin in lipoamide dehydrogenase appears to be merely as an effective coupler of the pyridine nucleotides to the active site disulfide. We have shown that the normal reduced state of the enzyme involves an oxidized flavin, which is thus viewed as funneling reducing equivalents into the disulfide. Over-reduction, i.e. reduction to form a reduced flavin, is to be avoided, as the disulfide substrate preferentially accepts electrons pairwise from a reduced protein disulfide. To this end, pyridine nucleotide binding site II has in part been developed. In addition, harnessing of the flavin is favoured by a planar aromatic structure. If transfer of two electrons to the disulfide is concerted, planarity need not be broken. When over-reduction does occur, the "flavin funnel" is

fully reduced and incapable of further accepting electrons, but 'reducing equivalents' may still be removed from the disulfide end of the chain by oxidized disulfide substrate and thus alleviate the situation. Only if the existence of EH_4 is prolonged or its formation repetitive do deleterious effects accrue, as discussed above.

Matthews and Williams (272) have measured the reduction potential of E/EH_2 to be -280 mV at pH 7.0, consistent with reduction of disulfide. For example, the corresponding value for lipoamide or lipoic acid is -286 mV (210). The value for EH_2/EH_4 is -346 mV, in keeping with the value of a flavin; the E_0' of FAD/FADH_2 at pH 7 is -410 mV (78). Thus when the flavin is reduced by NADH, the enzyme will spontaneously oxidize it, and we can conceive of this process as concerted. With this difference in redox potential, however, we can calculate that about one molecule in one hundred will have the flavin reduced, and this may account for the difference of an order of magnitude in the catalytic efficiency for forward and reverse directions in the DHase reaction (225). Furthermore, the redox potential of enzyme-bound flavin is the value for the loosened flavin in EH_2 . When the disulfide is oxidized, the value may be still higher, since reduction of the flavin appears disfavoured in E_{ox} . Then the real disproportionation may be actually more favourable to the sequence $(\text{SH})_2 \xrightarrow{2e^-} \text{FAD} \xrightarrow{2e^-} \text{NAD}^+$ than indicated by about 1% FADH_2 in EH_2 . Clearly, however, the preferred direction of electron flow is $\text{NADH} \rightarrow \text{FAD} \rightarrow -\text{S-S-} \rightarrow \text{R}$, where R is the disulfide substrate.

The DHase mechanism proposed by Williams, as outlined in the Introduction, would appear to be essentially correct. A plausible

reaction sequence consistent with our mechanistic interpretation of lipoamide dehydrogenase multifunctionality is given in Figure 102. The nature of the base deserves some attention. As mentioned, it has variously been suggested to be cysteine, lysine, or histidine. We have observed extensive modification of lysine residues without adversely affecting the DHase activity.* Deprotonation of a group with $pK_a \approx 6$ on binding of NAD^+ (245) supports the presence of an essential histidine residue at the active centre. We have found that protonation of a group with $pK_a \approx 6$ introduces a positive charge at the active centre, which is reflected in favouring reduction of the negatively charged phenoxide form of DCIP. This rules out a sulfhydryl residue, which becomes neutral upon protonation. Modification of lysine residues does not affect the protonation (as judged by an unchanged pH profile for DCIP species dependence). The pK_a 's of cysteine and lysine are 8.3 and 10.5 respectively (139). The evidence appears strong that there is an imidazole group at the active centre. That this may actually be the catalytic base of the mechanism is supported by a reduction of 65% in the DHase activity upon rose bengal sensitized photooxidation, shown to be specific for histidine. Only the DHase reaction is strongly affected by this modification, and only it employs base catalysis (cf. Figure 102).

*. In fact, we have observed an increase in the V_{max} of the reaction, although an explanation of this curious fact has eluded us. As a point of speculation, one may note that phosphate ion stabilizes the dimer but inhibits the DHase reaction, with increasing concentration, and an intersubunit salt bridge between lysine residues has been suggested (237). In light of our crosslinking results, a looser bridge between amidine residues might be considered.

Figure 102: Proposed multifunctional mechanistic pathways of lipoamide dehydrogenase. See text for details. Im represents protein-bound imidazole.

The transient enhancement of biological DHase activity by phosphoramidate modification remains unexplained. Due to the labile nature of the phosphorylated site(s), we have not attempted to characterize E_{phos} further at this time. We report the finding, however, due to the possibility of interesting biological significance, given the regulation of the PDC complex by inhibitory phosphorylation of pyruvate dehydrogenase, as described. The labile nature of the DHase-active species makes histidine a likely candidate for the site of phosphorylation. Hydroxylamine readily displaces the phosphate adduct, and the pH dependence of modification is consistent with an imidazole as the reactive group. The possibility of stabilization of the imidazolium cation of the active centre histidine by phosphorylation must then be considered. This cation is involved in the DHase mechanism and may also stabilize the thiolate of EH_2 by charge transfer complexation. Such an explanation is also consistent with the observed increase in V_{max} , as stabilization of the imidazolium cation affects an intermediate of the rate determining steps. In view of the initial rapid reaction followed by a decline in activity in the continued presence of phosphoramidate, we note the work of Hultquist *et al.* (347) on histidine phosphorylation by phosphoramidate. Rapid formation (<15 minutes) of 1-phosphohistidine as the kinetic product was observed, with eventual isomerization to 3-phosphohistidine as the thermodynamic product. The lability of these species, particularly in the presence of nitrogen heterocycles, was stressed.

The other substrates dealt with here are viewed as reacting with the flavin, and the low concentration of FADH_2 forming in the

regular catalytic cycle may in part account for the reduced activities of O_2 , $K_3Fe(CN)_6$, and DCIP as compared to free flavin. This may be especially true of O_2 and $K_3Fe(CN)_6$, which are not expected to face steric requirements in entry to the active centre. DPase activity is favoured by loosening the flavin and breaking the FAD - disulfide interaction, i.e. by conditions which obligatorily produce FADH quantitatively. The increased rate of DCIP reduction then appears to reflect a return to the rate with free flavin. That $K_3Fe(CN)_6$ does not behave likewise may indicate that this obligatory one electron transfer suffers from loss of stabilization of the flavin semiquinone experienced in a hydrophobic enzyme environment. If indeed Co^{2+} is chelated by flavin, E_{Co} may exhibit enhanced ETase activity due to just such a stabilization. The problem is of course circumvented by addition of riboflavin, which is rapidly reduced by enzyme and is therefore capable of (a) disproportionation reaction to make available the semiquinone and (b) provide reduced flavin free in solution. In view of enhancement by riboflavin of only the one-electron ETase and OXase reactions (a) appears the most likely explanation. It was observed that addition of riboflavin affected only the slope and not the intercept of the Lineweaver-Burk plot of ETase activity at constant NADH concentration. Therefore the V_{max} of the reaction at infinite concentration of iron is not affected, and this is consistent with riboflavin interposing between reduced enzyme and $K_3Fe(CN)_6$ in the reaction. This type of activation has been termed cooperative (407), by analogy with competitive inhibition.

Steric factors should not play a large role in this reaction, but may well account for the inability of the enzyme to reduce cytochrome c. This view is supported by the fact that when the flavin is loosened in E_{RCM} , and cytochrome c need no longer penetrate the active centre of EH_2 , NADH-linked cytochrome c reductase activity appears. If O_2 must react by a C(4a) peroxo adduct, the low OXase activity of the enzyme may be accounted for by the congestion in the neighbourhood of this part of the ring (cf. Figure 102). Electrostatic repulsion of O_2 by the thiolate anion of EH_2 may also be significant.

CONCLUSIONS

Lipoamide dehydrogenase has been shown to be an enzyme fulfilling the criteria of type C2 multifunctionality, as outlined in Part A of the Introduction. This has been demonstrated by many examples of independent variations and behaviour of the DHase, THase, ETase, DPase and OXase activities, and by supplementing the existing mechanistic and structural studies on the enzyme. The multifunctionality does not arise from the presence of distinct isozymes, nor does it depend directly on monomeric or dimeric species. Rather it is an inherent property of the active centre arrangement of the enzyme. The primary dependence of all activities upon cofactor chemistry typifies type C2 multifunctionality. DHase activity proceeds through an enzyme disulfide which is intimately linked with the flavin and requires reduction by flavin. The THase reaction is almost certainly flavin mediated. OXase, ETase, and DPase activities are properties of the free reduced flavin, which are greatly reduced in the enzyme, due in large part to the preferred thiolate structure of the normal EH_2 . O_2 may also face steric requirements in formation of a C(4a) adduct, and DCIP may also have difficulty reacting with flavin trapped in a hydrophobic pocket. This latter fact is sufficient to account for the one property which the enzyme flavin does not share with its unbound counterpart, namely its reactivity with pyridine nucleotides.

This work, then, represents a well documented case of type C2

multifunctionality conferred by a cofactor but harnessed and controlled by the protein molecule. Further, it illustrates exploitation of the multifunctional properties of this key enzyme to gain a better understanding into the structure and function of the system.

APPENDIX I

METHODS OF KINETIC ANALYSIS

The reactions dealt with are all bisubstrate, and use the terminology of Cleland (408) throughout. With products ignored (initial velocities only considered), the velocity is expressed in terms of the substrates A and B. (concentrations understood) as

$$v_i = \frac{V_{AB}}{K_{ia}K_b + K_bA + K_aB + AB} \quad (I-1)$$

for a sequential mechanism. When a ternary complex fails to form, the $K_{ia} = 0$ eliminates the constant term. In this ping pong mechanism, one product dissociates from the enzyme before the second substrate reacts. Equation (I-1) follows from a steady state Michaelis-Menten treatment (eg. see 409-412).

In practice, the measured v is plotted against one substrate (say A) in a double reciprocal plot, the second substrate being held constant. When the mechanism is obeyed, the plot is linear, as

$$\frac{1}{v_i} = \frac{K_a}{V} \left(1 + \frac{K_{ia}K_b}{K_aB} \right) \frac{1}{A} + \frac{1}{V} \left(1 + \frac{K_b}{B} \right) \quad (I-2)$$

and a family of converging lines, each at different B, is obtained. Secondary plots of the slopes or intercepts of these lines *versus*

1/B then yield the four parameters V , K_a , K_b , and K_{ia} . For the ping pong mechanism, $K_{ia} = 0$ necessitates that the primary plot is a family of parallel lines. As A and B become very large in (I-1), the rate equation reduces to $v_i = V$, and thus V is the maximum velocity at saturating substrate concentrations. In the Cleland nomenclature, V has units of amount of substrate converted per unit time, and depends on enzyme concentration. When this value is divided by enzyme concentration, the more meaningful catalytic efficiency, V/E_t , is obtained.

The K_a and K_b are evaluated as B and A respectively approach infinity (cf. equation (I-2)). Under these circumstances, equation (I-1) reduces, say for very large B, to

$$v_i = \frac{VA}{K_a + A} \quad (I-3)$$

In this Michaelis-Menten expression, K_a is the concentration of A necessary to give one-half maximal velocity at saturation of B, and *vice versa*.

When B approaches zero, equation (I-1) becomes

$$v_i = \frac{(VB/K_b)A}{K_{ia} + B} \quad (I-4)$$

and thus K_{ia} is the limiting value of K_a as B goes to zero. Further, as B goes to zero, reaction of B with the A-enzyme complex, EA, must go to zero. Then EA can be maintained in equilibrium, and K_{ia} is

the true dissociation constant of EA (see 411). The origin of the $K_{ia} = 0$ criterion for the ping pong mechanism is now clear, as there is no reaction of B with EZ, or no ternary complex. It should be noted that the nature of A and B cannot normally be distinguished by initial phase kinetics alone.

In the present work, regression analysis is used to fit experimental data with 4x4 points or more in the asymptotic (linear) region of the primary plot which are in turn fitted into secondary plots. The concern is primarily with qualitative mechanistic comparison.

Formulae for calculating standard errors for the kinetic parameters obtained by regression are given by Cornish-Bowden (411), and are typically $\pm 20\%$. As Cornish-Bowden points out, however, variation from one experiment to the next is often much greater, and expression of the true standard error of a kinetic parameter is an exaggeration of the certainty of the value. In the present work, variation by as much as an order of magnitude in some parameters with history of the enzyme sample necessitates comparison with a control specific for the given experimental conditions to which the treated enzyme was subjected, and which was assayed simultaneously. Except where noted, all kinetic parameters are the average of two to five such independent experiments.

APPENDIX II

LIGAND BINDING STUDIES

This Appendix treats more fully the methods of determining binding constants, and their applicability and limitation. Our starting point in all these studies is the Hill equation, a simple derivation of which has been given by Wold (12). For binding of a molecule A to a protein E, the association constant K_a is given by

$$K_a = \frac{[EA]}{[A][E]} \quad (\text{II-1})$$

We next define $r = [A_b] / [E_t]$ where the subscripts have the same meaning as in the text. Then

$$\begin{aligned} r &= \frac{[AE]}{[E] + [AE]} \\ &= \frac{K_a [A][E]}{[E] + [AE]} \\ &= \frac{K_a [A]}{1 + [AE]/[E]} \end{aligned}$$

But $[AE]/[E] = K_a [A]$, so

$$r = \frac{K_a [A]}{1 + K_a [A]} \quad (\text{II-2})$$

If there is a multiple equilibrium situation, that is n binding sites allowing species E , A , EA , EA_2 , ... EA_n all to exist in equilibrium, straightforward algebra extends the definition to yield the form of equation (19) in the text. Thus if n sites with similar association constants are present,

$$\bar{r} = \frac{nK_a[A]}{1 + K_a[A]} \quad (\text{II-3})$$

and we shall use the reciprocal form of Klotz (370),

$$\frac{1}{\bar{r}} = \frac{1}{n} + \frac{1}{nK_a[A]} \quad (\text{II-4})$$

Thus a plot of $1/\bar{r}$ versus $1/[A]$ yields values of n and K_a . This form has an inherent disadvantage in that it causes data points to group near the origin increasing the uncertainty of the fit. We use it here, however, as it separates n from the other unknowns, and when a value of n is assumed, eases data analysis.

In the set of experiments reported here, we are following binding by observing a change in absorption ΔA due to E , noting that the molar extinction coefficient of the enzyme chromophore is changed from ϵ to ϵ_b on binding. With a difference spectrum in cuvettes of path length l , and a single site present,

$$\frac{\Delta A}{l} = \frac{\epsilon_b[E_b] + \epsilon[E_f] - \epsilon[E_t]}{\epsilon_b E_b + \epsilon(E_f - E_t)} \quad (\text{II-5})$$

where we drop the square brackets for convenience and concentration will be understood. Since $E_f + E = E_t$, equation (II-5) becomes

$$\begin{aligned}\Delta A/l &= E_b(\epsilon_b - \epsilon) \\ &= \Delta\epsilon \cdot E_b\end{aligned}\quad (\text{II-6})$$

From equation (II-4),

$$\frac{E_t}{A_b} = 1 + \frac{1}{K_a} \cdot \frac{1}{(A_t - A_b)} \quad (\text{II-7})$$

Noting that with a single site $E_b = A_b$, substitution of (II-6) into (II-7) gives

$$\frac{E_t}{(\Delta A/\Delta\epsilon \cdot l)} = 1 + \frac{1}{K_a} \cdot \frac{1}{A_t - (\Delta A/\Delta\epsilon \cdot l)} \quad (\text{II-8})$$

where now all terms are known but $\Delta\epsilon$ and K_a .

We solve this equation by systematically varying $\Delta\epsilon$ and plotting $E_t \cdot \Delta\epsilon \cdot l / \Delta A$ versus $[A_t - (\Delta A/\Delta\epsilon \cdot l)]^{-1}$ until a straight line of intercept unity is obtained. This procedure is carried out by the program SCATCH included here.

If there are two binding sites, of association constants K_1 and K_2 for native enzyme, and we assume non-interaction of sites, there are species $E^I A$, $E^{II} A$, and $E^{I,II} A_2$ where the superscript denotes the site bound, I and II being distinguishable. An exact

C SCATCH

REAL INT,N,INC
 DIMENSION ET(15),DELX(15),AT(15),DEL(15),Y(15),X(15)

READ,NO

N=NO

OUTPUT,'ET'

READ,(ET(I),I=1,NO)

OUTPUT,'AT'

READ,(AT(I),I=1,NO)

OUTPUT,'DEL'

READ,(DELX(I),I=1,NO)

DO 5 I=1,NO

DELX(I)=DELX(I)/0.4375

5 CONTINUE

30 OUTPUT,'DEL EPS, INC'

READ,DPX,INC

DO 10 J=1,10

DP=DPX+INC*(J-1)

DO 15 I=1,NO

DEL(I)=DELX(I)*(1./DP)

15 CONTINUE

C LEAST SQUARES

SX=0

SY=0

SXY=0

SX2=0

DO 20 I=1,NO

Y(I)=ET(I)/DEL(I)

X(I)=1./(AT(I)-DEL(I))

SX=SX+X(I)

SY=SY+Y(I)

SXY=SXY+(X(I)*Y(I))

SX2=SX2+(X(I)**2)

20 CONTINUE

SL=((N*SXY)-(SX*SY))/((N*SX2)-(SX**2))

INT=(SY-(SL*SX))/N

```

WRITE,DP,INT.
C
C
IF(ABS(INT-1.0).GT.0.1)GOTO 10
GOTO 25
10 CONTINUE
25 OUTPUT, 'NEW DEL EPS, INC, INPUT 1'
READ,K
IF(K.EQ.1)GOTO 30
C
C STD = DEV.
C
SUM=0
DO 35 I=1,N0
SUM=SUM+(Y(I)-(SL*X(I)+INT))**2
35 CONTINUE
STD=(SUM/N)**0.5
C
C
WRITE40,DP,INT,SL,STD
40 FORMAT(1X,///,'DELTA EPSILON =',E10.5,/, 'INT =',
1,E10.5,/, 'SL =',E10.5, 'STD. DEV. =',E10.5)
WRITE45
45 FORMAT(5X, '1/(AT-AB)',10X, 'ET/AB')
DO 50 I=1,N0
WRITE55,X(I),Y(I)
55 FORMAT(5X,E10.5,10X,E10.5)
50 CONTINUE
** STOP
END
!OFF

```

treatment as above becomes algebraically complicated, and the final expression will have as unknowns K_1 , K_2 , and extinction coefficients for each of the three bound enzyme species. Thus we turn to approximate methods.

The first of these methods is due to Stockell (369). We must assume that when the value of the measured parameter (here ΔA) levels off at increasing concentration of A, we have reached saturation of all binding sites. Furthermore, we assume that at all intermediate values, the ratio of intermediate to final values, $\Delta A_i/\Delta A_f$, represents the fraction of sites bound. This will be the case if binding at both sites alters the extinction coefficient to the same degree. We next define

$$p_i = (\Delta A_i/\Delta A_f) \cdot E_t$$

and then $np = A_b$. In the Klotz equation (II-4),

$$\frac{E_t}{np} = \frac{1}{n} + \frac{K_d}{n(A_t - np)} \quad (\text{II-9})$$

where we have used the dissociation constant $K_d = K_a^{-1}$. This rearranges to the form

$$\frac{A_t}{p} = \frac{K_d}{(E_t - p)} + n \quad (\text{II-10})$$

and we then plot A_t/p versus $(E_t - p)^{-1}$ to solve for n and K_d . If $n > 1$, there are n straight line regions to the plot, each of slope K_{di} .

The second approximate method we use is that of Harbury and Foley (87) adapted to fluorescence work by Su and Wilson (246). We shall start with the form given by the latter authors (and misprinted in 246),

$$\frac{1}{\Delta F} = \frac{1}{(F_E - F_{EA})} + \frac{K_d}{(F_E - F_{EA})} \cdot \frac{1}{A_t} \quad (\text{II-11})$$

where ΔF is the observed change in fluorescence intensity on addition of A to a solution of E, F_E and F_{EA} are the relative fluorescence emission abilities of free and complexed enzyme, and the other symbols have the same meanings as above. In the present instance, a plot of $1/\Delta F$ versus $1/A_t$ is constructed, and division of the slope of the straight line obtained by its intercept yields K_d .

On inversion, equation (II-11) becomes

$$\Delta F = (F_E - F_{EA}) + (F_E - F_{EA}) \cdot A_t / K_d$$

or

$$\frac{\Delta F}{(F_E - F_{EA})} = 1 + A_t K_d \quad (\text{II-12})$$

If the assumption is made that the complex EA is non-fluorescent, then A is a quencher and $F_{EA} = 0$. Equation (II-12) becomes

$$\frac{\Delta F}{F_E} = 1 + [Q] K_a \quad (\text{II-13})$$

Equation (II-13) is quite similar to the Stern-Volmer equation (cf. equation (27) in the text). The left hand side expresses the decrease in fluorescence relative to the inherent fluorescence of the chromophore. This is intimately linked with the quantum yield decrease upon complexation in that, if $F_{EA} = 0$, the observed quantum yield varies with the change in fluorescence, provided the absorption remains constant.

REFERENCES

1. George R. Stark; TIBS (March 1977), pp. 64-66.
2. Kaspar Kirschner and Hans Bisswanger; Annu. Rev. Biochem., 45, 143-166 (1976).
3. G. Rickey Welch; Prog. Biophys. Mol. Biol., 32 (2), 103-191 (1977).
4. F. H. Gaertner; Trends Biochem. Sci., Ref. Ed., 3 (1), 63-63 (1978).
5. Sten Orrenius and Lars Ernster; in *Molecular Mechanisms of Oxygen Activation*, Osamu Hayaishi, ed.; Academic Press, New York, 1974. pp. 215-244.
6. A.P. Alvares and Attaliah Kappos; J. Biol. Chem., 252 (18), 6373-6378 (1977).
7. F. Peter Guengerich; J. Biol. Chem., 253 (21), 7931-7939 (1978).
8. M.J. Fasco, K.P. Vatsis, L.S. Kaminsky and M.J. Coon; J. Biol. Chem., 253 (21), 7813-7820 (1978).
9. Gisela Heidecker and Benno Muller-Hill; Mol. Gen. Genet., 155 (3) 301-307 (1977).
10. B. Mueller-Hill, G. Heidecker and J. Kania; Struct. Funct. Relat. Prot., Proc. John Innes Symp., 3rd. (1976), pp.167-179; quoted from Chem. Abstr., 87, 179530u (1977).
11. D.M. Bonner, J.A. DeMoss and S.E. Mills; in *Evolving Genes and Proteins*; V. Bryson and H.J. Vogel, eds.; Academic Press, London and New York, 1965. pp. 305-318.
12. Finn Wold; *Macromolecular Structure and Function*; Prentice-Hall, Englewood Cliffs, N.J., 1971.
13. Albert Light; *Proteins: Structure and Function*; Prentice-Hall, Englewood Cliffs, N.J., 1974.
14. Eugene Zeffren and Philip L. Hall; *The Study of Enzyme*

Mechanisms; John Wiley and Sons, N.Y., 1973.

15. W.G.J. Hol, P.T. van Duijnen and H.J.C. Berendsen;
Nature, 273, 443-448 (1978).
16. Robert L. Baldwin; TIBS, (March 1978), pp. 66-68.
17. G.E. Schulz, R.H. Schirmer, W. Sachsenheimer and E.F. Pai;
Nature, 273, 120-124 (1978).
18. M.G. Rossman, Anders Liljas, Carl-Ivar Branden and L.J.
Barnaszk; in *The Enzymes*, 3rd., vol. XI, pt. A, P.D.
Boyer, ed., Academic Press, New York, 1975. pp. 61-102.
19. Christian B. Anfinsen; Science, 181, 223-230 (1973).
20. Peter Y. Chou and Gerald D. Fasman; Ann. Rev. Biochem.,
47, 251-276 (1978).
21. J. Theze and I. Saint-Girons; J. Bacteriol., 118 (3),
990-998 (1974).
22. E. Schweizer, B. Kniep, H. Castorph and U. Holzner;
Eur. J. Biochem., 39, 353-362 (1973).
23. S.-L. Li, J. Hanlon and C. Yanofsky; Nature, 248, 48-50
(1974).
24. C. Yanofsky, V. Horn, M. Bonner and S. Stasiowsky;
Genetics, 69, 409-433 (1971).
25. Frank H. Gaertner, Mary C. Ericson, John A. Demoss;
J. Biol. Chem., 245 (3), 595-600 (1970).
26. G.R. Welch and F.H. Gaertner; Proc. Natl. Acad. Sci. USA,
72 (11), 4218-4222 (1975).
27. James Lumsden and John R. Coggins; Biochem. J., 161, 599-
607 (1977).
28. Ronald G. Duggelby, Margaret K. Sneddon and John F.
Morrison; Biochemistry, 17 (8), 1548-1554 (1978).
29. Rowland H. Davis; in *Organizational Biosynthesis*, H.J. Vogel,
J.O. Lampen and Vernon Bryson, eds.; Academic Press, N.Y. and
London, 1967. pp. 303-322.

30. J.C. Schmit, S.W. Artz and H. Zalkin; J. Biol. Chem., 245, 4019-4027 (1970).
31. Elizabeth Heyde and John F. Morrison; Biochemistry, 17 (8), 1573-1580 (1978).
32. Andrew C. Storer and Athel Cornish-Bowden; Biochem. J., 141, 205-209 (1974).
33. G.R. Welch and F.H. Gaertner; Proc. Natl. Acad. Sci. USA, 72 (11), 4218-4222 (1975).
34. G.R. Welch; Prog. Biophys. Mol. Biol., 32, 103-191 (1977).
35. Carl-Ivar Branden, Hans Jornvall, Hans Eklund and Bo Furugren; The Enzymes, 114, 104-190 (1975).
36. Thomas Cronholm and Christina Fors; Eur. J. Biochem., 70, 83-87 (1976).
37. Michael F. Dunn, J.F. Biellmann and Guy Branlant; Biochemistry, 14 (14), 3176-3182 (1975).
38. C.S. Tsai; Biochem. Biophys. Res. Commun., 86 (3), 808-814 (1979).
39. Y. Pocker, J.E. Meany, Brian C. Davis, James Arrigoni and John E. Stein; J. Am. Chem. Soc., 100 (9), 2883-2885 (1978).
40. L.J. Zatman, N.O. Kaplan, S.P. Colowick and M.-M. Ciotti; J. Biol. Chem., 209, 453-466 (1954).
41. ibid., pp. 467-484.
42. Biochim. Biophys. Acta, 429, 1-45 (1976).
43. Joseph Yourno, T. Kohno, and John R. Roth; Nature, 228, 820-824 (1970).
44. Thomas E. Barman; *Enzyme Handbook*, Supplement I; Springer-Verlag, Berlin, Heidelberg, 1974.
45. W.I. Wood, David O. Peterson, and Konrad Bloch; J. Biol. Chem., 253 (8), 2650-2656 (1978).

46. Thomas E. Barman; *Enzyme Handbook* (2 vols.); Springer-Verlag, Berlin, Heidelberg, 1969.
47. Cynthia F. Bearer and Kenneth E. Neet; *Biochemistry*, 17 (17), 3512-3516 (1978).
48. *ibid.* pp. 3517-3522.
49. *ibid.* pp. 3523-3530.
50. Thomas Kornberg and Arthur Kornberg; *The Enzymes*, 10, 119-144 (1974).
51. Randle C. White and Thomas E. Nelson; *Biochim. Biophys. Acta*, 400, 154-161 (1975).
52. Carol Taylor, A.J. Cox, J.C. Kernohan, and Philip Cohen; *Eur. J. Biochem.*, 51, 105-115 (1975).
53. D.E. Koshland, Jr., and H. Levitzki; *The Enzymes*, 10, 539-559 (1974).
54. James L. Paukert,; Ph.D. Thesis, University of Cal., Berkely, 1977.
55. Jesse C. Rabinowitz; *Biochem. Soc. Trans.*, 4 (5), 850-853 (1976).
56. LaVerne Schirch; *Arch. Biochem. Biophys.*, 189 (2), 283-290 (1978).
57. T. Tanabe, K. Wada, T. Okazaki, and S. Numa; *Eur. J. Biochem.*, 57, 15-24 (1975).
58. Norman H. Giles, Mary E. Case, C.W.H. Partridge, and S.I. Ahmed; *Proc. Natl. Acad. Sci. USA*, 58, 1453-1460 (1967).
59. C.A.F. Edwards and James C. Orr; *Biochemistry*, 17 (21), 4370-4376 (1978).
60. Rudolf Fluri, Alan Coddington, and Urs Flury; *Molec. Gen. Genet.*, 147, 271-282 (1976).
61. E.W. Nester, J.H. Lorence, and D.S. Nasser; *Biochemistry*, 6, 1553-1563 (1967).

62. Shlomo Eisenberg, Jack Griffith, and Arthur Kornberg; Proc. Natl. Acad. Sci. USA, 74 (8), 3198-3202 (1977).
63. Gunther Siebert, M. Carsiotis, and G.W.E. Plaut; J. Biol. Chem., 226, 977-991 (1957).
64. K. Erhard and R.C. Davis; J. Biol. Chem., 250, 5945-5950 (1975).
65. Donald J. Creighton and Irwin A. Rose; J. Biol. Chem., 251 (1), 61-68 (1976).
66. ibid., pp. 69-72.
67. Y. Pocker and S. Sarkanen; Advances Enzymol., 47, 149-274 (1978).
68. F. LeGoffic, N. Moreau, and N. Masson; Ann. Microbiol. (Paris) 128B (4), 465-469 (1977).
69. Rhoda I. Feldman and Henry Weiner; J. Biol. Chem., 247 (1), 267-272 (1972).
70. A.K.H. MacGibbon, S.J. Haylock, P.D. Buckley, and L.F. Blackwell; Biochem. J., 171, 533-538 (1978).
71. K. Ikura, R. Sasaki, H. Narita, E. Sugimoto, and H. Chiba; Eur. J. Biochem., 66, 515-522 (1976).
72. R. Sasaki, S. Utsumi, E. Sugimoto, and H. Chiba; Eur. J. Biochem., 66, 523-533 (1976).
73. Giovanna Colombo, Gerald M. Carlson, and Henry A. Lardy; Biochemistry, 17 (25), 5321-5329 (1978).
74. Gerald M. Carlson, Giovanna Colombo, and Henry A. Lardy; Biochemistry, 17 (25), 5329-5338 (1978).
75. D.M. Waisman, T.J. Singh, and J.H. Wang; J. Biol. Chem., 253 (10), 3387-3390 (1978).
76. Ryozi Sasaki; Nippon Nogei Kagaku Kaishi, 51 (10), R101-R107 (1977); quoted from Chem. Abstr. 88, 100694 q (1978).
77. P. Hemmerich, C. Veeger, and H.C.S. Wood; Angew. Chem. Internat. Edit., 4 (8), 671-686 (1965).
78. W.R. Weimar and A.H. Neims; in *Riboflavin*, R.S. Rivlin, ed., Plenum Press, N.Y., 1975. pp. 1-47.
79. G. Weber; Biochem. J., 47, 114-121 (1950).

80. Michael A. Slifkin; *Charge Transfer Interactions of Biomolecules*, Academic Press, London, 1971.
81. Kunio Yagi; in *Biochemical Fluorescence: Concepts*, vol. 2, R.F. Chen and Harold Edelhoch, eds., Marcel Dekker, New York, N.Y., 1972. pp. 639-658.
82. Donald Voet and Alexander Rich, in *Flavins and Flavoproteins: 3rd International Symposium*, Henry Kamin, ed. University Park Press, Baltimore, 1971. pp. 23-35
83. A. Bowd, P. Byron, J.B. Hudson, and J.H. Turnbull; *Photochem. Photobiol.*, 8, 1-10 (1968).
84. M. Kainosho and Y. Kyogoku; *Biochemistry*, 11 (5), 741-752 (1972).
85. R.H. Sarma, Priscilla Dannies, and Nathan O. Kaplan; *Biochemistry*, 7 (12), 4359-4367 (1968).
86. Matthew Raszka and Nathan O. Kaplan; *Proc. Natl. Acad. Sci. USA*, 71 (11), 4546-4550 (1974).
87. Henry A. Harbury and Kathryn A. Foley; *Proc. Natl. Acad. Sci. USA*, 44, 662-668 (1958).
88. H.A. Harbury, K.F. LaNoue, P.A. Loach, and R.M. Amick; *Proc. Natl. Acad. Sci. USA*, 45, 1708-1717 (1959).
89. A. de Kok, R.D. Spencer, and G. Weber, *Fed. Proc.*, 27, 298 (1968).
90. John R. Platt; *J. Chem. Phys.*, 17 (5), 484-495 (1949).
91. G.R. Penzer, G.K. Radda, J.A. Taylor, and M.B. Taylor; *Vitamins and Hormones*, 28, 441-466 (1970).
92. Edward M. Kosower; *Molecular Biochemistry*, McGraw-Hill, New York 1962.
93. Arpad G. Fazekas and Karoly Kokai; *Meth. Enzymol.* XVIII(B), 385-398 (1971).
94. Jacek Koziol; *Meth. Enzymol.*, XVIII(B), 253-285 (1971).
95. Bernard Pullman and Albert Pullman; *Proc. Natl. Acad. Sci. USA*, 45, 136-144 (1959).
96. J.L. Fox, S.P. Laberge, K. Nishimoto, and L.S. Forster; *Biochem. Biophys. Acta*, 136, 544-550 (1967).
97. Pill-Soon Song; *J. Phys. Chem.* 72 (2), 536-542 (1968).

98. J.M. Lhoste, A. Haug, and P. Hemmerich; *Biochemistry* 5, 3290-3300 (1966).
99. Pill-Soon Song; *Int. J. Quantum Chem.*, VIII, 303-316 (1969).
100. Pill-Soon Song; in *Flavins and Flavoproteins: 3rd International Symposium*, Henry Kamin, ed., University Park Press, Baltimore, 1971. pp. 37-61.
101. G. Weber, V. Massey, and G. Greull, (unpublished; quoted in ref. 103).
102. William E. Kurtin and Pill-Soon Song; *Photochem. Photobiol.*, 7, 263-273 (1968).
103. Graham Palmer and Vincent Massey; in *Biological Oxidations*, T.P. Singer, ed. Interscience, N.Y. 1968. pp. 263-300.
104. G. Weber; in *Flavins and Flavoproteins*, E.C. Slater, ed., Elsevier, Amsterdam, 1966. pp. 15-21.
105. Ming Sun, Thomas A. Moore, and Pill-Soon Song; *J. Am. Chem. Soc.*, 94 (5), 1730-1740 (1972).
106. G.R. Penzer and G.K. Radda; *Meth. Enzymol.* XVIII(B), 479-495 (1971).
107. Eddie C. Smith and David E. Metzler; *J. Am. Chem. Soc.*, 85, 3285-3288 (1963).
108. W.L. Cairns and D.E. Metzler; *J. Am. Chem. Soc.*, 93, 2772-2777 (1971).
109. S.P. Vaish and G. Tollin; *J. Bioenerg.* 1(2), 181-192 (1970).
110. Vincent Massey, Marian Stankovich, and Peter Hemmerich; *Biochemistry*, 17 (1), 1-8 (1978).
111. G.K. Radda; *Biochem. Biophys. Acta*, 112, 448-458 (1966).
112. Wilhelm R. Frisell and Cosmo G. MacKenzie; *Proc. Natl. Acad. Sci. USA*, 45, 1568-1572 (1959).
113. G.K. Radda and Melvin Calvin; *Biochemistry* 3(3), 384-393 (1964).
114. D.R. Kearns, R.A. Hollins, A.U. Khan, and P. Radlick; *J. Am. Chem. Soc.* 89 (21), 5456-5457 (1967).
115. Paul G. Johnson, Anne P. Bell, and Donald B. McCormick; *Photochem. Photobiol.*, 21 205-208 (1975).

116. Peter Hemmerich; *Vitamins and Hormones* 28 467-487 (1970).
117. Thomas C. Bruice; *Prog. Bioorg. Chem.*, 4, 1-87 (1976).
118. Ming Sun and Pill-Soon Song; *Biochemistry*, 12, 4663-4669 (1973).
119. R.F. Williams, S.S. Shinkai, and T.C. Bruice; *J. Am. Chem. Soc.*, 99(3), 921-931 (1977).
120. T.C. Bruice and Y. Yano; *J. Am. Chem. Soc.*, 97 (18), 5263-5271 (1975).
121. Michael Novak and T.C. Bruice; *J. Am. Chem. Soc.*, 99(24), 8079-8082, (1977).
122. P. Hemmerich; *Helv. Chim. Acta*, 43, 1942-1946 (1960).
123. W.C. Kenney and T.P. Singer; *J. Biol. Chem.*, 252(14), 4767-4772, (1977).
124. D.E. Edmondson, W.C. Kenney, and T.P. Singer; *Biochemistry*, 15(14), 2937-2945 (1976).
125. W.C. Kenney, D.E. Edmondson, T.P. Singer, D.J. Steenkamp, and J.C. Schobert; *Biochemistry*, 15(22), 4931-4935 (1976).
126. T.C. Bruice, T.W. Chan, J.P. Taulane, Ichiro Yokoe, D.L. Elliott, R.F. Williams, and M. Novak; *J. Am. Chem. Soc.*, 99(20), 6713-6720 (1977).
127. P. Hemmerich, S. Ghisla, V. Hartmann, and F. Muller; in *Flavins and Flavoproteins, 3rd International Symposium*, H. Kamin, ed.; University Park Press, Baltimore, 1971, pp. 83-105.
128. I. Isenberg and A. Szent-Gyorgyi; *Proc. Natl. Acad. Sci. USA.*, 47, 1229-1231 (1959).
129. I. Isenberg, S.L. Baird, and A. Szent-Gyorgyi; *Proc. Natl. Acad. Sci. USA.*, 47, 245-247 (1961).
130. Takashi Sakurai and Haruo Hosoya; *Biochim. Biophys. Acta*, 112, 459-468 (1966).
131. D.J.T. Porter, Gunter Blakenhorn, and Lloyd L. Ingraham; *Biochem. Biophys. Res. Commun.*, 52(2), 447-452 (1973).
132. Gunter Blakenhorn; *Biochemistry*, 14(14), 3172-3176 (1975).
133. R.T. Proffitt, L.L. Ingraham and G. Blakenhorn; *Biochim. Biophys. Acta*, 362, 534-548 (1974).
134. Gunter Blakenhorn; *Eur. J. Biochem.*, 50, 351-356

135. D.J.T. Porter, Harold J. Bright, and Donald Voet; *Nature*, 269, 213-217 (1977).
136. C.H. Suelter and D.E. Metzler; *Biochim. Biophys. Acta*, 44, 23-33 (1960).
137. Lloyd L. Ingraham; *Biochemical Mechanisms*, John Wiley & Sons, New York, 1962.
138. Lubert Stryer; *Biochemistry*, W.H. Freeman, San Francisco, 1975.
139. Albert L. Lehninger; *Biochemistry*, Worth Publishers, N.Y., 1970.
140. E.M. Kosower, in *Flavins and Flavoproteins*, E.C. Slater, ed., Elsevier, Amsterdam, 1966. pp. 1-14.
141. V. Massey, G. Palmer, and D. Ballou; in *Flavins and Flavoproteins, 3rd International Symposium*, H. Kamin, ed.; University Park Press, Baltimore, 1971. pp. 349-361.
142. Ronald J. Kill and David A. Widdowson; *J.C.S. Chem. Commun.* (1976), pp. 755-756.
143. J. Bryan Jones and Keith E. Taylor; *Can. J. Chem.*, 54(19), 2969-2973 (1976).
144. *ibid*, pp. 2974-2980.
145. M. Yamasaki, S. Fujii, and T. Yamano; in *Reactivity of Flavins*, Kunio Yagi, ed., University of Tokyo Press, Tokyo 1975. pp. 37-49.
146. C.T. Walsh, Agnes Schönbrunn, and R.H. Abeles; *J. Biol. Chem.*, 246(22), 6855-6866 (1971).
147. G. Tollin and F. Rizzuto, in *Reactivity of Flavins*, Kunio Yagi, ed.; University of Tokyo Press, Tokyo 1975. pp. 25-28.
148. A. Ehrenberg, F. Muller, and P. Hemmerich; *Eur. J. Biochem.*, 2, 286-293 (1967).
149. L. Michaelis; in *The Enzymes*, vol. 2 part 1; J.B. Sumner and K. Myrback, eds., Academic Press, New York, 1951. pp. 1-54.
150. H.R. Mahler and D.E. Green; *Science*, 120, 7-12 (1954).
151. H.R. Mahler, A.S. Fairhurst, and B. Mackler; *J. Am. Chem. Soc.* 77, 1514-1521 (1955).
152. L.L. Ingraham; *J. Chem. Phys.*, 18, 988 (1950).

153. Helmut Beinert; J. Am. Chem. Soc., 78, 5323-5328 (1956).
154. S. Ghisla, V. Massey, Jean-Marc Lhoste, and S.G. Mayhew; in *Reactivity of Flavins*, Kunio Yagi, ed., University of Tokyo Press, Tokyo, 1975. pp. 15-24.
155. P. Hemmerich and W. Haas; in *Reactivity of Flavins*, Kunio Yagi, ed., University of Tokyo Press, Tokyo, 1975. pp. 1-13.
156. P. Kierkegaard, R. Norrestam, P. Werner, I. Csoregh, M. Glehn, R. Karlsson, M. Leijonmarck, O. Ronnquist, B. Stensland, O. Tillberg, L. Torbjornsson; in *Flavins and Flavoproteins, 3rd International Symposium*, H. Kamin, ed., University Park Press, Baltimore, 1971. pp. 1-22.
157. G.I. Dmitrienko, V. Snieckus, and T. Viswanatha; Bioorg. Chem., 6, 421-429 (1977).
158. T.W. Chan and T.C. Bruice; J. Am. Chem. Soc., 99(7), 2387-2389 (1977).
159. C. Kemal, T.W. Chan, and T.C. Bruice; J. Am. Chem. Soc., 99(22), 7272-7286 (1977).
160. Christopher Walsh; Ann. Rev. Biochem., 47, 881-931 (1978).
161. P. Hemmerich and A. Wessiak; in *Flavins and Flavoproteins*, T.P. Singer, ed., Elsevier, Amsterdam, 1976. pp. 9-22.
162. H.I.X. Mager; Tetrahedron, 33, 981-989 (1977).
163. S. Ghisla, J.W. Hastings, V. Favaudon, and J.M. Lhoste; Proc. Natl. Acad. Sci. USA, 75(12), 5860-5863 (1978).
164. Cemal Kemal and T.C. Bruice; J. Am. Chem. Soc., 99(21), 7064-7067 (1977).
165. Marcia S. Flashner and Vincent Massey; in *Molecular Mechanisms of Oxygen Activation*, Osamu Hayaishi, ed., Academic Press, New York, 1974. pp. 245-283.
166. Vincent Massey and Peter Hemmerich; in *The Enzymes*, P.D. Boyer, ed., 3rd ed., vol. XII, part B; Academic Press, New York, 1975. pp. 191-252.
167. Harold J. Bright and D.J.T. Porter; in *The Enzymes*, P.D. Boyer, ed., 3rd ed., vol. XII, part B; Academic Press, New York, 1975. pp. 421-506.
168. Howard L. Levine, Y. Nakagawa, and E.T. Kaiser; Biochem. Biophys. Res. Commun., 76(1), 64-70 (1977).

169. Tetsuo Otsuki, Y. Nakagawa, and E.T. Kaiser; J.C.S. Chem. Commun., 1978, pp. 457-459.
170. Seiji Shinkai, Shinji Yamada, and Toyoki Kunitake; Macromolecules, 11, 65-68 (1978).
171. Wayne J. Spetnagel and Irving M. Klotz; Biopolymers, 17, 1657-1668 (1978).
172. Eduard Knobloch; Meth. Enzymol. XVIII(B) 305-381 (1971).
173. Seiji Shinkai, Reiko Ando, and Toyoki Kunitake; Biopolymers, 17, 2757-2760 (1978).
174. Malcolm Dixon and D.C. Webb; *The Enzymes*, Academic Press, New York, 1964.
175. T.P. Singer and D.E. Edmondson; FEBS Lett., 42, 1-14 (1974).
176. T.P. Singer and W.C. Kenney; Vitamins and Hormones, 32, 1-45 (1974).
177. E.B. Kearney and W.C. Kenney; Horizons Biochem. Biophys., 1, 62-96 (1974).
178. C. Veeger, H. Voetberg, J. Visser, J.E.G. Staal, and J.F. Koster; in *Flavins and Flavoproteins, 3rd International Symposium*, H. Kamin, ed., University Park Press, Baltimore, 1971. pp. 261-294.
179. N.K. Gupta and B. Vennesland; J. Biol. Chem., 239, 3787-3789 (1964).
180. P. Strittmatter; J. Biol. Chem., 236, 2329-2335 (1961).
181. Cees Veeger; in *Flavins and Flavoproteins, Proceedings of 2nd Conference*, Kunio Yagi, ed., University of Tokyo Press, Tokyo, 1968. pp. 252-267.
182. V. Massey and H. Ganther; Biochemistry, 4, 1161-1173 (1965).
183. P. Hemmerich, G. Nagelschneider, and C. Veeger; FEBS Lett., 8(2), 69-83 (1970).
184. Gregorio Weber; Biochem. J., 47, 114-121 (1950).
185. Sandro Ghisla, Vincent Massey, Jean-Marc Lhoste, and S.G. Mayhew; Biochemistry, 13(3), 589-597 (1974).
186. A.J.W.G. Visser, T.M. Li, H.G. Drickamer, and Gregorio Weber; Biochemistry, 16(22) 4879-4882 (1977).
187. Vincent Massey and Graham Palmer; Biochemistry, 5(10), 3181-3189 (1966).

188. D.L. Elliott and T.C. Bruice; J. Am. Chem. Soc., 95, 7901-7902 (1973).
189. Vincent Massey and Peter Hemmerich; J. Biol. Chem., 252(16), 5612-5614 (1977).
190. Vincent Massey and Peter Hemmerich; Biochemistry, 17(1), 9-16 (1978).
191. Franz Muller, Peter Hemmerich, and Anders Ehrenberg; in *Flavins and Flavoproteins, 3rd International Conference*; Henry Kamin, ed., University Park Press, Baltimore, 1971. pp. 107-122
192. Graham Palmer, Franz Muller, and Vincent Massey; in *Flavins and Flavoproteins, 3rd International Conference*; Henry Kamin, ed., University Park Press, Baltimore, 1971. pp. 123-140.
193. A. Ehrenberg, L.E.G. Erikson, and James S. Hyde; in *Flavins and Flavoproteins, 3rd International Conference*; Henry Kamin, ed., University Park Press, Baltimore, 1971. pp. 141-152.
194. P.A. Shaffer; J. Am. Chem. Soc., 55, 2169-2170 (1933).
195. Henry Kamin; in *Reactivity of Flavins*, K. Yagi, ed., University of Tokyo Press, Tokyo, 1975. pp. 137-143.
196. V. Massey, F. Muller, R. Feldberg, M. Schuman, P.A. Sullivan, L.G. Howell, S.G. Mayhew, R.G. Mathews, and G.P. Foust; J. Biol. Chem., 244(15), 3999-4006 (1969).
197. Franz Muller and Vincent Massey; Meth. Enzymol. XVIII(B), 468-477 (1971).
198. Charles H. Williams, Jr.; in *The Enzymes*, vol XIII part c, 3rd ed.; Paul D. Boyer, ed., Academic Press, New York, 1976. pp. 90-174.
199. Malcolm Dixon; Biochim. Biophys. Acta, 226, 241-258 (1971)
200. ibid, pp. 259-268
201. ibid, pp. 269-284
202. Ferenc Bruno Straub; Biochem. J., 33, 787-792 (1939)
203. H.S. Coran, D.E. Green, and F.B. Straub; Biochem. J., 33, 793-801 (1939).
204. H.R. Mahler, N.K. Sarkar, Leo P. Vernon, and Robert A. Alberty; J. Biol. Chem., 199, 585-597 (1952)

205. Leo P. Vernon, H.R. Mahler, and N.K. Sarkar; J. Biol. Chem., 199, 599-606 (1952).
206. Nerina Savage; Biochem. J., 67, 146-155 (1957)
207. Vincent Massey; Biochem. J., 69, 58R-59P (1958)
208. Vincent Massey; Biochim. Biophys. Acta, 30, 205-206 (1958)
209. Vincent Massey; Biochim. Biophys. Acta, 37, 310-314 (1960)
210. ibid; pp. 314-322
211. V. Massey and C. Veeger; Biochim. Biophys. Acta, 40, 184-185 (1960)
212. Robert L. Searls and D.R. Sanadi; J. Biol. Chem., 236(2), 580-583 (1961).
213. N.V. Bhagavan; *Biochemistry: A Comprehensive Review*, J.B. Lippincott, Philadelphia, 1974.
214. V. Massey, Q.H. Gibson, and C. Veeger; Biochem. J., 77, 341-351 (1960).
215. Robert L. Searls, James M. Peters, and D.R. Sanadi; J. Biol. Chem., 236(8), 2317-2322 (1961).
216. Vincent Massey and C. Veeger; Biochim. Biophys. Acta, 48, 33-47 (1961).
217. C. Veeger and V. Massey; Biochim. Biophys. Acta, 64, 83-99 (1962).
218. R.L. Searls and D.R. Sanadi; J. Biol. Chem., 225, PC 32 (1960).
219. R.L. Searls, and D.R. Sanadi; in *Light and Life*, W.D. McElroy and B. Glass, eds., Johns Hopkins Press, Baltimore, 1961. p. 157.
220. Vincent Massey and N.M. Atherton; J. Biol. Chem., 237(a), 2965-2967 (1965).
221. I.M. Gascoigne and G.K. Radda; J. Chem. Soc, Chem. Commun., (1965) pp. 211-212.
222. Luigi Casola, Philip E. Brumby, and Vincent Massey; J. Biol. Chem., 241(21), 4977-4984 (1966).
223. Vincent Massey and Graham Palmer; J. Biol. Chem., 237(7), 2347-2358 (1962).
224. C. Veeger and V. Massey; Biochim. Biophys. Acta, 67, 679-681 (1963).

225. Vincent Massey; in *The Enzymes*, 1st edit., vol. VII, Paul D. Boyer, ed., Academic Press, New York, 1963. pp. 275-306
226. V. Massey, T. Hofmann, and G. Palmer; *J. Biol. Chem.*, 237, 3820-3828 (1962).
227. C. Veeger and J. Visser; *Meth. Enzymol.* XVIII(B), 582-590 (1971)
228. V. Massey and Q.H. Gibson; *Federation Proc.*, 23, 18-29 (1964).
229. C. Veeger, H. Fehrmann, A.J.W.G. Visser, H.J. Grande, F. Muller, and J.S. Santema; in *Reactivity of Flavins*, Kunio Yagi, ed., University of Tokyo Press, Tokyo, 1975. pp. 119-136
230. George G Guilbault; David N. Kramer, and P. Goldberg; *J. Phys. Chem.*, 69(11), 3696-3699 (1965).
231. Luigi Casola and Vincent Massey; *J. Biol. Chem.*, 241(21), 4985-4993 (1966).
232. Cees Veeger; in *Flavins and Flavoproteins, Proceedings of 2nd Conference*, Kunio Yagi, ed., University of Tokyo Press, Tokyo, 1967. pp. 252-267.
233. J.F. Kalse and C. Veeger; *Biochim. Biophys. Acta*, 159, 244-256 (1968).
234. J. Visser, D.B. McCormick, and C. Veeger; *Biochim. Biophys. Acta*, 159, 257-264 (1968).
235. J. Visser and C. Veeger; *Biochim. Biophys. Acta*, 159, 265-275 (1968).
236. J. Visser and C. Veeger; *Biochim. Biophys. Acta*, 206, 224-241 (1970).
237. H. van Muiswinkel-Voetberg, Jaap Visser, and Cornelis Veeger; *Eur. J. Biochem.*, 33, 265-270 (1973).
238. H. van Muiswinkel-Voetberg and Cornelis Veeger; *Eur. J. Biochem.*, 33, 271-278 (1973).
239. *ibid.*, pp. 279-284
240. Vincent Massey; *Meth. Enzymol.* IX, 272-278 (1966)
241. C.H. Williams, Jr., Giuliani Zanetti, L.D. Arscott, and J.K. McAllister; *J. Biol. Chem.*, 242(22), 5226-5631 (1967).
242. A.M. Stein and G. Czerlinski; *Federation Proc.*, 26, 842 (1967)

243. J. Visser, H. Voetberg, and C. Veeger, in *Pyridine Nucleotide Dependent Dehydrogenases*, H. Sund, ed., Springer-Verlag, Berlin, 1970, pp. 359-373.
244. Jutta K. Reed; J. Biol. Chem., 248(13), 4834-4839 (1973).
245. H. van Muiswinkel-Voetberg and Cornelis Veeger; Eur. J. Biochem., 33, 285-291 (1973).
246. George Su and John E. Wilson; Arch. Biochem. Biophys., 143, 253-260 (1971).
247. C.S. Tsai; Arch. Biochem. Biophys., 159, 453-457 (1973).
248. William H. Scouten, Harry Knowles, Jr., Lisa C. Freitag, and William Obst; Biochim. Biophys. Acta, 482, 11-18 (1977).
249. Christopher R. Lowe; Eur. J. Biochem., 76, 391-399 (1977).
250. *ibid.*, pp. 401-409.
251. *ibid.*, pp. 411-417.
252. Philippe Wahl, Jean-Claude Auchet, A.J.W.G. Visser, and Cees Veeger; Eur. J. Biochem., 50, 413-418 (1975).
253. Philippe Wahl, J.C. Auchet, A.J.W.G. Visser, and F. Muller; FEBS Letters, 44(1), 67-7- (1974).
254. A.J.W.G. Visser, Hans J. Grande, Franz Muller, and Cees Veeger; Eur. J. Biochem., 45, 99-107 (1974).
255. K. Ogasahara, K. Koike, M. Hamada, and T. Hiraoka; J. Biochem., 79, 967-975 (1976).
256. Edwin G. Moore, Emilia Cardemil, and Vincent Massey; J. Biol. Chem., 253(18), 6413-6422 (1978).
257. Abraham M. Stein and Jeanne H. Stein; J. Biol. Chem., 246(3), 670-676 (1971).
258. Abraham M. Stein and Jeanne H. Stein; Biochemistry, 4(8), 1491-1500 (1965).
259. Paul G. Stecker, ed.; *The Merck Index*, 8th edit., Merck & Co. Rahway, N.J., 1968.
260. Colin Thorpe and Charles H. Williams, Jr.; Biochemistry, 13(16) 3263-3268 (1974).

261. Galen C.L. Huang and A.H. Brady; *Biochemistry*, 12(6), 1093-1099 (1973).
262. D.E. Edmondson and G. Tollin; *Biochemistry* 10, 113-123 (1971).
263. Colin Thorpe and Charles H. Williams, Jr.; *Biochemistry*, 14 (11), 2419-2424 (1975).
264. R.G. Mathews and C.H. Williams, Jr.; *Biochim. Biophys. Acta*, 370, 39-48 (1974).
265. C.H. Williams, Jr. and L.D. Arscott; *Z. Naturforsch.*, 27(b), 1078-1080 (1972).
266. R.G. Mathews, L.D. Arscott, and C.H. Williams, Jr.; *Biochim. Biophys. Acta*, 370, 26-38 (1974).
267. Joseph P. Brown and Richard N. Perham; *Eur. J. Biochem.* 39, 69-73 (1973).
268. Joseph P. Brown and Richard N. Perham; *Biochem. J.*, 137, 505-512 (1974).
269. Colin Thorpe and C.H. Williams, Jr.; *J. Biol. Chem.*, 251, 3553-3557 (1976).
270. *ibid.*, pp. 7726-7728
271. R.G. Mathews, D.P. Ballou, Colin Thorpe, and C.H. Williams, Jr.; *J. Biol. Chem.*, 252(10), 3199-3207 (1977).
272. R.G. Mathews and C.H. Williams, Jr.; *J. Biol. Chem.*, 251(13), 3956-3964 (1976).
273. T. Hayakawa, Y. Sakurai, T. Aikawa, Y. Fukuyoshi, and M. Koike; in *Flavins and Flavoproteins; Proceedings of 2nd Conference*, Kunio Yagi, ed., University of Tokyo Press, Tokyo, 1968. pp. 99-106.
274. Y. Sakurai, Y. Fukuyoshi, M. Hamada, T. Hayakawa, and M. Koike; *J. Biol. Chem.*, 245(17), 4453-4462 (1970).
275. Major L. Cohn and I. Rosabelle McManus; *Biochim. Biophys. Acta* 276, 70-84 (1972).
276. Ivy R. McManus and Major L. Cohn; in *Isozymes*, vol. 1, C.L. Markert, ed.; Academic Press, New York, 1974. pp. 621-636.
277. William C. Kenney; *ibid.* pp. 601-619.
278. Jaap Visser and Marijke Strating; *Biochim. Biophys. Acta*, 384, 69-80 (1975).

279. C. Stanley Tsai, Michael W. Burgett, and Lester J. Reed; J. Biol. Chem., 248(24), 8348-8352, (1973).
280. Peter J. Butterworth, C. Stanley Tsai, Michael H. Eley, Thomas E. Roche, and Lester J. Reed; J. Biol. Chem., 250 (5), 1921 - 1925 (1975).
281. Lester J. Reed; Accts. Chem. Res., 7, 40-46 (1974).
282. H. Nawa, W.T. Brady, M. Koike, and L.J. Reed; J. Am. Chem. Soc., 82, 896-903 (1960).
283. M. Koike, L.J. Reed, and W.R. Carroll; J. Biol. Chem., 238, 30-39 (1963).
284. D.A. Walsh, R.H. Cooper, R.M. Denton, B.J. Bridges, and P.J. Randle; Biochem. J., 157, 41-67 (1976).
285. T.E. Roche and L.J. Reed; Biochem. Biophys. Res. Commun., 48, 840-846 (1972).
286. S.J. Yeaman, E.T. Hutcheson, T.E. Roche, F.H. Pettit, J.R. Brown, L.J. Reed, D.C. Watson, and G.H. Dixon; Biochemistry, 17(12), 2364-2370 (1978).
287. Richard L. Cate and Thomas E. Roche; J. Biol. Chem., 253(2), 496-503 (1978).
288. David L. Bates, Michael J. Danson, Geoffrey Hale, Elizabeth A. Hooper, and Richard N. Perham; Nature, 268, 313-316
289. Gary B. Shepherd and Gordon G. Hammes; Biochemistry, 16, 5234-5240 (1977).
290. Perry A. Frey, Bryan H. Ikeda, Grace R. Gavinos, David C. Speckhard, and Shan S. Wong; J. Biol. Chem., 253(20), 7234-7241 (1978).
291. Michael J. Danson, Alan R. Fersht, and Richard N. Perham; Proc. Natl. Acad. Sci. USA., 75(11), 5386-5390 (1978).
292. Kimon J. Angelides and Gordon G. Hammes; Proc. Natl. Acad. Sci. USA., 75(10), 4877-4880 (1978).
293. H. Stokes; Am. Chem. J., 15, 198-214 (1893).
294. D. Corbridge and E. Lowe; J. Chem. Soc., 1954 part 1, pp. 493-502.
295. S.M. McElvain and J.P. Schroeder; J. Am. Chem. Soc., 71, 40-46 (1949).

296. L.J. Reed, M. Koike, M.E. Levitch, and F.R. Leach; *J. Biol. Chem.*, 232, 143-158 (1958).
297. Kohlrausch and Holborn; quoted in F. Daniels *et al.*, *Experimental Physical Chemistry*, McGraw Hill, New York, 1962. p. 476.
298. J. Leggett Bailey; *Techniques in Protein Chemistry*, Elsevier, Amsterdam, 1962. pp. 294-295.
299. P.D. Boyer; *J. Am. Chem. Soc.*, 76, 4331-4337 (1954).
300. G. Litwack; *Experimental Biochemistry*, John Wiley, New York, 1960. pp. 150-154.
301. G. Ceriotti and L. Spandrio; *Biochem. J.*, 66, 603-607 (1957).
302. Ernesto Scoffone and Angelo Fontana; in *Protein Sequence Determination*, Saul B. Needleman, ed., Springer-Verlag, New York, 1975. pp. 162-203.
303. Gary E. Means and Robert E. Feeney; *Chemical Modification of Proteins*, Holden-Day, San Francisco, 1971.
304. Robert Fields, *Meth. Enzymol.*, 25, 464-468 (1972)
305. A.F.S.A. Habeeb; *Anal. Biochem.*, 14, 328-336 (1966).
306. P.E. Hare; in *Protein Sequence Determination*, Saul B. Needleman, ed., Springer-Verlag, New York, 1975, pp. 204-231.
307. G.W. Kosicki; *Biochemistry*, 7, 4299-4302 (1968)
308. Herbert A. Sober, ed.; *Handbook of Biochemistry*, Chemical Rubber Co., Cleveland, Ohio, 1968
309. P-L Biochemicals, Inc., Circular no. OR-18.
310. Nathan O. Kaplan; *Meth. Enzymol.* II, 660-663 (1955)
311. *ibid.*, pp. 664-666
312. Edward W. Westhead; *Biochemistry*, 4 (10), 2139-2144 (1965).
313. Ivor Smith; *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 2; John Wiley and Sons, New York, 1968.
314. K. Weber, J.R. Pringle, and M. Osborn; *Meth. Enzymol.* XXVI, 3-27 (1972).
315. Robert G. Martin and Bruce N. Ames; *J. Biol. Chem.*, 236(5), 1372-1379 (1961).

316. J. Fahrenfort; *Spectrochimica Acta*, 17, 698-709 (1961).
317. P.A. Wilkes, Jr., and Tomas Kirschfeld; *Applied Spectroscopy Reviews*, 1(1), 99-130 (1967).
318. I.Z. Steinberg; *Ann. Rev. Biochem.*, 40, 83-114 (1971)
319. Lubert Stryer; *Ann. Rev. Biochem.*, 47, 819-846 (1978)
320. Elisha Haas, E.-K.-Katzir, and I.Z. Steinberg; *Biochemistry*, 17(23), 5064-5070 (1978).
321. J. Eisinger, B. Feuer, and A.A. Lamola; *Biochemistry*, 9(10), 3908-3915 (1969).
322. R. Dale and J. Eisinger; in *Biochemical Fluorescence: Concepts*, vol.1, R.F. Chen and H. Edelhoch, eds.; Marcel Dekker, New York, 1975..p. 115.
323. D.M. Rayner, A.E. McKinnon, A.G. Szabo, and P.A. Hackett; *Can. J. Chem.*, 54(20), 3246-3259 (1976).
324. A.E. McKinnon, A.G. Szabo, and D.R. Miller; *J. Phys. Chem.*, 81(16), 1564-1570 (1977).
325. D.M. Rayner, A.E. McKinnon, and A.G. Szabo; *Rev. Sci. Instrum.*, 48 (8), 1050-1054 (1977).
326. B. Holmquist, T.A. Kaden, and B.L. Vallee; *Biochemistry*, 14, 1454-1461 (1975).
327. F.R.N. Gurd; *Methn Enzymol.*, 11, 532-541 (1967).
328. H. Jornvall; *Eur. J. Biochem.*, 16(1), 25-40 (1970).
329. H. Susi; *Meth. Enzymol.* XXVI , 455-472 (1972).
330. Perkin-Elmer Infrared Accessories CAtalog, L-60 (1970).
331. L. D'Esposito and J.L. Koenig; in *FTIR - Applications to Chemical Systems*, vol.1, J.R. Ferraro and L.J. Basile, eds., Academic Press, New York, 1978. pp. 61-97.
332. Louis G. Lange, III, James F. Riordan, and Bert L. Vallee; *Biochemistry*, 13 (21), 4361-4370 (1974).
333. Frank Marcus; *Biochemistry* 15(16), 3505-3509 (1976).
334. C.L. Borders, Jr., and James F. Riordan; *Biochemistry*, 14(21), 4699-4704 (1975).

335. J.F. Riordan; *Biochemistry*, 12, 3915-3923 (1973).
336. F. Daemen and J.F. Riordan; *Biochemistry*, 13, 2865-2871 (1974).
337. S.G. Powers and J.F. Riordan; *Proc. Natl. Acad. Sci. USA.*, 72, 2616-2620 (1975).
338. E.R. Kantrowitz and William N. Lipscomb; *J. Biol. Chem.*, 251(a) 2688-2695 (1976).
339. Laszlo Patthy and Emil L. Smith; *J. Biol. Chem.*, 250(2), 565-569 (1975).
340. Kenji Takahashi; *J. Biol. Chem.*, 243(23), 6171-6179 (1968).
341. John V. Schloss, I. Lucile Norton, Claude D. Stringer, and Fred C. Hartman; *Biochemistry*, 17(26), 5626-5631 (1978).
342. Koji Toi, Edward Bynum, Claire Norris and Harvey Itano; *J. Biol. Chem.*, 242(5), 1036-1043 (1967).
343. W.J. Ray, Jr.; *Meth. Enzymol.* XI, 490-497 (1967).
344. G. Oster, J.S. Bellin, R.W. Kimball, and M.E. Schrader; *J. Am. Chem. Soc.*, 81, 5095-5099 (1959).
345. M. Tomita, M. Irie, and T. Ukita; *Biochemistry*, 8, 5149-5160 (1969).
346. G. Oster, G.S. Bellin, and B. Holmstrom; *Experientia*, 18, 249-253 (1962).
347. P.S. Song, E.C. Smith, and D.E. Metzler; *J. Am. Chem. Soc.*, 87, 4181-4184 (1965).
348. M.J. Hunter and M.L. Ludwig; *J. Am. Chem. Soc.*, 84, 3491-3504 (1962).
349. D.E. Hultquist, R.W. Moyer, and P.D. Boyer; *Biochemistry*, 5(1) 322-331 (1966).
350. Friedhelm Schneider; *Angew. Chemie, Int. ed., Eng.*, 17(8), 583-592 (1978).
351. Finn Wold; *Meth. Enzymol.* XI, 617-640 (1967).
352. Gregg E. Davis and George R. Stark; *Proc. Natl. Acad. Sci. USA.*, 66(3), 651-656 (1970).
353. Frederick H. Carpenter and Kathryn T. Harrington; *J. Biol. Chem.*, 247 (17), 5580-5586 (1972).

354. Barton Holmquist, Thomas A. Kaden, and Bert L. Vallee; *Biochemistry*, 14(7), 1454 (1975).
355. Sheldon W. May and Jong-Yuan Kuo; *Biochemistry*, 17(16), 3333-3338 (1978).
356. Harold E. VanWart and Bert L. Vallee; *Biochemistry*, 17(16), 3385-3394 (1978).
357. IUPAC-IUB(CBN); *J. Biol. Chem.*, 252(17), 5939-5941 (1977)
358. J. Eisinger; *Photochem. Photobiol.*, 9(3), 247-258 (1969).
359. Joel Janin and Cyrus Chothia; *Biochemistry*, 17(15), 2942-2948 (1978).
360. Terrell L. Hill; *An Introduction to Statistical Thermodynamics*, Addison-Wesley, Reading, Mass., 1960.
361. Gordon Tollin; *Biochemistry*, 7(5), 1720-1727 (1968).
362. Dale E. Edmondson and Gordon Tollin; *Biochemistry*, 10(1), 113-124 (1971).
363. Dr. C.S. Tsai, personal communication.
364. Robert C. Weast, ed.; *CRC Handbook of Chemistry and Physics*, 59th ed., CRC Press, West Palm Beach, Fla., 1978.
365. D.E. Koshland, Jr., G. Nemethy, and D. Filmer; *Biochemistry*, 5(1), 365-385 (1966).
366. Irving M. Klotz and D.L. Hunston; *Biochemistry*, 10(16), 3065-3069 (1971).
367. Irving M. Klotz; *Accts. Chem. Res.*, 7, 162-168 (1974).
368. Norma J. Greenfield; *CRC Critical Revs. Biochem.*, 1975 (May), pp. 71-110.
369. Anne Stockell; *J. Biol. Chem.*, 234(5), 1286-1292 (1959).
370. Irving M. Klotz; *Arch. Biochem.*, 9, 109-117 (1946).
371. A.J. Pesce, C.-G. Rosen, and T.L. Pasby; *Fluorescence Spectroscopy: An Introduction for Biology and Medicine*, Marcel Dekker, New York, 1971.
372. V. Massey and C.H. Williams, Jr.; *J. Biol. Chem.*, 240, 4470-4480 (1965).

373. G.E.J. Staal and C. Veeger; *Biochim. Biophys. Acta*, 185, 49-62 (1969).
374. James E. Bulger and Karl G. Brandt; *J. Biol. Chem.*, 246(18), 5578-5587 (1971).
375. G.E. Schulz, R.H. Schirmer, W. Sachsenheimer, and E.F. Pai; *Nature*, 273, 120-124 (1978).
376. Jorge R. Barrio, John A. Secrist III, and Nelson J. Leonard, *Proc. Natl. Acad. Sci. USA.*, 69(8), 2039-2042 (1972).
377. John A. Secrist III, Jorge R. Barrio, Nelson J. Leonard, and Gregorio Weber; *Biochemistry*, 11(19), 3499-3506 (1972).
378. J. William Lown, Soo-Khoon Sim, and Hsiao-Hsiung Chen; *Can. J. Biochem.*, 56(11), 1042-1047 (1978).
379. J. McD. Armstrong; *Biochim. Biophys. Acta*, 86, 194-197 (1964).
380. Ben'Ichiro Tonomura, Hiroshi Nakatani, Masatake Ohnishi, Junko Yamaguchi-Ito, and Kaitaro Hiromi; *Anal. Biochem.*, 84(2), 370-383 (1978).
381. C.J. Pouchert and J.R. Campbell, eds., *The Aldrich Library of NMR Spectra*, vol. II; Aldrich Chemical Co., Milwaukee, 1974. p. 140.
382. W. Mansfield Clark; *Oxidation-Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, 1960.
383. A.M. Crestfield, S. Moore, and W.H. Stein; *J. Biol. Chem.*, 238, 622-627 (1963).
384. H. Jornvall and J.I. Harris; *Eur. J. Biochem.*, 13, 565-576 (1970).
385. G.M. Whitesides, J.E. Lilburn, and R.P. Szajewski; *J. Org. Chem.*, 42, 332-338 (1977).
386. Bruce D. Burleigh, Jr. and Charles H. Williams, Jr.; *J. Biol. Chem.*, 247(7), 2077-2082 (1972).
387. Severino Ronchi and Charles H. Williams, Jr.; *J. Biol. Chem.*, 247(7), 2083-2086 (1972).
388. Karl F. Freed; *Accts. Chem. Res.*, 11(2), 74-80 (1978).
397. Donald B. McCormick; in *Excited States in Organic Chemistry and Biochemistry*, B. Pullman and N. Goldblum, eds., D. Reidel, Holland, 1977. pp. 233-245.

389. P.N. Schatz and A.J. McCaffery; *Quart Rev. Chem. Soc.*, 23, 552-584 (1970).
390. Barton Holmquist and Bert L. Vallee; *Meth. Enzymol.*, XLIX(6), 149-179 (1978).
391. Josef Michl; *J. Am. chem. Soc.*, 100(22), 6801-6811* (1978).
392. *ibid.*, pp. 6812-6818.
393. *ibid.*, pp. 6819-6824.
394. Bryan R. Hollebone and Martin J. Stillman; *J. Chem. Soc., Faraday Trans. II*, 74, 2107-2127 (1978).
395. Richard P. Steiner and Josef Michl; *J. Am. Chem. Soc.*, 100(22), 6861-6867 (1978).
396. F.A. Cotton and G. Wilkinson; *Advanced Inorganic Chemistry*, 3rd ed., John Wiley, New York, N.Y., 1972.
397. Donald B. Mc Cormick; in *Excited States in Organic Chemistry and Biochemistry*, B. Pullman and N. Goldblum, eds., D. Reidel, Holland, 1977, pp. 233-245.
398. Ichiro Yokoe and T.C. Bruice; *J. Am. Chem. Soc.*, 97(2), 450 - 451 (1975).
399. Edward L. Loechler and Thomas C. Hollocher; *J. Am. Chem. Soc.*, 97(11), 3235-3237 (1975).
400. V. Massey, B. Curti, and H. Ganther; *J. Biol. Chem.*, 241, 2347-2357 (1966).
401. J.F. Koster and C. Veeger; *Biochim. Biophys. Acta*, 167, 48-63 (1968).
402. Kwan-sa You, Lyle J. Arnold, Jr., William S. Allison, and Nathan O'kaplan; *TIBS* (Dec. 1978), pp. 265-268.
403. Morton M. Weber, Nathan O. Kaplan, Anthony San Pietro, and Francis E. Stolzenbach; *J. Biol. Chem.*, 237, 27-36 (1957).
404. Nathan O. Kaplan; in *The Enzymes*, vol. 3, part B. P.D. Boyer, H. Lardy, and K. Myrback, eds., Academic Press, N.Y., 1960.
405. Q.H. Gibson and J.W. Hastings; *Biochem. J.*, 83, 368-377 (1962).
406. M.J. Gibian and J.A. Rynd; *Biochem. Biophys. Res. Commun.*, 34, 594-599 (1969).
407. M.J. Dove and C.S. Tsai; *Can. J. Biochem.*, 54(5), 432-437 (1976).

- 408. W.W. Cleland; *Biochim. Biophys. Acta*, 67, 104-137 (1963).
- 409. Keith J. Laidler; *The Chemical Kinetics of Enzyme Action*, Oxford University Press, London, 1958.
- 410. Kent M. Plowman; *Enzyme Kinetics*, McGraw-Hill, New York, 1972.
- 411. Athel Cornish-Bowden; *Principles of Enzyme Kinetics*, Butterworths, London, 1976.
- 412. H.J. Fromm; *Initial Rate Enzyme Kinetics*, Springer-Verlag, Berlin, 1975.

END

29-12-80

FIN