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'A COMPARATIVE STUDY OF THE FLAVOPROTEINS LIPOAMIDE
DEHYDROGENASE AND GLUTATHIONE REDUCTASE'

by

© James Robert Paul Godin

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

Department of Chemistry

Carleton University
Ottawa, Ontario
September, 1982

The undersigned recommend to the Faculty of Graduate
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"A COMPARATIVE STUDY OF THE FLAVOPROTEINS LIPOAMIDE
DEHYDROGENASE AND GLUTATHIONE REDUCTASE"

submitted by James Robert Paul Godin, B.Sc.
in partial fulfilment of the requirements for
the degree of Master of Science

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ABSTRACT

Glutathione reductase from yeast (EC1.6.4.2) catalyses NADPH linked reduction of glutathione efficiently at acidic pH. Steady state kinetic studies suggest a Ping Pong mechanism is operating over the pH range of 5-8, unlike that found in the similar flavoprotein, lipoamide dehydrogenase. A pK of 5.8-6.0 has been associated with the pH dependence of glutathione reduction and has tentatively been assigned to a histidine residue.

Like lipoamide dehydrogenase, glutathione reductase also possesses electron transferase, transhydrogenase, oxidase and diaphorase activities. In general, glutathione reductase displays a suppressed catalytic efficiency for these activities compared to lipoamide dehydrogenase. Chromatofocusing and isoelectrofocusing display an absence of isozymes ruling this out as a possible explanation for the multifunctionality.

Both transhydrogenase reactions using thionicotinamide adenine dinucleotide phosphate (sNADP) and 3-acetylpyridine adenine dinucleotide phosphate (3-Ac-NADP) appear to proceed by a Ping Pong mechanism as supported by product inhibition studies. This would seem to indicate the presence of only one nucleotide binding site unlike lipoamide dehydrogenase which has at least two. Difference spectroscopy of glutathione reductase supports the one-site postulate.

Spectra of the half-reduced species (EH_2) of glutathione reductase displays a higher extinction coefficient for the 530 nm absorption band by approximately $1000-1500 \text{ M}^{-1} \text{ cm}^{-1}$. This may indicate a stronger thiolate to flavin charge transfer interaction after reduction in glutathione reductase. In addition, the diminished pH dependence of EH_2 in glutathione reductase (NADPH as

reductant) indicates a weaker thiolate to histidine ion pair.

Proton N.M.R. work on the oxidized and reduced species (EH_2) of the two flavoproteins supports this view.

An extensive dye-sensitized photooxidation study was performed on four (4) enzymes. In all cases, photoinactivation depended chiefly on the sensitizer used. Rates of inactivation varied in a similar manner to the $\Sigma g : \Delta g$ ratio of singlet oxygen with the triplet state energy of the sensitizer. A mechanism for the photoinactivation is proposed in light of the possible involvement of these two species of singlet oxygen.

Treatment of lipamide dehydrogenase and glutathione reductase with a 100-200 fold excess of N-bromosuccinimide (NBS) results in a near complete loss of all multifunctionality with a half-life of 15 minutes ($t_{1/2} \leq 15$ mins). Destruction of the nucleotide binding site (NAD(P)H) is proposed as the reason for the lost activities since initial binding of NAD(P)H is the first step necessary for all activities catalyzed by these two flavoproteins. Modification with tetranitromethane (TNM) produces similar results. Target amino acids seem to be tryptophan, tyrosine and histidine. The above aromatic amino acids are proposed to be involved in the nucleotide binding site of both flavoproteins. Other chemical modifications of glutathione reductase have been performed and the results are discussed in terms of the importance of certain amino acids at the active site.

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Production of Singlet Oxygen by Photochemical,
Chemical and Biological Systems

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DCIP	2,6-Dichloroindophenol
DHase	Lipoamide dehydrogenase activity
DPase	Diaphorase activity
DSS	Sodium-2,2-dimethyl-2-silapentane-5-sulfonate
EDTA	Ethylene diamine tetracetate
ETase	Electron transferase activity
FAD	Flavin adenine dinucleotide
FADH	Reduced form of FAD
FADH	Semiquinone form of FAD
Flox	Oxidized flavin
Flred	Reduced flavin
GSSG	Glutathione
GSH	Reduced glutathione
MCD	Magnetic Circular Dichroism
MO-NAG ₂	p-methylphenyl-2-acetamido-4-O-(2-acetamido- 2-deoxy- -D-glucopyranosyl)-2-deoxy- -D- glucopyranoside
NAD(P)	B-nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced form of NAD(P)
NADD	Deuterated form of NADH
3-acetyl-NAD(P)	3-acetyl pyridine analog of NAD(P)
3-acetyl-NAD	3-acetyl pyridine analog of NAD
s-NAD(P)	Thio analog of NAD(P)
NBS	N-bromosuccinimide
N.M.R.	Nuclear Magnetic Resonance
OXase	Oxidase activity
PLP	Pyridoxal-5'-phosphate

RDase	Glutathione reductase activity
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNM	Tetranitromethane
TRIS	Tris (hydroxymethyl) aminomethane
TNBS	Trinitrobenzene sulfonic acid
1E_g	Sigma singlet oxygen
$^1\Delta_g$	Delta singlet oxygen
Enzyme species:	
$E_{h\nu}$	Photooxidized enzyme
$E_{(CH_3)_3SO_3^+NH_3^+}$	Enzyme amidated with ethylamine derivatives
E_{EFA}	Enzyme modified with ethoxyformic anhydride
$E_{(R_1)_2C(=O)O-C_6H_4-C(=O)R_2}$	Enzyme modified with arginine reagents: phenylglyoxal, 1,2-cyclohexanedione or 2,3-butanedione
E_{TNBS}	Enzyme modified with trinitrobenzene sulfonic acid
$E_{MA,BA}$	Enzyme modified with methyl or benzylacetimidate
E_{PLP}	Enzyme pyridoxylated with PLP
E_{NBS}	Enzyme modified with N-bromosuccinimide
E_{TNM}	Enzyme modified with tetranitromethane

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INTRODUCTION

The flavoenzymes (14) display a wide variety of enzymic reactions involving both one and two-electron transfers and are sensitive to various environmental factors. The investigation of this multifunctionality can be fruitful in providing insights into the chemistry of the flavin by correlating flavin reactivity with perturbations induced by the peptide environment. The flavin can serve as a sensitive probe of enzymic structure, mechanism and conformational flexibility. Some of these principles will be illustrated in this thesis as the multifunctionality of lipoamide dehydrogenase and glutathione reductase are compared. Some basic principles of flavin chemistry will be introduced.

FLAVINS:

Riboflavin, vitamin B₂, is a yellow tricyclic (isocallooxazine) molecule which in biological systems is phosphorylated and then adenylated further to the two active redox coenzyme forms FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide, 15). The structure of these and other derivatives are shown in Figure 1.

Flavins and nicotinamide coenzymes (NAD^+/NADH , $\text{NADP}^+/\text{NADPH}$) are the primary acceptors for electron pairs removed from the large number of soluble metabolites undergoing cellular oxidation or in the reverse direction reduction. Their substrates include amines, amino acids, alcohol sugars, hydroxy acids, dithiols, aldehydes, ketones, and acids. These oxidations are net two-electron redox processes and in a stoichiometric sense either flavin or nicotinamide coenzymes can be used for the same reaction. However, the tricyclic flavins are much more versatile catalysts than the monocyclic nicotinamides, as evidenced by two related properties: (a) nicotinamides appear restricted to two(2) electron pathways

Figure 1. Nomenclature of the flavin ring systems, from Templeton(16)

while flavins can undergo both facile two-electron and one-electron chemistry; (b) dihydronicotinamides are inert to oxygen, but dihydroflavins, with a central dihydropyrazine ring system, are highly reactive for reductive oxygen metabolism (eq. 1)

These features place flavins at the crossroads of biological redox chemistry (88). In one instance they serve as redox switches between obligate two-electron donors (e.g. oxidation of $\text{NADH} \rightarrow \text{NAD}^+$, succinate $\rightarrow$ fumarate, lactate $\rightarrow$ pyruvate) and obligate one-electron acceptors (iron-sulfur cluster proteins or hemoproteins) in cellular redox metabolism. On the other hand, the reactivity with molecular oxygen, allows flavins to serve as a cofactor for both net two-electron reduction of O_2 to H_2O_2 and four-electron reductive activation and cleavage in monooxygenation reactions (eq. 2)

XH = reductant; SH = substrate. Both are key processes in aerobic organisms (17-19).

The electronic character of the isoalloxazine system has been probed both experimentally and theoretically. The yellow flavin chromophore has four solvent sensitive absorption bands around 220, 270, 375-330 and 450 nm (20). The two lower energy transitions have

received the most attention from the biochemist since higher energy absorptions are obscured in the protein environment. Polarization studies of immobilized FMN and lumiflavin (10-methyl-isoalloxazine) reveal three transition moments corresponding to the three lowest energy absorptions (21) (Figure 2). The 450 nm band is a single asymmetric absorption band in aqueous solution. Lowering solvent polarity results in shoulders at 425 nm and 475 nm (22).

Important in any mechanistic postulation is a knowledge of the reduced form(s) of flavin encountered during the catalytic event. In solution, the half-reduced semiquinone flavin is in general an unstable free radical species and exists in rapid equilibrium with its oxidized and reduced species. (eq. 3)

The comproportionation equilibrium is very rapid in solution and lies far to the left (23-25).

Reduction of flavin can proceed by several routes although reduction by NADH and NADPH will be considered more pertinent to the present work. Other reductants of interest include dithionite, dithiols and photoreduction by ethylenediaminetetraacetate (EDTA). For further details the reader is referred to two excellent reviews (27,28).

Photoreduction of flavin by EDTA results in the transient production of semiquinone radicals (29). Most photoreductions are thought to involve triplet flavin states due to the short lifetimes of the singlet (29). Radda (30) 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 $\cdot$ abstraction to form a

Figure 2. Charge redistribution in flavin upon absorption of light, from Johansson(21).

flavin semiquinone as the initial post-excitation step (31).

Interesting is the fact that triplet flavin will transfer energy to ground state $^3\Sigma_g^+ O_2$ converting it to the $^1\Delta_g O_2$ state (32, 33), a powerful oxidant which will be dealt with later. The reaction is fast and in the presence of excess O_2 the flavin semiquinone is rapidly reoxidized. The various reduced flavin structures are shown in Figure 3.

The chemical events taking place during flavin reduction by reduced nicotinamides (NAD(P)H) seems to occupy the interest of most flavin biochemists and to date still remains an enigma. In 1959, Isenberg and Szent-Gyorgyi (34) provided low-temperature spectroscopic evidence supporting a charge-transfer complex between NADH and FMN. Later Isenberg's room temperature studies supported the view (34).

However, 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. An isotope effect of $k_H/k_D = 3.2$, and the rate dependence on ionic strength, indicate C-H bond cleavage as the rate determining step in an ionic transition state (35). Traditionally two-electron (i.e. hydride) transfer has been (36,37) proposed, though others argue a sequential one-electron process (15), as does Kosower (38). It seems as though both the mechanism and initial products of reduction of free flavins by NADH are far from resolved, and a number of competing routes still exist.

FLAVOENZYMES:

In 1964, Dixon and Webb (39) listed some forty known flavoenzymes, and at least as many more have been discussed in the literature since. The complexity of the 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 soluble and insoluble enzymes.

With such a wide range of diverse reactions being performed by flavoenzymes, it becomes evident that the protein is playing a key role in the chemistry of the flavin. At present, about 18 flavoproteins are known where the flavin is covalently attached to the protein, (40), always through histidine, cysteinyl or tyrosine residues normally at the C(8)- α -methyl group. In the majority of flavoproteins, however, the flavin is non-covalently bound with apparent dissociation constants ranging from 10^{-8} - 10^{-10} M, though D-amino-acid oxidase is more readily dissolvable ($K_d \sim 10^{-7}$ M).

Electrostatic interaction between flavin and protein have been invoked to explain the diverse but individually restricted chemistry of flavoenzymes (41). The blue neutral radical (Figure 3) appears to be favored by blocking of the N5 position by either alkylation or protonation (41). So, flavoenzymes containing the blue neutral radical may reflect a hydrogen bridge from protein to flavin at position N5 (Figure 4). In fact, X-ray diffraction studies of the blue semiquinone form of flavodoxin reveals that the N5 position of the flavin is within hydrogen bonding distance of a carbonyl component of the polypeptide chain (42).

One thing seems important in order to understand a flavoprotein. The protein environment profoundly affects flavin chemistry and the flavin can effect protein conformation. In addition to the electrostatic and steric restraints placed on the flavin ring system during catalysis, another perturbation of the flavins' chemical behaviour is possible. It involves the transfer of

Figure 3. Redox scheme of the flavin ring system, from Templeton(16).

Figure 4. Hydrogen bonding to reduced flavin semiquinones as envisaged by Hemmerich and Massey(41).

reducing equivalents from the isoalloxazine ring system to some acceptor on the protein. Glutathione reductase (EC 1.6.4.2) and Lipoamide dehydrogenase (EC 1.6.4.3) are prime examples of this situation. This statement will be used as a means of introducing a brief review of these two enzymes.

LIPAMIDE DEHYDROGENASE AND GLUTATHIONE REDUCTASE

Lipoamide dehydrogenase and glutathione reductase, last reviewed by Williams (43), are closely related flavoproteins which catalyze chemically similar reactions are shown:

Lipoamide dehydrogenase is part of a 3 enzyme complex (pyruvate dehydrogenase complex) catalyzing the oxidative decarboxylation of α -ketoacids, pyruvate or α -ketoglutarate (44). Glutathione reductase catalyzes the virtually irreversible reduction of GSSG by NADPH, re-oxidation of GSH being entropically less favorable. Glutathione is the most abundant thiol-disulfide in the cell by more than an order of magnitude; in most cases the GSH:GSSG ratio is about 10-20:1 (45).

In addition to the non-covalently bound FAD, each enzyme contains a redox-active cystine residue (43). Both enzymes, therefore, can accept a total of 4 electrons when reduced anaerobically by dithionite. Figure 5 shows spectra of oxidized, 2-electron-reduced, and 4-electron-reduced lipoamide dehydrogenase. The analogous spectra for glutathione reductase are almost identical in shape although a major difference in the EH_0 extinction coefficient at 530 nm exists that will be dealt with in

Figure 5. Spectra of oxidized(E), 2-electron reduced(EH_2) and 4-electron reduced(EH_4) lipoamide dehydrogenase at pH 7.6. Taken from reference 46.

the results section. The spectra for the oxidized and 4-electron reduced enzyme are typical for FAD and FADH_2 having little absorbance beyond 500 nm. The 2-electron-reduced enzyme (EH_2), retains the 2-banded spectrum of FAD but also has a third long wavelength absorption band centered about 530 nm (43,46).

The origin of the 530 nm absorption generated upon reduction was originally postulated to be a radical pair between flavin semiquinone and a sulfur radical (I) (43,47). However, this has since been discarded in favour of a full thiolate anion interacting with FAD (II) (48,49,50).

While the spectrum of the fully reduced and oxidized enzyme species were pH independent (51) that of half-reduced (EH_2) lipoamide dehydrogenase is not (52). Charge-transfer between thiolate and FAD is enhanced by high pH (51). At the time of the last review, Williams (43) postulated the presence of a base at the active site to provide electrostatic stabilization to the thiolate anion and to act as an acid-base during catalysis.

Templeton (16) has provided more concrete evidence for the existence of such a base by showing that 2-electron-reduced enzyme gives rise to a magnetic circular dichroism (MCD) Faraday A term whose energy and intensity is independent of the redox state of the flavin. Glutathione reductase behaved similarly although the A term was much weaker in intensity (16). This led Templeton and Tsai to argue for the presence of a protein acceptor. In addition they have shown that rose bengal sensitized photooxidation of lipoamide

dehydrogenase removes both the enzyme's long wavelength absorption band (characteristic of EH_2) and the MCD A term (53). Amino acid analysis revealed destruction of 5 histidines. Wand (26) provided support for the possible involvement of histidine(s) in EH formation by demonstrating the removal of the Faraday A term in native enzyme at pH 8.6. Additional evidence for a thiolate-histidine ion pair will be revealed from proton N.M.R. data in the results section which demonstrates an interesting difference between lipoamide dehydrogenase and glutathione reductase.

Lipoamide dehydrogenase and glutathione reductase share a fair degree of similarity in amino acid composition and in their sequence of amino acids surrounding the redox active disulfide (Figure 5). Many investigators use this and other evidence to assume that the two enzymes operate by essentially identical mechanisms (43, 46 and references therein).

Pig heart Lipoamide Dehydrogenase

Glu-Lys-Asn-Glu-Thr-Leu-Gly-Gly-Thr-Cys-Leu-Asn-Val-Gly-Cys-ile-Pro

Yeast Glutathione Reductase

Lys-Ala-Gly-Lys-Ala-Leu-Gly-Gly-Thr-Cys-Val-Asn-Val-Gly-Cys-Val-Lys

Figure 6 Amino acid sequence around the active site disulfide

TABLE I: AMINO ACID COMPOSITION OF LIPOAMIDE

DEHYDROGENASE AND GLUTATHIONE REDUCTASE (43)

Amino Acid	Lipoamide Dehydrogenase	Glutathione
	(Pig Heart)	Reductase (Yeast)
Cysteic acid	10	6
Aspartic acid	44	49
Threonine	26	23
Serine	24	27
Glutamic acid	47	45
Proline	18	13
Glycine	53	43
Alanine	46	33
Valine	44	41
Methionine	9	7
Isoleucine	36	29
Leucine	31	31
Tyrosine	8	17
Phenylalanine	15	16
Lysine	36	39
Histidine	11	14
Arginine	14	16
Tryptophan	2	5
FAD	1	1
Total amino acid	474	454
residues		
Minimum mol.wt./FAD	51,000	50,600

In fact, the recently determined X-ray diffraction study performed on glutathione reductase (54,55) is often used in discussions of structure and substrate binding sites in lipoamide dehydrogenase.

Kinetically, lipoamide dehydrogenase has been the more extensively studied of the two enzymes (16,26,56,57,58). In addition to its physiological role, lipoamide dehydrogenase also displays a number of other NADH-linked activities. They include hydrogen transfer between nicotinamide nucleotides, electron transfer to inorganic acceptors, reduction of quinone dyes and molecular oxygen (58,59). (Scheme two). Tsai (58) has examined the multifunctionality of this enzyme over a wide range of substrate concentrations and has argued for a multiple pathway kinetic model presented in Scheme Three that is consistent with early work (56,60) performed on the dehydrogenase reaction.

Kinetics of hydrogen transfer between reduced nicotinamide adenine dinucleotide and its oxidized analogues (snAD^+ and 3-Ac-NAD^+) reveal a catalytic sensitivity to the nicotinamide ring (60). Tsai has shown that thionicotinamide dinucleotide (snAD^+) and NADH react via a Ping Pong mechanism at low NADH concentrations while a ternary complex mechanism becomes significant at higher concentrations. However, reduction of 3-Ac-NAD^+ by NADH proceeds by a ternary complex route alone (60). Binding studies performed by several workers (16,61,62) have argued that lipoamide dehydrogenase contains two distinct nucleotide binding sites with only one site being available for snAD^+ while 3-Ac-NAD^+ has variable affinity for both sites.

NADH reduction of quinone dyes (DPase) and electron transfer to inorganic acceptors (ETase) are catalyzed by ternary complex mechanisms (75). Although premature, NADH-linked reduction of

Scheme Two

(DHase)

(DPase)

(ETase)

(OXase)

Scheme Three

molecular oxygen (16,63) has also been argued to proceed via a ternary complex mechanism.

Glutathione reductase on the other hand, has not undergone such an extensive kinetic analysis. Early kinetic studies on this enzyme were restricted to the forward reductase reaction and were void of product inhibition studies, so vital to a proper interpretation of kinetic data in the elucidation of mechanisms (64,65).

Reexamination of the kinetics of erythrocyte glutathione reductase (64) confirmed the substrate and product inhibition patterns and salt effects of an earlier study (65). Product inhibition by NADP was found to be competitive with NADPH and non-competitive towards GSSG. This is in conflict with predicted inhibition patterns for a binary complex mechanism, so the authors proposed that the mechanism was mixed, ordered sequential and binary complex (64). These results have since been confirmed (66). However, to date a pH profile of the kinetics has not been performed to gain insight on the groups participating in catalysis as was done in lipoamide dehydrogenase (26).

Despite homologies between the two enzymes (43), they both use radically different substrates (Figure 7) and clearly must possess some active-site chemistry or characteristic unique to that enzyme. As discussed, the protein environment around the flavin has a major influence on the chemistry the flavin is capable of performing, 1-electron-transfer, 2-electron-transfer, or both.

The non-physiological activities of lipoamide dehydrogenase have been used in our laboratory to infer changes in active-site structure. For example, modification with arsenite and iodoacetamide clearly demonstrated that the active-site disulfide

LIPOIC ACID

Figure 7. Structures of oxidized glutathione and lipoic acid

is not required for these activities (16). Fluctuations in the diaphorase activity has been useful in monitoring changes in flavin environment and mobility (67). Numerous chemical modifications have been performed (16,26) to determine the functional group(s) necessary for a given activity in the eventual hope of indirectly mapping the flavin environment.

Despite the availability of an X-ray mapping of glutathione reductase (54,55) little work until recently (40) has emerged to define catalytically important residues in this enzyme. The literature also lacks a kinetic investigation of its multifunctionality which would be of use in comparison with lipoamide dehydrogenase. Such knowledge would contribute to an understanding of these similar yet different enzymes and to the chemistry of flavoproteins in general. This aspect of flavoenzymes will constitute the major thrust of this work. However, dye-sensitized photooxidation is also a main component of this thesis, and a brief review of it will follow.

SINGLET OXYGEN

Credit for the discovery of oxygen is usually given to both Scheele and Priestley who independently isolated the species in about 1772. In 1811, Avogadro recognized that oxygen is a diatomic molecule and in 1848 Faraday demonstrated that it is unique among diatomic molecules with an even number of electrons: it is paramagnetic in its ground state. However, a satisfactory explanation for its paramagnetism was presented only in 1928 by Mulliken (68). The details will not be discussed here. For the purpose of this work, it is sufficient to note that although O_2 has a 'double bond', its outermost pair of electrons are in different orbitals and have their spins parallel, making the ground state a

'triplet' ($^3\Sigma_g$). Furthermore, two other arrangements of these two electrons are possible and should result in the presence of two low-lying singlet states ($^1\Delta_g$ and $^1\Sigma_g^+$) (Figure 8). Three years after Mulliken's paper, Childe and Mecke (69) were able to verify these predictions with the spectroscopic observation of the $^1\Sigma_g^+$ state 37.51 kcal above the ground state, and in 1934 Herzberg reported the observation of the $^1\Delta_g$ state (70). This singlet delta state lies only 22.54 kcal above the ground state and has a remarkable lifetime of 1 hour in the absence of collisions with other molecules. It is this species of oxygen that has come to be called singlet oxygen.

The role of singlet oxygen in a variety of photo-oxygenation processes has been demonstrated by many workers in the last 20 years. Much of the work done in this field however, was initiated by Hans Kautsky (71,72) who in 1931 correctly proposed that this species was responsible for the photo-oxygenation of a variety of unsaturated molecules. Unfortunately, Kautsky's work, despite his careful reasoning, did not convince his colleagues. However, in 1964 new evidence for a singlet oxygen mechanism began to appear through the work of Foote and Wexler (73) and Corey and Taylor (74). Since then a great deal of work has flooded the literature concerning singlet oxygen and the interested reader is referred to two excellent reviews (75,76).

Dye sensitized photooxidation has long been employed to investigate the structure-function relationship of proteins (78,80). However, the interpretation of experimental results is hindered by the lack of knowledge concerning the primary events of photosensitization and rationales for a choice of dyes with respect to their selectivity and effectiveness as photosensitizers. Two

THREE LOW LYING ELECTRONIC STATES OF MOLECULAR OXYGEN

Figure 8. Electronic states of molecular oxygen along with the orbital occupancy of these states, from Ranby and Rebek(77).

mechanisms have been proposed to account for kinetic observations of sensitized photooxidations (82). The first mechanism involves the interaction between the triplet state of the sensitizer and triplet oxygen to form a complex which reacts with substrate. The second mechanism involves the reaction between substrate and singlet oxygen which is produced by an energy transfer from triplet sensitizer to oxygen.

Singlet oxygen is also responsible for the photooxidation of unsaturated hydrocarbons (75,76). The same reactive oxygen is implicated in the sensitized photooxidation of amino acids and proteins (84,85,86,87). However, the particular species of singlet oxygen ($^1\Sigma_g^+$ or $^1\Delta_g$) remains somewhat an enigma. We have attempted to resolve this dilemma by studying the photoinactivation of lipoamide dehydrogenase, glutathione reductase, lysozyme and horse liver alcohol dehydrogenase. A mechanism is proposed to account for our results in light of the involvement of singlet oxygen.

MATERIALS AND METHODS

1. Enzymes

Lipoamide dehydrogenase (porcine heart, grade 111, EC 1.6.4.3) and glutathione reductase (yeast, grade 111, EC 1.6.4.2) were obtained from the Sigma Chemical Co. The preparations existed as suspensions in 3.2 M ammonium sulfate and were desalted (for chemical modifications) by dialysis or by Bio-gel P-6 exclusion chromatography.

Horse liver alcohol dehydrogenase (EC 1.1.1.1) and Lysozyme (EC 3.2.1.17) were also obtained from Sigma. The protein bovine serum albumin was obtained from Miles Laboratories.

2. Biochemicals

D,L-Lipoamide, glutathione and their reduced forms were from Sigma. NAD(P)^+ , NAD(P)H , SNAD(P)^+ , 3-Ac-NAD(P) $^+$, FAD and pyridoxal-5'-phosphate were also from Sigma. Riboflavin was from Eastman Organic Chemicals.

3. Chemicals

Rose Bengal (80%), Fluorescein, ErythrosinB (88%), Eosin Y (81%) Acridine Orange, sodium lauryl sulfate, cupric sulfate pentahydrate and phenol reagent were from Fisher Scientific Co.

Anachemia supplied sucrose, ammonium persulfate, sodium carbonate and methylene blue (84%). Methyl acetimidate and N-bromosuccinimide were from the Pierce Chemical Co.

Sigma provided acrylamide, guanidine-HCl, N,N,N',N'-tetramethylethylene diamine (TEMED), picryl sulfonic acid (TNBS) and sodium potassium tartrate.

J.T. Baker supplied sodium and potassium phosphate buffers.

Potassium ferricyanide and sodium azide were supplied by The British Drug Houses Ltd.

Tetranitromethane (TNM) was provided by the Nutritional Biochemicals Corporations, Cleveland, Ohio.

Chromatofocusing polybuffers (96 and 74) and polybuffer exchanger (PBE 94) were products from Pharmacia Fine Chemicals.

Merke Sharp & Dohme provided D₂O (99.7%) and absolute ethanol was from Consolidated Chemicals Co.

Propylamine, ethylene diamine, 2-aminoethane sulfonic acid and pyrazole were purchased from Eastman Kodak Co.

Cellulose-CM and Cellex-T cationic and anionic exchangers were from Bio-Rad Laboratories.

4. pH Determinations

Aqueous pH determinations were done using a Radiometer PHM62 pH meter. The instrument was calibrated daily with pH 7.0 ± 0.02 (25 C) standard buffer from Anachemia. Solutions were prepared with deionized water from a Milli-QTM water purification system, at a measured resistivity of 18 M Ω ·cm.

5. Ultraviolet-Visible Spectroscopy

All spectra were obtained on a Beckman 25 Spectrophotometer. Helma quartz cuvettes of 1 cm path length were employed. Thunberg cells were used when spectra were recorded anaerobically.

To determine difference spectra, cells with a quartz partition were used, each compartment bearing a path length of 0.4375 cm. Two cells were placed in the paths of a Beckman 25 spectrophotometer as in Scheme I. To record a spectrum of molecule A bound to enzyme E, buffer would be placed in compartments 1 and 3 and enzyme in compartments 2 and 4. Enough of A is added to compartments 1 and 4 to give equivalent concentrations. Buffer is added to compartment 2 to correct for enzyme dilution.

Scheme One

6. Fluorescence Spectroscopy

Fluorescent spectra of flavoproteins were performed on a Perkin-Elmer 204S Spectrophotometer. Spectra were obtained using 3 ml quartz cuvettes having a path length of 1 cm. Enzyme concentrations of 0.5-1 mg/ml were normally employed.

7. N.M.R. Spectroscopy

Proton N.M.R. of lipoamide dehydrogenase and glutathione reductase were performed as follows. Desalted and deuterated enzyme (10 mg) was dissolved in D_2O and lyophilized. The procedure was repeated once more to obtain deuterated enzyme which was dissolved in 0.10 M deuterated phosphate buffers (15-20 mg/ml) at the appropriate pH. The deuterated photooxidized enzyme was prepared by illuminating lipoamide dehydrogenase to 28% residual reductase activity in protonic buffer followed by deuterations. The reduced enzyme was prepared by an anerobic reduction of the deuterated lipoamide dehydrogenase with NADD in a N_2 filled glove bag. Spectra in the histidine region were recorded on a Varian XL100-12 N.M.R. spectrometer equipped with Nicolet Fourier transform and TT-100 Data systems. The proton signal from H_2O was used as an internal standard which was checked using tetramethylsilane as the external standard.

Proton N.M.R. of native and photooxidized lysozyme were recorded on a recently delivered Varian XL-200 instrument. Sample preparation was as follows. Lysozyme (0.15 gms) was dissolved in 6 mls of deionized H_2O (1.7 mM). The sample was then divided into two three millilitre portions. One sample was lyophilized, exchanged with D_2O (2 mls), lyophilized and repeated again. The other sample was photooxidized using methylene blue ($100\mu M$) to a residual activity of 10-12%. Methylene blue could be removed by

batch treatment with cellex-CM cation exchanger. The sample was then lyophilized and exchanged twice with D_2O as above. Native and photooxidized enzyme were then each dissolved in 1.5 mls D_2O and clarified by centrifugation at 10,000 r.p.m. for 10 minutes if necessary. Enzymes were then transferred to 10 mm N.M.R. tubes using 3% DSS in D_2O as external reference. One hundred transients were accumulated in a period of about 10 minutes and spectra recorded.

8. Buffer Preparations

Protic buffers were made from appropriate weights of basic and acidic components and made up to the desired volume with deionized water. To prepare buffer solutions in D_2O , the original weight ratios of protic buffer were dissolved in D_2O and lyophilized. The required volume of D_2O was then added. Adjustments of pH were made by adding solid deuterated buffer salts. Apparent pH readings in D_2O were converted to pD

according to: $pD = pH \text{ reading} + 0.4.$

9. Protein Determinations

Lipoamide dehydrogenase and glutathione reductase concentrations were determined spectrometrically assuming an extinction coefficient of $11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 455 nm and 463 nm respectively. (2) Low protein or apo-enzyme concentrations were determined by the method of Lowry et al (3) as modified by Miller (4). The procedure was as follows. A solution labelled mixture I was prepared by mixing 49 mls of 2% w/v sodium carbonate, with 0.5 mls of 2% w/v sodium potassium tartrate and 1% w/v cupric sulfate pentahydrate. To 5 mls of mixture I was added 1 ml of enzyme preparation or stock bovine serum albumin (BSA) standard (5-100 g/ml). The solution was left to stand for 10 minutes whereupon 0.5

mls of freshly prepared phenol reagent (diluted 1:1 with H_2O), was added. After mixing well, the solution was left to stand for 30 minutes. Absorbances were measured at 750 nm. Protein concentrations were determined from a standard curve with known quantities of bovine serum albumin (BSA).

10. Anaerobic Reductions

Anaerobic reduction of flavoproteins was performed by deoxygenating samples by purging with N_2 gas in an inflatable glove bag for about 5-6 minutes. Vigorous bubbling was avoided, since it led to sample loss. Samples were then transferred to Thorberg cuvettes.

11. Amino-Acid Analysis

Salt free lyophilized enzymes (0.5-2 mg) were dissolved in 1-1.5 mls of 6M HCL and transferred to a Pierce Chemical Co. Reacti-Therm vacuum hydrolysis system, evacuated and incubated in a Lab Line Instruments controlled heating block at 110 C for 22-24 hours. Hydrolysates were reduced to dryness and amino acid analysis performed on a Beckman 119 BL instrument by Cathy Shay (Biology Department, Carleton)

12. Apo and NBS-Apoenzyme Preparations

Desalted Lipoamide Dehydrogenase (3 mg) was made to a volume of 4 mls with 0.2 M potassium phosphate buffer pH 7.5 and then divided into two equal portions. One portion was made 5 M in guanadine-HCl and placed on ice for 15-20 minutes. To the other portion was added a 100-fold excess of NBS (8×10^{-4} M). The DHase and DPase activity was monitored and reached a level of < 5% in 5-10 minutes. This portion was then made 5 M in guanadine-HCl and placed on ice for 15-20 minutes. Both samples were concentrated to 0.7-1 ml by Millipore CX-10 units. Samples were then eluted

from a 25 x 1 cm Bio-Gel P-6 column, pre-equilibrated with potassium phosphate buffer (0.2 M, pH 7.5). Fractions were collected in amounts of 25 drops/tube.

13. Chromatographic and electrophoretic techniques

a) Polyacrylamide gel (Bio gel) exclusion chromatography was used routinely for desalting or for preparation of apoenzymes. Porous polyacrylamide beads were hydrated at room temperature, deaerated and poured into Pharmacia columns of required dimensions. Columns were then equilibrated with either deionized water or potassium phosphate buffers of appropriate pH.

b) Chromatofocusing of glutathione reductase was performed as follows. A chosen amount of polybuffer exchanger PBE94 (Pharmacia) was filtered and washed with 300-mls of deionized water. It was then dispersed in 50 mls of start buffer (25 mM TRIS, pH 8.3) to make a slurry. The slurry was then degassed prior to packing in a 1 x 30 cm column. The packed exchanger was then equilibrated by passing 200-300 mls of start buffer through the column. Equilibrium was checked by ensuring that the pH and conductivity of the eluant matched that of the start buffer.

Glutathione Reductase (0.35 mls, 11.5 mg/ml) was dialyzed for 0.5 hour against 1 litre of deionized water and then 1 hour against starting buffer. The enzyme was then applied to the top of the column and allowed to run in about 1 cm. The column was then topped off with starting buffer and eluted with a mixture of polybuffer 96 (6 mls) and polybuffer 74 (4 mls) in 200 mls of deionized water adjusted to pH 5.0 with 2 M acetic acid. The pH, protein absorption and activity of the 90 fractions (25 drops/fraction) were monitored.

c) Isoelectrofocusing was carried out on a LKB 8100-1 (110

mls) column using LKB Ampholine carriers (polyamino-polycarboxylic acids) of pH 5-8. Experiments were performed at 18 C with deionized water. A dense gradient cathode solution (10.4 mls monoethanolamine + 14 mls distilled water + 12 g sucrose) was poured into the column. A 10% solution of Ampholines was prepared by removing 2.5 mls of a 40% stock solution (Pharmacia) and diluting it to 10 mls with distilled water. The sucrose gradient in the column was established in the following manner. One heavy solution (7.5 ml ampholine solution + 34.5 mls distilled water + 28 gm sucrose) and one light solution (2.5 mls ampholine solution + 57.5 mls distilled water) were prepared and added to separate burets. Twenty-four test tubes were then set up with varying amounts of heavy and light solution being titrated into each to establish a sucrose gradient series. (Table II) Each test tube or fraction is then added in succession to the column, being careful to avoid much agitation. Midway through the procedure (fraction 12), light solution is replaced by an equivalent volume of enzyme. (25 mg/ml glutathione reductase). When all fractions have been added, a light solution is layered on top (0.1 ml H_3PO_4 (85%) + 10 mls distilled water). Samples were focused for 15 hours at 400 V using an Ortec Power Supply with the anode at the top of the column. The column was water cooled at 17-18 C. Protein absorptions, pH, and activity were monitored as 36 (42 drops/fraction) fractions were collected.

d) Polyacrylamide slab-gel electrophoresis was performed on an apparatus supplied by Dr. Neelin (Biology Department, Carleton) and constructed by Carleton's Science Workshop. To prepare the slab-gel, the following stock solutions were prepared.

Solution A: dissolve 0.4 gms Tris base in 0.5 mls 1N HCl. Add 0.46 mls TEMED and make up to 100 mls with distilled water.

TABLE II

A Volumetric aid for the preparation of a
constant density gradient in a 110 ml column

Fraction No.	Dense solution		Light solution	
	ml	sum (ml)	ml	sum (ml)
1	4.6	4.6	0.0	0.0
2	4.4	9.0	0.2	0.2
3	4.2	13.2	0.4	0.6
4	4.0	17.2	0.6	1.2
5	3.8	21.0	0.8	2.0
6	3.6	24.6	1.0	3.0
7	3.4	28.0	1.2	4.2
8	3.2	31.2	1.4	5.6
9	3.0	34.2	1.6	7.2
10	2.8	37.0	1.8	9.0
11	2.6	39.6	2.0	11.0
12	2.4	42.0	2.2	13.2
13	2.2	44.8	2.4	15.6
14	2.0	46.2	2.6	18.2
15	1.8	48.0	2.8	21.0
16	1.6	49.6	3.0	24.0
17	1.4	51.0	3.2	27.2
18	1.2	52.2	3.4	30.0
19	1.0	53.2	3.6	34.2
20	0.8	54.0	3.8	38.0
21	0.6	54.6	4.0	42.0
22	0.4	55.0	4.2	46.2
23	0.2	55.2	4.4	50.6
24	0.0	55.2	4.6	55.2

Solution B: dissolve 30 gm acrylamide and 0.9 gm Tris in water and make up to 100 mls with distilled water.

Solution C: Ammonium persulfate. 0.3% w/v in distilled water (prior to use)

Solution D: Electrode buffer. Dissolve 6 gms TRIS base and 2.9 gms glycine in 1 litre of water. Adjust to pH 7.4.

A typical preparation involved mixing 10 mls of solution A + 20 mls of solution B + 10 mls of distilled water + 40 mls of solution C. When SDS was used, it was included in solution C at a concentration of 2% w/v. During polymerization, a layer of water was carefully added to the top of the slab to produce a flat surface. Once solidified, 10-50 μ l of protein (1-5 mg/ml in sucrose) were applied to the slots. When SDS was used, proteins were digested in vials with 1% SDS at 80 C for 15 minutes before being applied to the slab. Electrophoresis was performed at 50-100 mA and a potential of 250-300 V.

Proteins were visualized using a sensitive silver stain in a procedure described below. Proteins were fixed in a solution of 50% methanol and 12% acetic acid overnight. When SDS was used, it was subsequently removed from the gels by soaking for several hours in a 10% ethanol, and 5% acetic acid solution.

Gels were then soaked for 5 minutes in a 200 ml solution of 0.004 M potassium dichromate and 0.0034N nitric acid. They were washed 3-5 times with 200-300 mls of deionized water, and then placed in 300 mls of 0.012 M silver nitrate for 30 minutes.

Staining seemed to be improved if gels were illuminated by fluorescent lighting for the 30 minutes. This was followed by rapid rinsing with two 300 ml portions of the image developer solution which contained 0.28 M sodium carbonate and 0.5 ml of commercial

formalin solution. The gels were gently agitated in a third solution until the image developed to the desired intensity. Development was stopped by discarding the developer and soaking the gel in 200 ml of 1% acetic acid.

14. Dye-sensitized photooxidation

Enzymes placed in water-jacket cell were illuminated with a 250-Watt tungsten lamp in the presence of a series of dye-sensitizers (0-100 μ M). The pH (potassium phosphate buffer, 0.2 M) was varied to find the optimum conditions for photoinactivation. Catalytic activity was measured by withdrawing aliquots at suitable time intervals and using them without further purification (6). Pseudo-first order decays with respect to sensitizer were observed. For spectroscopic analysis, dyes of appropriate charge could be removed from the enzymes by batch treatment with either Cellex-CM or Cellex-T ion-exchangers.

15. Carbodiimide Condensation of Primary Amines

Glutathione Reductase (1-1.5 mg/ml) was extensively dialyzed against deionized water (pH 5.2-5.5). To 0.5 mls of enzyme was added 0.25 mls of 0.4 M 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide and 0.25 mls of 0.4 M amine. (Taurine, ethylamine or 1,2-diamino ethane, all at the same pH of 5.2, $\pm$ 0.2). Condensation with the enzyme was monitored by the increase in electron transferase activity which appeared complete after 60-80 minutes.

16. Acetimidation of Glutathione Reductase

Desalted enzyme (2 mg) was made up to a volume of 2 mls with 0.2 M potassium phosphate buffer pH 8.1. A stock solution of methylacetimidate HCl and benzylacetimidate HCl was prepared by dissolving the appropriate imidoester (100 mg) in 1 ml of the same

buffer and readjusting to pH 8.1. Addition of $100\mu\text{l}$ of this reagent to the enzyme was followed by gentle stirring at room temperature. Multifunctionality was scanned up to a time of 1 hour after initiation and expressed as a percent of the control value.

17. Phenylglyoxal and 1,2-cyclohexanedione Modification

Desalted enzyme (1-1.5 mg) was dissolved in 1 ml of 0.1 M potassium phosphate buffer, pH 8.2. A stock solution of phenylglyoxal was prepared by dissolving 70 mg in 0.5 mls of the same buffer. To the enzyme was added $20\mu\text{l}$ of stock phenylglyoxal. When 1,2-cyclohexanedione was used, it was prepared in a similar manner, only dissolved in 0.5 mls of absolute ethanol. Again, $20\mu\text{l}$ of this stock solution was added to the enzyme. Modification was monitored over an hour period. The same was also performed in borate buffer (0.2 M; pH 7.0).

18. NBS Modification

A stock NBS solution was prepared by dissolving 20 mg of NBS in 1.5 mls of 0.2 M potassium phosphate buffer, pH 7.0. Desalted flavoenzyme (3 mg) was made to a volume of 3 mls using 0.2 M potassium phosphate buffer, pH 7.5. $100\mu\text{l}$ of NBS stock solution was added to the enzyme solution. Activities were monitored and fell to 5% of control values in less than 1/2 hour. Unreacted NBS was removed by dialysis or passage through a Bio-Gel P-6 column.

19. TNBS Modification

Flavoproteins (0.5 mg-1 mg) were desalted and made up to 1 ml with potassium phosphate buffer (0.1 M), pH 7.0. A 0.5 M stock solution of TNBS in water was prepared and $10\mu\text{l}$ of it added to the enzyme. The absorbancy could be monitored at 420 nm and compared to a run identical to the first except protein was omitted. Using an extinction coefficient of $E_{420} = 22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ will enable one

to calculate the number of lysines modified. Reactions could be terminated by the addition of 2.0 mls of 0.1 M NaH_2PO_4 containing 1.5 mM sodium sulfite (prepared fresh) (9).

20. Tetranitromethane Modification of Flavoenzymes

Flavoenzymes (0.1 ml; 10 mg/ml) were desalted by dialysis against deionized water and then made to a concentration of ~ 0.9 mg/ml ($\text{O.D.}_{455} = 0.12$) using 0.05 M TRIS (pH 8.0).

All activities were then monitored. A stock solution of TNM was prepared by dissolving $10\mu\text{l}$ of TNM in 1 ml of absolute ethanol (0.084 M). Then, $30\mu\text{l}$ of this diluted stock solution was added to the enzyme (1.6 mls) to give a final $[\text{TNM}] = 1.57 \times 10^{-3}$ M. The mixture was left to stand at room temperature for 35 minutes. Activities were re-monitored. The enzyme was passed through a Bio-gel P-6 column to remove TNM (13).

21. Pyridoxalation using PLP

A procedure adapted from Redman was used (63). Enzyme (1-2 mg/ml) was prepared in 0.1 M phosphate buffer, pH 7.0 and combined with a slight excess (1-3 fold) of aldehyde prepared in the same buffer. After 1 hour of incubation at room temperature a $5\mu\text{l}$ aliquot of octanol was added followed by 1 mg of NaBH_4 . After 15 minutes enzyme was separated by gel filtrations on Bio-gel P-6 followed by dialysis against deionized water. Care was taken to omit light from the preparation.

22. Kinetic Studies

All steady-state kinetic studies were carried out on a Beckman Model 25 Spectrophotometer. Unless otherwise stated, reagents were prepared in 0.2 M potassium phosphate buffer at the desired pH. D,L-Lipoamide being water insoluble was dissolved in absolute ethanol.

Any kinetic studies carried out with Lipoamide Dehydrogenase were performed as described (8). With ADH, NAD^+ reductions were monitored by following the increase in absorption of NADH at 340 nm ($E_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Typically ethanol concentrations varied from 1-10 mM while NAD^+ varied from 0.03-3 mM. Reactions were initiated by the addition of 0.07 mM enzymes.

Lysozyme reactions were monitored by following the decrease in turbidity at 540 nm when lysozyme (0.4 mM) was added to a suspension of dried cells in 0.1 M potassium phosphate buffer to give an $\text{O.D.}_{540} \sim 0.5 (\pm 0.05)$.

Multifunctional activities of Glutathione Reductase were assayed in 0.2 M potassium phosphate buffer at pH 7.0 unless stated otherwise. Kinetic studies of electron transfer to ferricyanide were monitored by following the decrease in absorption at 420 nm ($E_{420} = 0.87 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction mixture (1 ml) contained $\text{K}_3\text{Fe}(\text{CN})_6$ (0.05-0.3 mM) and NADPH (0.03-0.2 mM). Reactions were initiated by the addition of 20 nM enzyme. DCIP reduction by NADPH was followed by decreased absorbancy at 600 nm. ($E_{600} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). DCIP varied from 0.016 mM - 0.07 mM, while NADPH varied from 0.01 mM-0.1 mM. Enzyme (10-20 nM) initiated the reaction. Transfer of reducing equivalents between NADPH and SNADP was followed by the appearance of sNADPH (0.02 - 0.14 mM) at 395 nm. ($E_{395} = 11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Concentrations of NADPH ranged from 0.74 mM - 2.2 mM. Transfer of reducing equivalents to 3-acetyl-pyridine-NADP (0.034 - 0.2 mM) was determined by the appearance of the reduced NADP^+ analog at 365 nm ($E_{365} = 5.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). NADPH varied from $0.32 \mu\text{M}$ - $1.28 \mu\text{M}$. Enzyme (10 nM) initiated both transhydrogenase reactions. Reduction of glutathione by NADPH was followed at 340 nm ($E_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

NADPH concentrations ranged from $3\mu\text{M}$ - $9.6\mu\text{M}$ while GSSG varied from $6\mu\text{M}$ - $40\mu\text{M}$. Enzyme (0.8 nM) started the reaction.

Velocities in the asymptotic region were fitted to:

$$v = \frac{V_{AB}}{AB + K_b A + K_d B} \quad (10)$$

for parallel plots or

$$v = \frac{V_A}{A B + K_b A + K_{ia} K_b + K_d B}$$

For converging reciprocal plots. V , K_a , K_b and K_{ia} are maximum velocity, Michaelis constant for substrate A, Michaelis constant for substrate B and inhibition constant for NADPH respectively. Product and dye inhibition studies were analyzed according to linear competitive or non-competitive inhibition as described by

$$v = \frac{VS}{K_S(1 + I/K_{is}) + S} \quad (11)$$

$$v = \frac{VS}{K_S(1 + I/K_{is}) + S(1 + I/K_{ii})} \quad (12)$$

where K_{is} and K_{ii} are inhibition constants for inhibitor I, which affects the slope and intercept, respectively, of reciprocal plots of initial rates versus varying substrate, S (A or B).

RESULTS

I. Kinetic Studies of Glutathione Reductase

The kinetics of lipoamide dehydrogenase has been investigated (89,90). To account for the chemistry of lipoamide reduction, Williams (43) postulated the involvement of an active site base (A). Since then, this lab has

provided kinetic (26) and spectroscopic evidence (16,26,85,91,92) to indicate that this catalytic group is a histidine.

Recent X-ray diffraction analysis of glutathione reductase (54) reveals the presence of a group near the active site disulfide that is the right size and shape to be a histidine residue. It would be of interest to determine if this active site base serves a similar role both in catalysis and in providing electrostatic stability to the nascent thiolate anion (26,52,53).

A study of the pH dependence of the physiological reaction of lipoamide dehydrogenase is a logical and direct probe of the involvement of a catalytic base at the active site. Earlier kinetic studies on this enzyme (64,65) reveal a family of parallel lines from primary double reciprocal plots. Such a condition indicates a Ping Pong mechanism ($K_{ia} = 0$) is operating although product inhibition patterns are in disagreement with this postulate (64,66) and instead a branching mechanism has been proposed by Mannervik (93).

In performing the kinetics, we have obtained a series of parallel lines over the range pH 5.1-8.0. Examples are shown in figure 9. The pH dependence of the kinetic parameters are shown in figure 10. The parameters are derived from secondary plots from the linear region of the double reciprocal plots. Catalytic efficiency is somewhat random but appears at a maximum below pH 6.5.

Both $\log (K_a/V)$ and $\log (K_b/V)$ versus pH show positive points of inflection indicating that a positive group associated with free enzyme is catalytically relevant. The group appears to have a pK of 5.5-6.0. The monophasic pattern of these profiles is in contrast to results obtained with lipoamide dehydrogenase which are biphasic displaying two points of inflection (26). However an apparent pK of 5.5-6.0 would seem to indicate the involvement of a catalytic histidine residue during glutathione reduction.

The involvement of an active site base in lipoamide dehydrogenase has preoccupied much of the work on this enzyme, (26,91,92). Additional evidence for the involvement of such a residue during catalysis was obtained from N.M.R. data (85).

The unique properties of the imidazole ring of histidine permits it to be conveniently studied by proton N.M.R. since it is the only amino acid whose side chain can serve as either an acid (I) or as a base (II) in the physiological pH range.

Figure 9. Primary double reciprocal plots of glutathione reductase activity catalyzed by native enzyme in 0.2M phosphate buffer at:

- A = pH 5.5
- B = pH 6.5
- C = pH 7.5

Glutathione was varied from 6-40 μ M

Figure 10. pH dependence of glutathione reductase catalyzed by native enzyme.

In 1964, as part of the first proton N.M.R. investigation of proteins at 100 MHz, Mandel reported the detection of a composite signal from the C-2 imidazole protons of the histidine residues in ribonuclease A (94,95). Since then N.M.R. has been used abundantly to probe the involvement of histidine in protein structure and function (96,99).

At 100 MHz, one sharp resonance peak (9.18 ppm) with a distinct shoulder corresponding to the C-2 protons of histidine (9.2 ppm) is seen in the oxidized form of native lipoamide dehydrogenase (figure 11).

Anaerobic reduction with NADH causes an upfield shift of the shoulder region to ~ 9.1 ppm. The shoulder protons in oxidized enzyme are also eliminated by rose bengal sensitized photooxidation. The observed upfield shift upon reduction is in agreement with deprotonation of histidines during PMR titration (100,101) or in the present case, a reduced cationic character of the imidazole ring which interact with the thiol anion in the EH form of the enzyme. The absence of the PMR shift and the MCD faraday A term (26) of EH_2 at $\text{pD}(\text{H})$ 8.64 (figure 12) which deprotonates the interacting histidine, supports the existence of an ion pair between the thiolate anion and the imidazole cation of histidine, thus, the stabilization provided by such an ion pair provides the favored intermediate in the reductase reaction catalyzed by lipoamide dehydrogenase.

In contrast, PMR studies on glutathione reductase fail to indicate a strong thiolate to histidine ion pair (figure 13,14). This was found to be the case over a range of pD 's investigated. Evidence for a weak thiolate histidine ion pair in 2-electron reduced glutathione reductase, has also been demonstrated by

Figure 11. Proton N.M.R. of lipoamide dehydrogenase at 100MHz.

A= oxidized enzyme at pD 6.94

B= 2-electron reduced enzyme at pD 6.94(EH₂)

C= Enzyme photoinactivated to 10% residual activity(oxidized form)

Figure 12. Proton N.M.R. of oxidized(A) and 2-electron reduced(B) lipamide dehydrogenase at pD 8.64

Figure 13. Proton N.M.R. of oxidized(A) and 2-electron reduced(B) glutathione reductase. NADPH used as the reductant in 100-fold excess.

Figure 14. Proton N.M.R. of oxidized and 2-electron reduced glutathione reductase at pD 7.44(A,B) and at pD 8.44(C,D).

Templeton (16,52) who showed the formation of much weaker MCD faraday A term compared to lipoamide dehydrogenase.

These results indicate a major difference between the two flavoproteins. The histidine in lipoamide dehydrogenase appears to function both in catalysis (26) and in providing stability to the nascent thiol anion generated during the catalytic cycle (85, 52, 53). In glutathione reductase, histidine appears to be predominantly involved in catalysis than as a stabilizer of the nascent thiol anion generated during glutathione reduction.

In glutathione reductase the thiolate anion generated in EH_2 appears to be involved in a more efficient charge transfer with the flavin. The 530 nm absorption band, characteristic of 2-electron-reduced enzyme (figure 5) displays a higher extinction coefficient in glutathione reductase by 1000-1500 $\text{M}^{-1} \text{cm}^{-1}$ and also a smaller pH dependence compared to lipoamide dehydrogenase (figure 15). This tends to support the concept of an efficient charge transfer between thiolate anion and the flavin in glutathione reductase. Diminished 530 nm absorption in EH_2 at low pH is attributed to gradual protonation of the thiolate anion resulting in a loss of interaction with the flavin.

Like lipoamide dehydrogenase, glutathione reductase catalysis a number of non-physiological NADPH-linked reactions. (Scheme 2) This indicates that the 2-electron reduced form of the enzyme (EH_2) is a heterogeneous species existing in forms other than a reduced disulfide. Indeed, with lipoamide dehydrogenase, Wilkinson and Williams (102,103) having demonstrated that EH_2 is a mixture of species of which the mentioned charge transfer complex is one; the other two are a form with the thiolate presumably protonated and hence unable to charge transfer with the oxidized FAD and a form

Figure 15. pH dependence of the 530nm extinction coefficient of 2-electron reduced glutathione reductase using a 10-fold excess of NADPH as reductant(o). Enzyme was typically 1-2mg/ml. Data for lipoamide dehydrogenase(●) was taken from Williams(51).

with the flavin reduced and the disulfide oxidized.

Those additional reactions catalyzed by glutathione reductase include transhydrogenase, electron transferase, diaphorase and oxidase activities as previously defined (Scheme 2). The kinetics of the transhydrogenase reaction is particularly interesting since reduction of both sNAD^+ and 3-Ac-NAD^+ by NADPH appear to proceed by Ping Pong mechanisms since double reciprocal plots of initial velocities give a family of parallel lines (figures 16,17). If a Ping Pong mechanism is operating then NADP^+ should be non-competitive with varied NADPH. This was found to be the case with sNAD^+ and 3-Ac-NAD^+ reductions (figures 18,19). Intercept and slope inhibition constants have been calculated assuming NADPH as the leading substrate.

These results suggest the presence of a single nucleotide binding site unlike that found in lipoamide dehydrogenase (16). In this latter enzyme, sNAD^+ reduction by NADH proceeds by a Ping Pong mechanism whereas reduction of 3-Ac-NAD^+ proceed by a BiBi mechanism. Templeton (16) in discovering this, postulated the presence of two nucleotide binding sites, one catalytic and one regulatory. Difference spectroscopy reveal positive and negative absorbancies when the enzyme is titrated with NAD^+ (figure 20). Titration of native lipoamide dehydrogenase with 3-Ac-NAD^+ was able to produce a similar spectra as titration with NAD^+ . However, titration with sNAD^+ produced only the negative deflection (420-450 nm) seen in the titration with NAD^+ (figure 20). These results supported the 2-site hypothesis. However, addition of NADP^+ to glutathione reductase does not reveal both positive and negative absorbancies by difference spectroscopy (figure 32) as seen in lipoamide dehydrogenase. The negative absorbances appear to

Figure 16. Reduction of sNADP catalyzed by native glutathione reductase at pH 7.0 in 0.2M phosphate buffer. Points are the average of two runs and agree to within 10%.

Figure 17. Reduction of 3-Ac-NADP catalyzed by native glutathione reductase at pH 7.0 in 0.2M phosphate buffer. Points shown are the average of two runs and agree to within 9%.

Figure 18. NADP inhibition of sNADP reduction catalyzed by native glutathione reductase in 0.2M phosphate buffer at pH 7.0.

$$K_{ii} = 168\mu\text{M} \quad K_{is} = 39\mu\text{M}$$

Figure 19. NADP inhibition of 3-Ac-NADP reduction catalyzed by glutathione reductase at pH 7.0.

$$K_{ii} = 53\mu\text{M} \quad K_{is} = 54\mu\text{M}$$

NADP = 0, 24, 52 and 104 μ M

predominate in glutathione reductase (400-500 nm). This may indicate a one-site nucleotide binding domain in this enzyme. Indeed, recent X-ray studies on erythrocyte glutathione reductase reveal only one nucleotide binding site per subunit (54,55). However, this evidence may be misleading since the erythrocyte enzyme will be shown to differ in some respect to the yeast enzyme.

Kinetic studies have also been performed on the diaphorase and electron transferase reactions catalyzed by glutathione reductase. In both cases, a family of non-parallel lines result from initial velocity double reciprocal plots (figures 21,22). This indicates a BiBi mechanism is operating and similar results have been obtained with lipoamide dehydrogenase (58). Table (III) gives a kinetic comparison of the 2 enzymes. In general, the non-physiological transhydrogenation and diaphorase reactions catalyzed by glutathione reductase are suppressed in comparison to lipoamide dehydrogenase and are a result of a reduced catalytic efficiency. A suppressed catalytic efficiency (V/E_T) would result either from a poor binding affinity of a substrate for the enzyme or to a reduction in the population of the reduced flavin species that will transfer reducing equivalents to the acceptor. The enhanced thiolate to flavin charge transfer interaction (figure 15) along with these kinetic observations may support the postulate that glutathione reductase contains a smaller population of reduced flavin species other than the reduced disulfide form. (i.e. suppressed $FADH\cdot$ or $FADH_2$) (102,103). The kinetics of the OXase reaction was not investigated, however, its reaction rate was 1/70-100 that of lipoamide dehydrogenase under identical conditions of NAD(P)H (80 μ M) and enzyme (90 μ M). Again this may be in agreement with the above proposal.

TABLE III

Kinetic Parameters for reactions catalyzed by pig heart lipoamide dehydrogenase at pH 7.5

Reactions	Temp (C)	V (μ M/min)	K _a (μ M)	K _b (μ M)	K _i (μ M)	V/Et (min)
DHase: NADH+LipS ₂ NH ₂	25	356	59.5	893	0	17.8×10^3
THase: NADH+sNAD	25	379	183	263	0	19.0×10^3
ETase: NADH+K ₃ Fe(CN) ₆	25	277	277	495	27.5	2.77×10^4
DPase: NADH+DCIP	25	37.7	33.6	127	5.49	1.86×10^3

Kinetic Parameters for reactions catalyzed by yeast glutathione reductase at pH 7.0

Reactions	Temp (C)	V (μ M/min)	K _a (μ M)	K _b (μ M)	K _i (μ M)	V/Et (min)
DHase: NADPH+GSSG	25	6.2	68.1	70.3	0	15.8×10^3
THase: NADPH+sNADP	25	1.58	0.58	34	0	152
NADPH+3-AC-NADP	25	1.54	1.18	295	0	147
ETase: NADPH+K ₃ Fe(CN) ₆	25	90.9	154	445	0.68	4.5×10^3
DPase: NADPH+DCIP	25	9.5	106	297	44.4	909

Duplicate runs with Glutathione Reductase give kinetic parameters agreeing to within 8-11%. The above value are averages of the two runs.

Figure 20. Difference spectrum of addition of NAD to enzyme (1.5×10^{-4} M) in phosphate buffer (pH 7.0, 0.1 M). NAD added to final concentrations of 9.96×10^{-5} M, 3.92×10^{-4} M, 1.31×10^{-3} M, 1.82×10^{-3} M and 2.14×10^{-3} M. Inset shows difference spectra when titrations performed with NAD analogs. Taken from Templeton (16).

Figure 21. Primary and secondary double reciprocal plots of the diaphorase reaction catalyzed by native glutathione reductase in 0.2M phosphate buffer at pH 7.0.

Figure 22. Primary and secondary double reciprocal plots for the electron transferase reaction catalyzed by native glutathione reductase in 0.2M phosphate buffer at pH 7.0

Chromatofocusing (figure 23) and isoelectrofocusing (figure 24) of glutathione reductase do not demonstrate the existence of isozymes ruling this out as a possible source of enzyme multifunctionality. In contrast, lipoamide possesses 6 isozymes and all are multifunctional. (16)

Figure 23. Chromatofocusing profile of yeast glutathione reductase.

Figure 24. Isoelectric focusing profile of glutathione reductase.

II Chemical Modifications

In order to gain insight into how an enzyme functions mechanistically, enzymologists normally employ a series of chemical modifications followed by determination of whether or not such a modification causes a loss or change in the characteristic activity. A weakness of such studies often results from their failure to distinguish between absolutely vital residues such as those in an active site or those otherwise affecting the protein structure directly associated with the biological property of interest. Unfortunately, a clear distinction often cannot be made. Nonetheless, a number of chemical modifications have been performed on glutathione reductase in order to assess the role of certain amino acids in catalysis.

Perhaps the most striking difference between glutathione reductase and lipoamide dehydrogenase is that they are specific for their own nucleotides in addition to their substrates. Lipoamide dehydrogenase, for example, is less than 1% as active with NADPH as with NADH (104) while yeast glutathione reductase shows virtually no cross-reactivity with NADH (105). It seemed plausible, therefore, that the additional phosphate on NADPH may cause an enhanced binding affinity of this nucleotide for the enzyme giving rise to its specificity over NADH. As a result of chemical modifications there has been an increased awareness of the participation of lysine and arginine residues in or near the phosphate binding sites of many enzymes (106-109). The recognition of arginine residues at the active site of enzymes which act upon anionic substrates is largely due to the work of Riordan and co-workers (107,108) who have introduced the use of monomeric 2,3-butanedione as a highly selective reagent for the modification of

arginine residues (110).

Modification of yeast glutathione reductase with typical arginine reagents (2,3-butanedione, 1,2-cyclohexane-dione and phenylglyoxal) fail to alter the activity of the enzyme by more than 20%. Modifications carried out in borate rather than phosphate buffer gave similar results (Table IV). This data tends to indicate that arginine(s) are non-essential to the nucleotide binding domain in yeast glutathione reductase. However, Boggaram and Mannervik have shown that the same three reagents irreversibly inactivate the erythrocyte enzyme (40). NADPH, NADP^+ and other adenosine-2'-phosphate nucleotides partially protected the enzyme against inactivation by the dicarbonyl reagents. The authors suggest that an arginine residue in the nucleotide binding site of glutathione reductase interacts with the 2'-phosphate moiety of adenosine. The presence of such an arginine has been verified by recent sequencing of the NADPH domain of glutathione reductase from human erythrocytes (55). It would appear then that a structural difference exists between the yeast and erythrocyte enzyme.

A lack of sensitivity of the yeast enzyme towards the dicarbonyl reagents would suggest that an alternative residue(s) occupy the nucleotide binding site. Insensitivity towards arginine reagents has also been demonstrated in lipoamide dehydrogenase (16). Lysine, however, being cationic at physiological pH, could also serve as a binding residue for the anionic phosphate of nucleotides (106-109). However, treatment of glutathione reductase with methylacetimidate or benzylacetimidate (eqn. 4)

resulted in minor changes in enzyme activity (after 1 hour reaction

TABLE IV

Residual Activities upon Chemical
Modification of Glutathione Reductase

Enzyme form	Target Amino Acid	RDase	THase (sNADP ⁺)	THase (3-Ac-NADP ⁺)	ETase	DPase	OXase
E_h	Met, His, Trp	2	11	8	74	119	120
E_h	Met, His, Trp	3	7	9	88	126	138
E_{FA}	His	93	91	98	101	92	90
E_{NH}	Glu, Asp	19	200	-	724	92	-
E_{CH}	Glu, Asp	41	155	-	344	110	-
E_{SH}	Glu, Asp	38	133	-	198	98	-
E_{gh}	Arg	84	90	92	96	91	100
E_{CO}	Arg	90	83	92	100	97	103
E_Y	Arg	79	97	101	105	92	96
E_{NHS}	Lys	15	108	90	88	100	-
E_{MA}	Lys	100	104	96	106	91	-
E_{BA}	Lys	92	91	85	93	83	-
E_{PLP}	Lys	91	88	95	91	91	89
E_{NBS}	Trp, Tyr	<1	<1	<1	<1	<1	-
E_{TMM}	Tyr	4	16	3	9	6	3

All experiments were done in duplicate and results quoted are averages with deviations of $\pm 5 - 10\%$. Results shown are % of control activities.

time) (Table IV). Treatment of the yeast enzyme with pyridoxal-5'-phosphate, a recognized lysine modifier, (114,115) produced similar results causing little change in multifunctional activity. Interestingly, however, modification of the yeast enzyme with TNBS (eqn.5)

caused a 80-90% drop in the reductase activity while other activities were mildly affected (Table V). It would seem reasonable that the lysines attacked by this reagent are not those involved in nucleotide binding since it is only the reductase reaction that is drastically effected by this modification. According to Scheme 2, modification of the NAD(P)H binding site would result in a change in each of the activities since binding of the nucleotide is common to each reaction. Since glutathione is a very polar and anionic substrate, it is conceivable that the reduction in activity resulted from modification of lysine(s) involved at the glutathione binding site. Lysine residues do exist around the active site disulfide of glutathione reductase (figure 6) and their role in binding polar glutathione has been suggested by Williams as well (43). Work is currently under way in our lab to synthesize the methyl ester of glutathione which should disrupt charge interaction with cationic lysine and render this ester a poorer substrate. It is interesting to note that treatment of lipoamide dehydrogenase with the same reagent has little effect on any of the multifunctional activities (Table V). This would seem reasonable since lysines are unlikely to be involved in the binding of such a non-polar substrate as lipoamide (figure 7).

The aromatic residues, tryptophan, tyrosine and phenylalanine

TABLE V
Residual Activities upon Modification
with TNBS and NBS

enzyme	reagent	DHase (SNAD(P))	THase (3-Ac-NAD(P))	DHase	ETase	OXase
Lipoamide Dehydrogenase	TNBS	104	102	97	89	-
Glutathione Reductase	TNBS	15	108	100	88	-
Lipoamide Dehydrogenase	NBS	0	0	0	0	-
Glutathione Reductase	NBS	0	0	0	0	-

Modification with TNBS appeared complete in about 65-80 minutes. At this point 16-17 residues/subunit were modified in the case of glutathione reductase while with lipoamide dehydrogenase 10-11 residues/subunit were modified. See methods. Results shown are % of control activities.

would appear to be the only remaining candidates to assist in the binding of nucleotides to the enzyme. Recently, Inoue et al (111) have demonstrated favorable base stacking interactions between FMN and the amino acids tryptophan and tyrosine by X-ray and solution N.M.R. spectroscopy. In fact, information gained by diverse spectral studies of native and chemically modified flavoproteins, has provided unequivocal evidence for the interaction of the flavin isoalloxazine ring with aromatic amino acids residues within flavin binding sites of numerous flavoproteins (110,112,113). We have investigated the existence of these residues at the nucleotide binding site of lipoamide dehydrogenase and glutathione reductase by using NBS and TNM as chemical modifiers (13,116).

Treatment of the 2 flavoproteins with NBS resulted in a loss of all activities to less than 5% in less than a half hour reaction time. Complete inactivation results if reactions were left for an hour or more (Table V). NBS is known to be fairly specific for tryptophan but can also react with tyrosine (116). Suppression of all activities supports the belief that tryptophan and/or tyrosine may be present at the nucleotide binding site since initial binding of NAD(P)H is required for each reaction (Scheme 2). Amino acid analysis of NBS treated lipoamide dehydrogenase reveals that 2-3 tyrosines and 1-2 histidines are affected. (Table VI). Damage to the nucleotide binding site is also supported by the inability of lipoamide dehydrogenase and glutathione reductase to produce the long wavelength absorption band upon anaerobic reduction with their respective nucleotides. The spectra of the oxidized enzymes are also drastically affected displaying a loss in the shoulder region (470-490 nm) and a blue shift from their normal absorption maxima (figures 26-29). Damage done to the flavin is ruled out (figure 25).

Figure 25. Visible spectra of FAD before(o) and after(●) treatment with NBS.

$$\begin{aligned} [\text{FAD}] &= 4.5 \times 10^{-5} \text{ M} \\ [\text{NBS}] &= 2.8 \times 10^{-3} \text{ M} \end{aligned}$$

Figure 26. Visible spectra of native(-) and NBS modified(--)
glutathione reductase. The reaction was performed at pH 8.0 in
0.2M phosphate buffer.

Enzyme = $2.7 \times 10^{-5} \text{M}$
NBS = $6.2 \times 10^{-2} \text{M}$

Figure 27. Visible spectra of oxidized NBS modified glutathione reductase(-) and its(--) reduced form when 100-fold excess of NADPH is added anaerobically at pH 8.0 in 0.2M phosphate buffer. The spectra reveal an absence of the 530nm absorption band characteristic of the 2-electron reduced species.(EH₂)

Figure 28. Spectra of native(1) and NBS modified(2) lipamide dehydrogenase at pH8.0 in 0.2M phosphate buffer. Enzyme was 2.2×10^{-5} M and NBS was 5.9×10^{-3} M. Unreacted NBS was removed by passage of the enzyme through a P-6 column.

Figure 29. Visible spectra of oxidized NBS modified lipoamide dehydrogenase(-) and its reduced form(--) when a 100-fold excess of NADH is added anaerobically at pH 8.0 in 0.2M phosphate buffer. The spectra reveal an absence of the 530nm absorption band characteristic of the 2-electron reduced species(EH_2).

TABLE VI
Residual Activities upon Modification
with TNM (% of Control)

enzyme	DHase	THase (sNADP)	THase (3-AC-NADP)	ETase	DPase	OXase
lipoamide dehydrogenase	3	0.80	0.50	1.1	0.6	0.6
glutathione reductase	5	16.8	2	8	6	0.9

Duplicate runs are in agreement to within 5-12%.

TABLE VII
Amino Acid Analysis of Native
and NBS treated Lipoamide

Dehydrogenase		
Residue	Native (%)	NBS treated (%)
Asp	12.4	11
Thr	3.9	3.4
Ser	8.7	6.9
Glu	6.6	7.5
Pro	-	3.6
Cys	-	-
Gly	13.8	13.2
Ala	10.0	10.3
Val	4.3	5.7
Met	.61	1.2
Ile	1.6	2.2
Leu	5.1	5.3
Tyr	3.5	2.7
Phe	3.07	3.2
His	1.7	1.1
Lys	5.8	5.1
NH	16.5	15.4
Avg	2.2	2.1

Since tryptophan cannot be determined by amino acid analysis, its involvement at the nucleotide binding site must be determined by other means. Oxidation of the indole side chain of tryptophan upon treatment with NBS, can be monitored in the UV region but such spectral changes are complicated by the flavin absorption in the same region. However, this problem is overcome by treatment of the flavoprotein with 5M guanidine-HCl after reaction with NBS. The apoprotein and NBS-apoprotein can be easily separated from free flavin on a bio-gel P-6 column. Figures 30 and 31 show spectral changes in the UV region when L-tryptophan and lipoamide dehydrogenase are treated with NBS. Both give rise to long wavelength absorptions after NBS treatment. This supports the hypothesis that tryptophan is one of the amino acids likely effected by the NBS modification. The difference spectrum of lipoamide dehydrogenase also infers damage done to the nucleotide binding by a decreased binding affinity of NAD^+ to the enzyme (figure 32). Similar results were obtained with glutathione reductase (figure 33).

X-ray diffraction studies (54,55) of erythrocyte glutathione reductase reveal a tyrosine residue situated in the NADPH domain. Such a residue may also be present in the yeast enzyme and it may have a role in nucleotide binding in addition to the postulated tryptophan(s). This does not discount the possibility that tyrosine may also be involved in one or both of the nucleotide binding sites of lipoamide dehydrogenase.

Tetranitromethane (TNM) is one of the few reagents known to be fairly specific in chemically modifying tyrosine (117). The degree of nitration can be determined based on the absorption of the nitrotyrosyl residue ($E_{381\text{m}\mu} = 2200$). Treatment of lipoamide

Figure 30. Spectral changes resulting when L-tryptophan is treated with NBS. Modification was performed in 0.2M phosphate buffer at pH 7.0.

- (●) - L-tryptophan(0.12mM)
- (○) - L-tryptophan + 0.19mM NBS
- (●) - L-tryptophan + 0.36mM NBS

Figure 31. Lipamide dehydrogenase (1.4 mg/ml) was treated with a 200-fold excess of NBS. The flavin was later removed and the spectra of apo-protein(o) and NBS apo-protein recorded. Inset shows the elution profile of the apo-proteins as they elute from a P-6 polyacrylamide column.

Figure 32. Difference spectra of lipoamide dehydrogenase before and after treatment with NBS. Before (—), after (---). Enzyme was $1.2 \times 10^{-5} \text{ M}$ and NAD 20 mM (pH 7.0, 0.2M phosphate buffer). Inset shows spectral changes of the flavin after modification (---).

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Figure 33. Difference spectra of glutathione reductase when treated with NADP.
 (—) $2.1 \times 10^{-5} M$ enzyme + $15 mM$ NADP
 (---) $2.1 \times 10^{-5} M$ enzyme (NBS treated-see methods) + $15 mM$ NADP
 Inset shows lack of protection when native enzyme (●) is treated with NBS in the presence of $5 mM$ NADP. (○)

dehydrogenase with TNM resulted in a near loss of all activities in about thirty minutes. Spectral changes accompanied the modification in addition to the 80% drop in the fluorescent intensity of the enzyme. Results are shown in figure 34. Duplicate runs reveal 4.0 ± 0.4 tyrosines modified per mole of enzyme (2 subunits). Glutathione reductase displayed similar results (Table VI) with calculations indicating that 2.4 ± 0.4 residues modified per mole of enzyme (figure 35). Lipoamide dehydrogenase was partially protected from TNM inactivation by the addition of 1 mM NAD^+ , supporting the interaction of TNM with the nucleotide binding site (figure 36). The results obtained with TNM and NBS indicate that tryptophan and/or tyrosine play crucial roles at the nucleotide binding sites of both flavoproteins.

The electron transfer reaction which transfer reducing equivalents to ferricyanide via a one-electron transfer mechanism is particularly interesting since the physiological reactions of lipoamide dehydrogenase and glutathione reductase proceed presumably via a 2-electron pathway (43,118). A knowledge of the factors affecting the electron transfer reaction would undoubtedly be valuable to an understanding of the multifunctionality and redox chemistry of both flavoproteins.

The one electron transfer reaction catalyzed by these flavoproteins proceed via a semiquinone radical intermediate. (119). The neutral radical is favored by a base or an anionic residue whereas the anionic radical is stabilized by an acidic or cationic residue.

Figure 37 illustrates progress curves as glutathione reductase is amidated with amine nucleophiles bearing differently charged groups. Ethylenediamine offered the greatest enhancement in the

Figure 34. Changes in the visible spectra when lipoamide dehydrogenase(-) is treated with TNM(--) at pH 8.1 in 0.2M TRIS buffer. Inset shows the drop in fluorescent intensity of the enzyme which accompanied the modification.

Figure 35. Visible spectra of native(-) and TNM(---) treated glutathione reductase. Chemical modification was performed in 0.2M TRIS buffer at pH 8.1

Figure 36. Lipoamide dehydrogenase (1-1.2mg/ml) inactivated upon addition of $2 \times 10^{-3} \text{ M TMM}$ (o). Protection provided when the enzyme was supplemented with 1 mM NAD^+ (●).

Figure 37. Changes in the electron transfer activity when glutathione reductase is amidated with a series of substituted ethylamines.

- - $\text{NH}_3\text{CH}_2\text{CH}_2\text{NH}_3$
- - $\text{NH}_3\text{CH}_2\text{CH}_2\text{CH}_3$
- Δ - $\text{NH}_3\text{CH}_2\text{CH}_2\text{SO}_3^-$

TABLE VIII

Multifunctional Activities of
Amidated Glutathione Reductase

	% Control Activity			
	$E_{NH_3^+}$	E_{CH_3}	$E_{SO_3^-}$	$E_{carbo.}$
Reductase	12	38	41	61
Transhydrogenase	200	155	130	168
Electron Transferase	724	344	198	200
Diaphorase	106	91	98	92

Note: The control activities were based on 100% for controls in the presence of respective amines. Amidations were carried out at pH 5.2 for 75 + 10 minutes or until maximum activity was reached. The last column refers to reaction just with carbodiimide.

ETase activity. SDS slab electrophoresis revealed that no cross-linking had taken place. 2-aminoethylsulfonic acid slightly activated the ETase reaction due primarily to the effect of EDC which, as the control reagent, paralleled that of 2-aminoethylsulfonic acid (Table VIII). Similar results have been observed in lipoamide dehydrogenase (63,83). Steady-state kinetic studies reveal that the charge reversal of the carboxyl group by the amino ethyl group in $E_{NH_3}^+$ facilitates not only interaction of $Fe(CN)_6^{3-}$ (decreased K_b) but also the catalytic efficiency (V/E_T) for the transfer reaction (26). Presumably, glutathione reductase is operating in an analogous manner.

III Photooxidation

Photooxidation is a well established chemical modification in assessing the structure-function relationship in proteins (117,120). In most cases, however, it lacks specificity since histidine, tyrosine, methionine and cystine have been shown to react (77,78). Singlet oxygen is believed to be the species responsible for the photooxidation of proteins (84-87) and the chemical changes observed in non-enzymatic systems (76,121,122). However, the interpretation of experimental results is often hindered by the lack of our knowledge concerning the primary events of photosensitization and rationales for a choice of dyes with respect to their selectivity and effectiveness as photosensitizers.

Kearns et al (121) have shed some light on this dilemma in their dye-sensitized photooxidation of cholest-4-en-3 β -ol (figure 38a). Photooxidation of this compound yields only two products, an epoxy ketone (II) and an enone (III). However, in contrast to other sensitized photooxidation which are believed to involve singlet oxygen molecules (presumably $^1\Delta_g$) (123-127) this reaction gave an enone: epoxy ketone (III:II) product distribution ratio which varied from 30:1 to 1:5 depending upon the nature of the sensitizer used. The authors proposed that both $^1\Sigma_g^+$ and $^1\Delta_g$ oxygen molecules were involved as reaction intermediates, that these two species exhibited different chemical reactivities and that the observed variation in product distribution with sensitizer used results from a variation in the relative amounts of $^1\Sigma_g^+$ and $^1\Delta_g$ generated. The dramatic change in the III:II ratio parallels the increase in the $^1\Sigma_g^+ : ^1\Delta_g$ ratio as E_T (from fluorescence emission maximum of dye) is increased from 35 to 50 kcal. High energy sensitizers (fluorescein, acridine orange) appear to mainly generate $^1\Sigma_g^+$

Figure 38a. Dye sensitized photooxidation of cholest-4-ene-3-one. Ratio of II/III depended on the sensitizer used. Taken from Kearns (121).

Dyes employed include:

1. Methylene blue
2. Chlorin e6
3. Hematoporphyrin
4. Rose bengal
5. Erythrosin B
6. Sulforhodamin B
7. Eosin Y
8. Acridine orange
9. Fluorescein
10. Triphenylene

Figure 38b. Structure of a variety of dye-sensitizers.

oxygen, and this species reacted primarily to give the enone. Greenstock (122) has also observed a dye dependence in the photooxidation of a chemical carcinogen.

We have noticed a variation in the rate of inactivation of lipoamide dehydrogenase and glutathione reductase depending on the dye used and have undertaken a study to investigate this trend further. Meaningful data from rates of photoinactivation with sensitizer used, required each dye to be studied under conditions that would give a maximum rate of inactivation. This necessitated a concentration and pH profile for each dye (figure 38). Figure 39 gives a typical concentration profile when lipoamide dehydrogenase is illuminated in the presence of varying amounts of rose bengal. Wand (26) has shown that photoinactivation using rose bengal is pH-dependent with maximum rates of inactivation occurring between pH 5.0-6.0. An apparent pK of 6.5 is associated with the pH dependence and amino acid analysis revealed that 5 histidines were oxidized by the modification.

Photoinactivation with methylene blue also displays a significant pH dependence again with an apparent pK of 6.5-7.0 (figure 40). However, maximum rates of inactivation occur at pH's above neutrality. This may be explained in light of the charges on the sensitizer. Being anionic, rose bengal is likely to form more favorable dye-histidine complexes at pH's below histidine's pK where it will be protonated. Cationic methylene blue should cause rapid rates of inactivation at higher pH's for the opposite reasoning. This argument is made assuming both dyes are interacting at or near the active site. Indeed, Wand (26) has demonstrated that rose bengal competitively inhibits lipoamide reduction with respect to varied lipoamide in addition to suppressing flavin fluorescence.

Figure 39. Rates of inactivation of lipoamide dehydrogenase when illuminated (from 25cm) in the presence of varying amounts of rose bengal as shown. Illumination in the absence of sensitizer caused less than 2% inactivation over the same time period.

Figure 40. pH dependence of the rate of methylene blue sensitized photoinactivation of native lipoamide dehydrogenase as measured by the loss in reductase activity. Inset shows the pseudo-first order nature of the inactivation at pH 7.0. Sensitizer was 50-60 μM and enzyme 0.9 μM .

Figure 41. Suppression of lipamide dehydrogenase(0.7mg/ml) fluorescence after addition of 0, 33, 82, 147, 228 and 322uM methylene blue.

Figure 42. Rate of inactivation of lipoamide dehydrogenase with a series of sensitizers. Sensitizers used from low energy to high include: methylene blue, hematoporphrin, rose bengal, erythrosin B, eosin Y, acridine orange, fluorescein, riboflavin, and pyridoxal-5-phosphate. Points shown are the average of two runs and are typically ± 0.04 - 0.10 .

TABLE IX

Multifunctionality of Lipoamide

Dehydrogenase after photooxidation

% Control Activity						
dye	DHase	THase (sNAD ⁺)	THase ⁺ (3-AC-N)	ETase	DPase	OXase
Methylene blue	6	25	32	65	257	202
Rose Bengal	5	28	48	85	160	188
Acridine Orange	91	89	92	94	93	89
Fluorescein	95	92	98	95	98	99

Methylene blue behaves similarly, decreasing flavin fluorescence with increasing dye concentration supporting interaction near the active site flavin (figure 41).

All other dyes studied do not show the marked pH dependence that rose bengal and methylene blue do. In any event, rates of inactivation were determined under optimal conditions for each dye and the multifunctionality of the enzyme was monitored during modification. Typical results using 2 high energy and 2 low energy sensitizers is shown in Table (IX). Rates of inactivation observed versus sensitizer used are shown in figure 42. High energy sensitizers such as riboflavin, fluorescein and acridine orange were ineffective in causing significant photoinactivation of lipoamide dehydrogenase. Slower rates of inactivation by these high energy sensitizers is unlikely to result from a lack of dye-enzyme interaction since these sensitizers improve their effectiveness in D_2O (figure 43). High energy sensitizers also cause little damage to the flavin environment since a fluorescein sensitized enzyme is still capable of forming the 536 nm absorption band characteristic of the 2-electron reduced enzyme (figure 44).

Dyes are believed to be interacting near the lipoamide binding site since it offers protection during photooxidation (figure 45). NAD^+ does not offer such protection (figure 46). Nonetheless, multiple binding sites may exist on the enzyme surface.

Wand (26) has provided indirect support for the involvement of singlet oxygen by demonstrating enhanced rates of inactivation in D_2O and suppression by NaN_3 . However, one cannot rule out the possible involvement of a type I radical mechanism (122). Protection against photoinactivation was provided to some degree when reaction mixtures were supplemented with $Fe(CN)_6^{2-}$. This

Figure 43. Photoinactivation of lipoamide dehydrogenase (0.4 μ M) (●) when illuminated with fluorescein (70 μ M) in deuterated buffer pD 6.7 (96% D₂O), compared to its ineffectiveness in protic buffer (Δ). Circles demonstrate protection provided in D₂O when supplemented with 1mM NaN₃.

Figure 44. Spectra of native lipoamide dehydrogenase(1) and its fluorescein photosensitized form(2) obtained at pH 7.5 in 0.2M phosphate buffer. Spectra (3) is obtained when (2) is reduced anaerobically with a 100-fold excess of NADH. The reduced fluorescein derivative still maintains the long wavelength absorption band characteristic of the EH_2 species. Photooxidation of the native enzyme(1.5 mg/ml) was carried out for 25 minutes in the presence of 100uM fluorescein at a distance of 30cm.

Figure 45. Lipoamide dehydrogenase (0.9 μ M) photoinactivated at pH 5.5 (●) using 50 μ M rose bengal (A). Protection provided using 240 μ M lipoamide (2xK_b) (○). Similar protection provided at pH 8.0 (B).

Figure 46. Lipoamide dehydrogenase(0.9uM) photoinactivated with 50uM rose bengal(●) at pH 5.5(A) and pH 8.0(B). Little protection provided by 240uM(Δ) or 600uM NAD(o)

result may indicate a complex reaction mixture of radicals and singlet oxygen either or both of which may be causing the inactivation (figure 47).

Photoinactivation of glutathione reductase was also studied. A significant pH dependence was observed when photooxidations were carried out using rose bengal (figure 48) and methylene blue (figure 49). Hematoporphrin was always studied at pH 8.0 because of its insolubility at acidic pH. High energy sensitizers showed little pH dependence (figure 50) and like lipoamide dehydrogenase caused little inactivation (figure 51b).

Substrates provided protection for glutathione reductase but only at acidic pH (figure 51b). This may be explained by the reduced binding affinity of GSSG and NADPH with increasing pH (figure 9) or perhaps the requirement of a protonated residue at respective binding sites. Destruction of an essential histidine is believed to be the cause for inactivation as was observed in lipoamide dehydrogenase. Boggaram et al (128) have in fact demonstrated the presence of an essential histidine in erythrocyte glutathione reductase. The enzyme was modified with ethoxyformic anhydride and caused a > 95% loss in reductase activity. However, the yeast enzyme is less than 10-15% affected by this reagent and we have verified this result, though lack of inactivation does not negate histidine's presence.

It would appear that rates of inactivation for these two flavoproteins (k_{obs}) vary in a manner similar to the calculated ratio of singlet oxygens ($\sum_g^1 \Delta_g$) versus the triplet state energies (E_T) of the sensitizer (figures 42,50). This has been seen in non-enzymatic systems (121,122). The most effective dyes appear to be the low energy sensitizers (methylene blue, rose

TABLE X

Multifunctionality of Glutathione
Reductase after Photooxidation

% of Control Activities						
Dye	RD _{ase}	TH _{ase} (sNADP)	TH _{ase} (3-Ac-NADP)	ET _{ase}	DP _{ase}	OX _{ase}
rose bengal	2	28	14	81	116	122
riboflavin	95	101	99	91	95	89

Figure 47. Photoinactivation of liposamide dehydrogenase (●) at pH 8.0 (A) using 50μM methylene blue and pH 5.5 (B) using 50μM rose bengal. In both cases protection was provided by 0.5mM $K_3Fe(CN)_6$. (○) 1mM ferricyanide offered no further protection.

Figure 48. pH dependence of the rose bengal (50uM) sensitized photoinactivation of glutathione reductase. Inset shows the pseudo-first order nature of the reaction at pH 5.95. Enzyme was 0.8-0.9uM.

Figure 49. pH dependence of methylene blue sensitized photooxidation of glutathione reductase. Inset shows pseudo-first order nature of inactivation at pH 6.4. Dye and enzyme concentrations were 50uM and 0.9uM respectively. Samples were illuminated 30cm from the lamp source.

Figure 50. pH dependence of fluorescein, acridine orange and riboflavin photooxidation of glutathione reductase. (●) - 50uM of dye used. (○) - 100uM of dye used. Enzyme was 0.9uM in each case.

bengal, hematoporphrin) which cause rapid rates of inactivation ($t_{1/2} < 10$ min.).

Two other enzymes were investigated in an attempt to determine if this type of trend can be generalized to most enzyme systems containing reactive 'ene' moieties (histidine, tryptophan) at their active sites.

The first of these was horse liver alcohol dehydrogenase (ADH). ADH is a dimeric enzyme with identical subunit weights of 42,000, both of which contain 2 zinc ions (129). The second catalytic zinc is bound deep inside the molecule at the wall of a hydrophobic pocket by Cys-46, 174, his-67 and a water molecule in the pocket (130). The presence of these photosensitive residues makes this enzyme an ideal candidate to study.

Dye concentration and pH effects were conducted with ADH as with the previous two flavoproteins. An example is shown in figure 52. Other dyes demonstrated a similar pH dependence as rose bengal and erythrosin B. Methylene blue is the oddity in that it displays increasing rates of inactivation with increasing pH. An apparent $pK \sim 7.0$ is associated with the pH dependence. As with the other enzymes, this may indicate the presence of at least one dye binding site on the enzyme, binding being dependent on the structure of the particular dye. Support for this arises from inhibition studies which demonstrate that rose bengal competitively inhibits the enzyme with varied NAD^+ whereas methylene blue displays non-competitive inhibition. Both dyes exhibit non-competitive patterns towards varied ethanol concentrations (figures 53-56). Acridine orange, a high energy sensitizer, causes little inactivation ($< 10\%$) but does exhibit inhibition patterns similar to methylene blue (figures 57,58). Inhibition constants for these

Figure.51a. Rate of inactivation of glutathione reductase as a function of sensitizer used. Sensitizers used from low energy to high include: methylene blue, hematoporphrin, rose bengal, erythrosin B, eosin Y, acridine orange, fluorescein, roboflavin and pyridoxal-5-phosphate. Points shown are the average of two runs and are typically ± 0.05 -0.1. Enzyme was typically 0.8-0.9 μM .

Figure 51b. A- Photoinactivation of glutathione reductase using (●) methylene blue (50 μ M) at pH 7.5. Protection in presence of (○) 10mM GSSG or 1mM NAD (●). B- Same performed at pH 5.0 using 50 μ M rose bengal.

Figure 52. pH dependence of methylene blue, rose bengal and erythrosin B sensitized photoinactivation of horse liver alcohol dehydrogenase. Dyes were 60uM and samples of enzyme (3.7mM $\pm$ 0.3mM). Samples were illuminated from 28cm.

Figure 53. Competitive rose bengal inhibition of ethanol oxidation catalyzed by horse liver alcohol dehydrogenase at pH 8.0 in 0.2M phosphate buffer. NAD was varied as shown while ethanol was fixed at 3mM. Rose bengal varied at 0, 15, 67 and 117 μM .

Figure 54. Rose Bengal inhibition of ethanol oxidation catalyzed by native horse liver alcohol dehydrogenase. (pH 8.0)

NAD was fixed at 0.2mM. Rose bengal was varied at 0, 30, 67, 90 and 118μM.

Figure 55. Methylene blue inhibition of ethanol oxidation catalyzed by horse liver alcohol dehydrogenase as a function of varied NAD and ethanol. (pH 8.0 in 0.2M phosphate buffer). Inhibition constants are given in Table XI. Methylene blue was varied at 4, 8, 12 and 32 μ M versus varied NAD and 9, 19 and 27 μ M versus varied ethanol.

Figure 56. Secondary plots of methylene blue inhibition of ethanol oxidation catalyzed by horse liver alcohol dehydrogenase at pH 8.0 (0.2M phosphate buffer). Inhibition constants versus varied ethanol and NAD are given in Table XI.

Figure 57. Acridine orange inhibition of ethanol oxidation catalyzed by horse liver alcohol dehydrogenase (pH 8.0, 0.2M phosphate buffer). Ethanol was fixed at 3mM while NAD was varied as shown. Acridine orange varied at 20, 68 and 128μM. Inhibition constants are given in Table XI.

Figure 58. Acridine orange inhibition of ethanol oxidation catalyzed by horse liver alcohol dehydrogenase. (pH 8.0). NAD was fixed at 0.2mM and ethanol varied as shown. Acridine orange varied at 20, 42 and 63 μM . Inhibition constants are given in Table XI.

dyes are given in Table (XI).

Photoinactivation of ADH followed the same trend as the previous enzymes as a function of sensitizer used (figure 59). Lack of significant photoinactivation caused by high energy sensitizers would not appear to stem from poor dye-enzyme interaction since high energy sensitizers such as acridine orange display similar inhibition patterns as low energy sensitizers such as rose bengal or methylene blue (Table XI). However, low energy sensitizers do appear to be more effective in inactivating ADH. The photoinactivation would appear to stem from histidine oxidation as amino acid analysis reveals a loss of 7 histidines along with a concurrent increase in aspartate residues (87). One tyrosine is also lost (Table XII). Acridine orange, a high energy sensitizer, fails to significantly inactivate ADH over the same time period as rose bengal or methylene blue and has a minimal effect on photosensitive histidine (Table XII). Ethanol and NAD^+ alone provide little protection against photoinactivation. However, NAD in the presence of pyrazole, a potent ADH inhibitor (131), provides significant protection during rose bengal photo-inactivation (figure 60). Singlet oxygen would appear to be involved as an intermediate since D_2O enhances inactivation (77) whereas NaN_3 provides protection (figure 61). However, the system may be complicated again by a radical involvement since $\text{Fe}(\text{CN})_6^{3-}$ also provides protection against photoinactivation (figure 62).

Lysozyme was the fourth enzyme investigated for its sensitivity towards photoinactivation. Its bacteriolytic activity is defined by its attack on the 1,4-glycosidic bonds of mureins (N-acetyl-2-aminodeoxyglucans) in bacterial cell walls. The center of enzymic activity is divided into six subunits designated A, B,

TABLE XI

Dye Inhibition Parameters

for ADH

dye	E_T (kcal)	(vs. NAD^+)		(vs. EtOH)	
		K_{ii} (μM)	K_{is} (μM)	K_{ii} (μM)	K_{is} (μM)
Methylene Blue	34.3	21	13	20	6
Rose Bengal	44.4	—	46	44	81
Acridine Orange	48.3	36	42	62	52

Figure 59. Rate of inactivation of horse liver alcohol dehydrogenase as a function of sensitizer used. Sensitizers used from low energy to high include: methylene blue, hematoporphrin, rose bengal, erythrosin B, eosin Y, acridine orange, fluorescein, roboflavin and pyridoxal-5-phosphate. Points shown are the average of two runs and are typically ± 0.06 - 0.11 . Enzyme was $3.7\text{mM} \pm 0.3\text{mM}$.

Figure 60. Substrate protection of horse liver alcohol dehydrogenase against rose bengal sensitized photoinactivation at pH 7.0 in 0.2M phosphate buffer.

- - native enzyme
- - + 4mM NAD
- △ - + 5mM ethanol
- - + 5mM NAD and 15uM pyrazole

Figure 61. Variation in the rate of eosin Y sensitized photoinactivation of horse liver alcohol dehydrogenase in protic buffer, deuterated(96%) buffer and the protection provided by 10mM NaN_3 . Enzyme was 3.5mM and eosin Y was 100uM. Illumination was from 25cm.

Figure 62. Protection of horse liver alcohol dehydrogenase against methylene blue photoinactivation(●) by 0.1mM $K_3Fe(CN)_6$ (○), and by 0.5mM $K_3Fe_3(CN)_6$ (●). Studies performed at pH 7.5 in 0.2M phosphate buffer.

TABLE XII

ADH Amino Acid Analysis

before and after

Photooxidation

Residue	Native (%)	Rose Bengal (%)	Acridine Orange (%)
Asp	7.8	9.4	8.5
Ser	15.4	14.2	13.9
Glu	8.5	7.5	9.1
Pro	6.7	5.5	6.6
Cys	.65	1.01	1.2
Gly	13.5	13.1	13.2
Ala	9.9	9.52	9.5
Val	5.8	7.4	6.3
Met	2.8	2.2	2.7
Ile	2.0	2.6	2.5
Leu	6.4	7.2	6.7
Tyr*	2.2	1.9	1.9
Phe*	5.5	5.9	5.3
His*	2.3	2.0	1.9
Lys	7.1	8.9	7.6
Avg	3.2	3.4	3.1

* Photosensitive residues.

C, D, E and F. The hydrolysis of the glucans takes place at sub-site D and is catalyzed by a proton originating from the carboxyl group of a glutamic acid side chain. Three photosensitive tryptophan residues 62, 63 and 108 are also essential for enzymic activity because the saccharide residues are hydrogen bonded to these three side chains (132). The tryptophans have been shown to play an important role in photodynamic reactions (133,134).

For the most part, dye sensitized photooxidations of lysozyme are pH independent even with low energy sensitizers such as methylene blue or rose bengal (figures 63, 64, and 65). This may indicate a lack of interaction between the ionic dyes and any ionizable residues on the enzyme surface. Once again, rates of inactivation versus sensitizer used followed a predictable pattern analogous to the $^1\Sigma_g^+ : ^1\Delta_g$ ratio produced as a function of the triplet-state energy of the sensitizer (figure 66).

Photoinactivation is thought to stem from destruction of one or more essential tryptophans at the active sub-site D which would also help explain the near pH independence of the inactivation rates since tryptophan is not ionic. Indeed, spectral changes have been shown to accompany the modification which resemble spectral changes for L-tryptophan when it is photooxidized (figure 67).

Further support for damage done to the saccharide binding site is provided by protection experiments (figure 68) which shows that MO-NAG₂ suppresses rates of photoinactivation of lysozyme.

Disaccharides such as MO-NAG₂ and others have been shown to form non-productive complexes with lysozyme by difference spectroscopy (135). The inset of figure 67 demonstrates how this ability to form a non-productive complex is altered upon photooxidation. NMR spectroscopy also reveals changes in the aromatic region (probably

Figure 63. pH dependence of riboflavin, acridine orange and fluorescein sensitized photoinactivation of lysozyme. 50($\circ$) and 100($\bullet$) μM dye used in each case. Enzyme was 15mM($\pm$ 2mM). Reactions carried out in 0.2M phosphate buffer.

Figure 64. pH dependence of eosin Y, erythrosin B and PLP sensitized photo-inactivation of lysozyme. 50(o) and 100uM dye(o) used and enzyme was 15mM(± 2 mM). Reactions carried out in 0.2M phosphate buffer.

Figure 65. pH dependence of methylene blue and rose bengal sensitized photoinactivation of lysozyme. In each case 50 μ M dye was employed. Enzyme concentration was typically 15mM($\pm$ 2mM).

Figure 66. Rate of photoinactivation of lysozyme with a series of sensitizers. Sensitizers used from low energy to high include: methylene blue, rose bengal, erythrosin B, eosin Y, acridine orange, fluorescein, riboflavin and pyridoxal-5-phosphate. Rates of inactivation for each of the dyes demonstrated little pH dependence. The points shown are the average of two runs carried out at pH 6.5 and have an associated error of ± 0.06 - 0.09 .

Figure 67. Spectral changes observed when lysozyme is photooxidized with rose bengal (40 μ M). Photooxidation was performed at pH 7.0 in 0.2M phosphate buffer.

(o) - native enzyme
 (◐) - enzyme reduced to 50% residual activity by photooxidation
 (●) - enzyme reduced to 17% residual activity by photooxidation.
 Rose bengal was removed prior to spectral recording by treatment with cellex-T anion exchanger. Inset shows spectral changes when l-tryptophan(o) is photooxidized with rose bengal for 10 minutes (30 cm).

Figure 68. Substrate protection and spectral changes encountered when lysozyme is photooxidized with methylene blue at pH 6.5.(▲). Protection is provided in the presence of 0.35mM MO-NAG₂(○) and 0.9mM MO-NAG₂(●). Inset shows changes in the difference spectra of native lysozyme before(—) and after(---) treatment(photooxidation) with methylene blue to a residual activity of 15%. Difference spectra with MO-NAG₂ according to reference 135.

tryptophan) after photooxidation. Broad peaks at ~ 7.75 – 8.0 ppm appear removed or hidden by an upfield shift after modification (figure 69).

Four enzymes then, have been studied for their behaviour towards dye-sensitized photooxidation. Each enzyme is photoinactivated in a predictable manner depending on the dye used. The implication of these results in light of the involvement of singlet oxygen is reserved for the discussion section.

Figure 69. Proton N.M.R. of lysozyme before (A) and after (B) photooxidation. Residual enzyme activity was 15% when (B) was recorded. Inset shows resonance peaks of some L-amino acids in this region.

DISCUSSION

The goal of a study of enzyme catalysis is to provide an assignment of the various structures or interactions responsible for the kinetic behavior of the protein. In the following section we attempt to define the kinetic pathways responsible for catalysis performed by glutathione reductase.

Kinetic Properties of Glutathione Reductase

Steady state kinetic studies of glutathione reductase in the forward direction indicate that a Ping Pong mechanism is operating under conditions of low substrate concentration (figure 9). This condition appears to hold over the pH range 5.0-8.0. However, product inhibition studies are in conflict with an existing Ping Pong mechanism.

Staal et al (64) in studying the erythrocyte enzyme found that ⁺NADP was competitive with NADPH and non-competitive towards GSSG. Since this was in conflict with predicted inhibition patterns for a binary complex mechanism, the authors proposed that the mechanism was mixed, ordered sequential and binary complex (64,65). These results have been confirmed by Mannervik (66). It is claimed that the sequential mechanism applies at high concentrations of GSSG and that the binary complex (Ping Pong) mechanism applies at low GSSG concentrations (136). Our results seem to apply to the latter condition and the positive inflections obtained from the pH dependence of K_a/V is associated with an ionizing group of pK 5.8-6.0. This cationic residue is believed to be a histidine and its presence at the active site of the erythrocyte enzyme has been verified by X-ray analysis (54,55).

Active site histidine in Glutathione Reductase

Other workers support the involvement of histidine in

catalysis and propose an additional role in the stabilization of the nascent thiolate anion generated during the catalytic cycle (40,137). However, our N.M.R. results indicate that if an ion-pair exists (figures 13,14) it is very weak at least relative to lipoamide dehydrogenase (figure 11) since little upfield shift in histidine resonances occurs upon formation of EH_2 with glutathione reductase. Templeton (16) has also demonstrated that glutathione reductase possesses a weak MCD Faraday A term compared to lipoamide dehydrogenase. It was later shown that this Faraday A term arose from interaction of a thiolate anion and a protein residue, later identified as a histidine. The weak Faraday A term obtained with 2-electron reduced glutathione reductase may reflect a weaker thiolate to histidine ion pair. The N.M.R. work would tend to support this.

The weaker thiolate to histidine but strong thiolate to flavin interaction in glutathione reductase is supported by the higher extinction coefficient of the charge transfer absorption band at 530 nm characteristic of the 2-electron reduced form of the enzyme (figure 15). Singer et al (46) have also demonstrated that alkylation of 2-electron reduced glutathione reductase causes small spectral changes in the EH_2 form of the enzyme. This is in marked contrast to the complete loss of charge transfer interaction observed with lipoamide dehydrogenase indicating again perhaps a weaker thiolate to flavin charge transfer in lipoamide dehydrogenase.

Additional support of an efficient thiolate-flavin interaction in glutathione reductase can be seen from the kinetic investigation of this enzyme. In general, the non-physiological reactions proceed with a suppressed catalytic efficiency (V/E_T) compared to

lipoamide dehydrogenase. A reduced catalytic efficiency could be due to a number of factors one of which is the availability of the enzyme form with which the substrate reacts (FADH_2 or $\text{FADH}^\cdot$).

Of the reactions catalyzed by these two flavoproteins, only the physiological reductase reaction proceeds through an intermediate EH_2 species where the disulfide is reduced. The other non-physiological reactions proceed through alternative EH_2 species ($\text{FADH}^\cdot$ or FADH_2 , 102, 103). An efficient charge transfer interaction between the thiolate anion and the flavin may lower the reduction potential of this disulfide and decrease the population of these alternative EH_2 species. This would reduce the catalytic efficiency of these reactions, other parameters (K_a , K_b) being equal.

Evidence for histidine being catalytically important is derived from photooxidation data which demonstrate destruction of histidines (amino acid analysis of photoinactivated enzyme shows destruction of 9 histidines and 1 methionine). The rate of inactivation is associated with a protein residue having a pK 5.8-6.4 (figures 47,48) in close agreement with the pK observed in the pH dependent kinetic studies of glutathione reductase (figure 9). Loss in activity is associated with a loss in ability of this histidine to function as an acid catalyst (138) (figure 70).

Other cationic residues that may serve the role as an acid catalyst include lysine and arginine. Phenylglyoxal and 1,2-cyclohexanedione fail to alter enzyme multifunctionality which suggests that arginine is not involved in the catalytic cycle of the yeast enzyme. The opposite result has been found in the erythrocyte enzyme. The mammalian enzyme has also been inactivated by ethoxyformic anhydride modifying 1-1.5 histidines (128). This

Figure 70. Proposed catalytic cycle of erythrocyte glutathione reductase, from Schultz([38).

modification was ineffective towards the yeast enzyme demonstrating a significant difference between the yeast enzyme and those from mammalian sources.

Modification of glutathione reductase with TNBS diminishes reductase activity of the yeast enzyme to about 15-20%. Other activities are marginally affected. If lysine were the cationic residue involved during catalysis then modification should reduce enzyme activity to near zero. However, cationic lysine(s) may serve as a binding residue for anionic glutathione. This is currently under investigation in our lab. Approaches include a kinetic investigation of the reductase reaction after modification in order to assess changes in kinetic parameters. We are also preparing the methyl ester of glutathione which should disrupt electrostatic interaction with cationic lysine if such interactions previously existed. This should render the ester a poorer substrate than the free acid. Other lysine modifying reactions included pyridoxylation with PLP and amidation with methyl and benzylacetimidate all of which had a minimal effect on enzymic activity. No spectral changes of the enzyme accompanied these reactions. It is possible under the conditions employed (materials and methods) that modification did not occur. Further investigation of these reactions was not undertaken.

Nucleotide binding site

Outside the presence of a flavin, an active site disulfide and a histidine, little additional information is available on amino acids near or around the active site influencing either catalysis or binding of substrates in lipoamide dehydrogenase. However, monitoring of enzymic activity during chemical modification provides an indirect means of obtaining such information. To date

no information is available on the residue(s) responsible for the binding of NADH, the first step in the catalytic cycle.

Electrostatic interaction between the guanidine moiety of cationic arginine and the anionic phosphate of the nucleotide would be a reasonable choice for a residue present at the nucleotide binding site. Templeton (16) has shown that phenylglyoxal and 1,2-cyclohexanedione do not alter enzyme activity in lipoamide dehydrogenase. Similar results have been obtained for yeast glutathione reductase. However, modification with NBS eliminated all multifunctional activity from both flavoproteins. Since initial binding of NAD(P)H is required for each reaction catalyzed by these enzymes (Scheme 2), it was assumed that the nucleotide binding site of these enzymes had been affected. Tryptophan being the main target with this reagent is implicated as one of the essential residues occupying its nucleotide domain. Tyrosine may have the same role since modification with TNM produced a similar result. The nucleotide NADP^+ protected glutathione reductase against the modification supporting interaction of TNM with the nucleotide binding site. These two residues are thought to provide base stacking interactions with the incoming nucleotide and this has been supported in model systems (III). Difference spectroscopy and attempts to form the half reduced EH_2 species support damage done to the nucleotide binding domain.

Lipoamide dehydrogenase and Glutathione reductase (A comparison)

Lipoamide dehydrogenase and glutathione reductase have both mechanistic and structural properties in common. Both flavoproteins contain a cystine residue which undergoes reduction-oxidation during catalysis. In catalysis, both cycle between the oxidized and two-electron reduced (EH_2) forms. Both enzymes also share a high

degree of homology around this active site cystine (43).

Based on chemical modification studies on the 2-electron reduced enzymes, the thiol nearer the amino terminus of each protein interacts with the respective substrate in thiol-disulfide interchange while the other thiol interacts with the FAD (46,153). Unlike Williams et al (40) however, we do not feel the nascent thiols in EH_2 of these enzymes share a mechanistic and structural equivalence.

N.M.R. of lipoamide dehydrogenase displays an upfield shift in its histidine resonance when reduced to the EH_2 form with NADH. This indicates a significant thiolate-histidine ion pair may exist in EH_2 providing electrostatic stabilization to the generated thiolate. This idea has been supported by the appearance of a Faraday MCD A term when reduced to EH_2 . This A term is thought to arise from the same thiolate-histidine ion pair (16, 52, 53). However, the existence of such an ion-pair in glutathione reductase is less convincing. Proton N.M.R. does not display the upfield shifts seen in lipoamide dehydrogenase. The MCD A term is also very weak. Instead, a stronger thiolate-FAD interaction exists in glutathione reductase since it has a much higher ($1000\text{--}1500 \text{ M}^{-1} \text{ cm}^{-1}$) extinction coefficient at 530 nm in EH_2 which arises from such an interaction. The histidine residue in glutathione reductase would appear to have a more catalytic role than in providing electrostatic stability to the nascent thiol.

Both flavoproteins appear to possess a carboxyl group(s) near the active site flavin that has a major influence on the ETase reaction. Modification of the carboxyl with ethylene diamine increases the activity of this reaction 6-7 fold over control runs (figure 37). The one electron transfer reaction (ETase) catalyzed

by the flavoprotein proceeds via a semiquinone radical intermediate (41) and the carboxyl being anionic is believed to function as a destabilizer of this radical EH_2 species.

Arginine does not appear to have a significant role at the nucleotide binding site of either flavoprotein. Instead the aromatic amino acids tryptophan and tyrosine are argued to be the residues responsible for the binding of the nucleotides NADH and NADPH to the enzymes. Upon chemical modification of these residues, such a postulate would require a significant decrease in all activities catalyzed by the flavoproteins since initial binding of the nucleotide is the first step in each reaction. This was found to be the case.

The role of histidine in each flavoprotein appears to differ and has already been discussed in some length. Modification of lysine in lipoamide dehydrogenase does not significantly alter any of the activities but TNBS modified lysine in glutathione reductase reduces the reductase activity of this enzyme to about 15-20%. Lysine is believed to have a role in binding anionic glutathione and this is currently under further investigation.

In general, the active site components of both flavoproteins are quite similar but reveal some differences. Both contain a flavin, redox-active disulfide and a catalytic histidine although the role of this histidine is not the same in each enzyme. Evidence has been provided by N.M.R. and MCD (16,26) that the histidine in glutathione reductase functions more as an acid catalyst than in providing additional electrostatic stability to the generated nascent thiol in EH_2 . The latter has been demonstrated in lipoamide dehydrogenase. The more efficient thiolate-flavin interaction in glutathione reductase supports this view. Both

enzymes also possess aromatic residues (tryptophan and tyrosine) apparently crucial to the nucleotide binding domain. Lipoamide dehydrogenase differs from glutathione reductase, however, in that the former appears to have two nucleotide binding sites, the latter only one. Both enzymes also possess a carboxyl group (2) capable of suppressing formation of radical semiquinone. Both enzymes catalyze reactions according to the same overall kinetic scheme (Scheme three). However, non-physiological reactions catalyzed by glutathione reductase proceed at a reduced catalytic efficiency compared to lipoamide dehydrogenase and this may be linked to the stronger thiolate-flavin interaction in glutathione reductase.

Photooxidized enzyme

There are now several biochemical systems in which the evidence appears reasonable for singlet oxygen (1O_2) involvement. Photosensitized oxidations can result in several types of cellular damage as reviewed recently (139). Numerous examples exist but often little mention is made of the particular species of singlet oxygen ($^1\Sigma_g^+$ or $^1\Delta_g$) involved in the photodynamic process.

In most cases it would appear that $^1\Delta_g$ rather than $^1\Sigma_g^+$ is the reacting species. This is to be expected on the basis of physical quenching studies which shows that $^1\Sigma_g^+$ is collisionally deactivated, whereas $^1\Delta_g$ is relatively immune to collisional quenching (142,143). Therefore, simply on the basis of lifetimes, $^1\Sigma_g^+$ is not expected to survive long enough to react in a condensed phase (lifetime $\sim 10^{-10}$ sec). Additional experimental evidence against the involvement of $^1\Sigma_g^+$ has been provided by Gollnick's study (144). He used a wide variety of photosensitizers to effect singlet oxygen reactions with acceptors which formed more than one type of oxygenation product and in this way hoped to see whether or not there was a change in

product distribution when sensitizers of varying triplet energy were used. For a given acceptor, there was no variation in product distribution regardless of whether the triplet state energy of the sensitizer was above or below the minimum energy required to produce $^1\Sigma_g^+$ (37 kcal). Another reason for believing that $^1\Sigma_g^+$ is not involved in most photooxygenation reactions is that it cannot, for theoretical reasons, enter into a concerted addition reaction, and yet most singlet oxygen reactions do appear concerted (145,146,147). However, Kearns et al (121) demonstrate results opposite to those obtained by Gollnick. Photooxidation of cholest-4-en-3 β -ol gave rise to two products whose ratio varied as much as 30:1 to 1:5 depending on the sensitizer used. Since a dramatic change in the product ratio occurs when the triplet state energy of the sensitizer falls below 45 kcal, it was proposed that both $^1\Sigma_g^+$ and $^1\Delta_g$ were involved as reactive intermediates and that the variation in product distribution with sensitizer used resulted from a variation in the relative amounts of $^1\Sigma_g^+$ and $^1\Delta_g$ generated.

We have conducted a similar study on four enzymes and in each case have shown that rates of inactivation depend mainly on the sensitizer used. Rate enhancement in D₂O and rate suppression by NaN₃ support involvement of singlet oxygen as a possible intermediate responsible for enzyme inactivation. Results tend to indicate that low energy sensitizers ($E_T < 45$ kcal) are effective because they generate a larger population of $^1\Delta_g$ which is more reactive and has a longer lifetime than $^1\Sigma_g^+$ towards the unsaturated side chains of proteins. The observed variation in rates of enzyme inactivation may arise from the relative amounts of $^1\Sigma_g^+$ and $^1\Delta_g$ generated with the sensitizer chosen. If $^1\Sigma_g^+$ is generated it is not likely to be involved because of earlier arguments. In retrospect

it appears that if evidence for $^1\Sigma_g^+$ reactions is to be found, it will undoubtedly come from gas-phase experiments where $^1\Sigma_g^+$ will have a longer lifetime.

In closing our results demonstrated that effective photoinactivation of the enzymes studied occur when low-energy sensitizers are used ($E_T < 45$ kcal). High energy sensitizers cause little inactivation although their effectiveness is improved in D_2O . Trends with sensitizer used parallel ratios of $^1\Sigma_g^+ / ^1\Delta_g$ generated with the triplet state energies of the same sensitizers (121). If $^1\Sigma_g^+$ is being generated it would not appear to be very reactive (lifetime $< 10^{10}$ sec) or has collapsed to the ground state through collisions before it has a chance to react. The lower energy $^1\Delta_g$ would appear to be responsible for the inactivation although the system may be complicated by competing radical reactions since Fe^{3+} has been shown to provide protection against photoinactivation. However, if radicals were involved, there formation (via hydrogen abstraction) should proceed independently of oxygen concentrations. However, rates of photoinactivation are decreased when solutions of enzyme and dye are purged with N_2 . So, an apparent inconsistency still remains concerning the exact mechanism of enzyme photoinactivation. More work in this area will contribute to an understanding of the photoinactivation process with enzymes and should contribute to the study of singlet oxygen chemistry in general.

CONCLUSION

It is hoped that this thesis has demonstrated how the protein environment around the flavin can influence its chemistry. Chemical modifications lead to perturbations of the flavin environment and the resultant changes (if any) in the multifunctionality of the flavoprotein can provide insight into the residues necessary for a given activity.

The homologies between the two flavoproteins, lipoamide dehydrogenase and glutathione reductase indicate an evolutionary relationship exists between the two and the pair have probably diverged slowly. Yet, interesting differences do exist and further work will hopefully reveal more.

The photooxidation study remains somewhat unsettling. Evidence appears in the literature for and against the involvement of $^1\Sigma_g^+$ in dye sensitized photooxidation. Other methods exist to generate singlet oxygen (figure 71) and perhaps they could be looked at to help solve the dilemma (120). However, the results do demonstrate a significant difference in the effectiveness of certain dyes to photoinactivate enzymes and should provide protein chemists with an added rationale in choosing a particular sensitizer.

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