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**THE NAD⁺ AND NADP⁺-LINKED ACTIVITIES OF ALCOHOL
DEHYDROGENASES: ISOLATION, CHARACTERIZATION
AND MECHANISTIC STUDY**

by

Loola S. Al-Kassim, B.Sc.

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

Doctor of Philosophy

Department of Chemistry
Carleton University
Ottawa, Ontario
28/8/89

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Submitted by Lcola S. Al-Kassim

in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Chairman, Chemistry Department

Thesis Supervisor

External Examiner

Carleton University
1989

TO IHSAN, ALI, NADIA AND NAMIR

ABSTRACT

Alcohol dehydrogenases were purified to electrophoretic and chromatographic homogeneity from rat liver, pickerel fish liver, and thermophilic bacteria *Thermoanaerobium brockii*. Purification of these enzymes employed a common procedure that included anion exchange, gel filtration and affinity chromatographies. Partial characterization and a comparison of physical, catalytic and mechanistic properties is made with respect to horse liver and baker's yeast alcohol dehydrogenases.

Alcohol dehydrogenases (rat and fish) are both dimers with molecular weights similar to horse liver alcohol dehydrogenase. These two enzymes display multifunctional activities. The alcohol dehydrogenase from rat and fish liver have an aldehyde oxidizing and esterolytic activities in addition to alcohol oxidizing activity. Non-mammalian alcohol dehydrogenase isolated from *Thermoanaerobium brockii* on the other hand exists as a tetramer and shows only the oxidative activity toward alcohols similar to yeast alcohol dehydrogenase.

Alcohol dehydrogenases from eukaryotic organisms are mainly NAD⁺ specific, while those from prokaryotic organisms are either NAD⁺ or NADP⁺ dependent. Purified alcohol dehydrogenases from animal and bacteria differ in their coenzyme as well as their substrate specificity.

Thermoanaerobium brockii alcohol dehydrogenase showed a preference for NADP⁺ and secondary alcohols. Rat and fish

liver alcohol dehydrogenases exhibit preference for NAD^+ and primary alcohols. The resemblance in physical properties, substrate and coenzyme specificity of rat and fish liver alcohol dehydrogenase to horse liver alcohol dehydrogenase may indicate evolutionary connection. The fish liver alcohol dehydrogenase showed preference for NAD^+ , but contrary to horse liver alcohol dehydrogenase, it can use either NAD^+ or NADP^+ almost equally as a coenzyme for alcohol oxidizing activity.

Steady state kinetic analyses of fish liver alcohol dehydrogenase revealed that NAD^+ and NADP^+ -linked activities are catalyzed by two different mechanisms. The NAD^+ -linked activity proceeds by Steady-State Random BiBi mechanism, whereas the mechanism of NADP^+ -linked activity is Rapid Equilibrium Random BiBi with dead-end ternary complex. The different mechanisms influence ways in which NADP^+ -linked activity is controlled and may correlate with the different physiological requirements for NAD^+ and NADP^+ .

Kinetic analysis of bacterial alcohol dehydrogenase indicate that oxidation of secondary alcohol in presence of NADP^+ proceeds by an Ordered BiBi mechanism with NADP^+ as leading substrate. Steady State behaviour of NAD^+ -linked activity of this bacterial enzyme is inconsistent with a simple ordered mechanism and exhibits a higher degree of dependence on coenzyme concentration. A high association constant for enzyme- NADP^+ binary complex obtained by ^{31}P -NMR and higher turnover rate for NADP^+ -linked reaction

contribute to the preference of *Thermoanaerobium brockii* alcohol dehydrogenase for NADP⁺ as a coenzyme.

In comparison to the known effects of certain chemical modifications on horse liver alcohol dehydrogenase, these same modifications were performed on rat, fish and bacterial alcohol dehydrogenase, in an effort to identify those amino acid residues that are essential for catalysis. The differential effect of chemical modifications on oxidative and esterolytic activities in rat and fish liver enzymes implicates these activities are not associated with same active site regions. Cysteine and histidine residues were found to be essential for the NAD⁺ and NADP⁺-linked activities of rat, fish and bacterial alcohol dehydrogenases. Arginine modification was detrimental to the NADP⁺-linked activity in rat liver alcohol dehydrogenase and is in agreement with the importance of arginine residue(s) to horse liver alcohol dehydrogenase activity. Marginal effects of arginine modification on the NAD⁺ and NADP⁺-linked activities in fish liver and bacterial alcohol dehydrogenases was observed. It suggests arginine residues in the active site are not accessible for modification, and those arginine residue(s) that were modified are not involved in coenzyme binding. This conclusion is supported by ³¹P-NMR analysis of α -phosphate resonance in the presence of phenylglyoxalated enzymes.

Activation of NADP⁺-linked activity in fish liver alcohol dehydrogenase is not caused by amidation of a

specific active site carboxylic group(s). This activation is likely induced by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). Incorporation of radioactive amine and ^{31}P -NMR analysis of α -phosphate resonance in presence of amidated fish liver alcohol dehydrogenase support the EDC involvement. The same argument regarding EDC's role in amidation of the bacterial alcohol dehydrogenase is supported by ^{31}P -NMR analysis of modified enzyme.

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ABBREVIATIONS

ac Py-AD(P) ⁺	3-acetyl pyridine adenine dinucleotide (phosphate), oxidized form
ADH	Alcohol dehydrogenase(s)
ATP	Adenosine triphosphate
AG-NAD ⁺	Agarose-hexane NAD ⁺ , type 1 and 3 affinity gel
AG-NADP ⁺	Agarose-hexane NADP ⁺ , type 3 affinity gel
Å	Angstroms
Bis	N, N'-methylene-bis-acrylamide
BSA	Bovine serum albumine
cm	centimeter
CM-52	carboxymethyl cellulose
DCl	Deuterium chloride
DEAE	Diethylamino ethyl ion exchange
Deamino-NAD ⁺	Deamino adenine dinucleotide (analogue of NAD ⁺)
DHase	Dehydrogenase activity
Dmase	Dismutase activity
D ₂ O	Deuterated water
DTE	Dithiocerythritol (Cleland's reagent)
DTT	Dithiothreitol (Cleland's reagent)
EDC	1-Ethyl-3-(3-dimethylamino propyl) carbodiimide
Esase	Esterase activity
E _t	Total enzyme concentration
EtOH	Ethanol
FLADH	Fish (pickerel) liver alcohol dehydrogenase

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G6PD	Glucose-6-phosphate dehydrogenase
g	gram
xg	centrifugal force (x unit gravitational field)
HLADH	Horse liver alcohol dehydrogenase
hν	Light radiation
K_{av}	Relative partition coefficient (gel filtration)
L	Litre
LDH	Lactate dehydrogenase
M	Molar
MES	2- (N-morpholino)-ethansulfonic acid
MW	Molecular weight
NAD(P)⁺	Nicotinamide adenine dinucleotide (phosphate), oxidized form
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced form
NaOD	Sodium deuteroxide
NaPB	Sodium phosphate buffer
NBT	Nitro blue-tetrazolium [2,2' di-nitrophenyl-5, 5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride]
NMN	Nicotinamide mononucleotide
NPO	p-nitrophenyl caprylate (octanoate)
NMR	Nuclear magnetic resonance
PEG	Polyethyleneglycol
pI	Isoelectric point
PMS	Phenazine methosulfate (N-Methyl-phenazolum methyl sulfate)

PyA-AD ⁺	3-Pyridine aldehyde adenine dinucleotide
R _r	Relative distance
RLADH	Rat liver alcohol dehydrogenase
SDH	Sorbitol dehydrogenase
SDS	Sodium dodeccyl sulfate
S-MDH	Soluble malate dehydrogenase
Ta.Brockii	<i>Thermoanaerobium brockii</i>
TBADH	<i>Thermoanaerobium brockii</i> alcohol dehydrogenase
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylene diamine
TNM	Tetranitromethane
Thio-NAD(P) ⁺	Thio (analogues of NAD(P) ⁺)
tris	Tris (hydroxymethyl) amino methane
U	Enzyme activity unit
UV	Ultraviolet radiation
v	Initial reaction rate (velocity)
V _{max}	Maximum velocity
V _{max} /E _t	Turnover number
WSC	Water soluble carbodiimide
YADH	Yeast alcohol dehydrogenase
	Wavelength (in nanometers)
δ	Chemical shift (in ppm)

CHAPTER ONE

INTRODUCTION

More than two hundred and fifty dehydrogenases that employ nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate (NADP^+), or both for the transfer of redox equivalents to various metabolites are known. One tenth of this number has been studied kinetically to give sufficient information regarding their mechanism. Of these dehydrogenases, alcohol dehydrogenase has been under extensive investigation, thus there has been a great deal of advance in the knowlegde, especially of horse liver enzyme. Historically, yeast alcohol dehydrogenase (YADH) from brewer's yeast was the first pyridine nucleotide dependent dehydrogenase to be crystallized by Negelin and Wulff in 1943 (1). In 1948 Bonnichsen and Wassen (2) isolated the second alcohol dehydrogenase in crystalline form from horse liver. Besides these two enzymes which were obtained in pure state, the alcohol dehydrogenase (NAD^+ : alcohol oxidoreductases, EC 1.1.1.1, ADH) has a widespread occurrence in nature. The enzyme occurs in a variety of organisms (3) including different types of bacteria, yeasts, fungi, insects, fishes, amphibians, birds and mammals (4). Apart from horse liver and yeast enzymes, the alcohol dehydrogenase has been partially purified and characterized from other sources, including rainbow trout (5), chicken

liver (6), wheat germ (7), cotton seeds (8), peanuts (9), thermophilic bacteria (10), insects, as well as many others (4).

Estimation of functional, structural similarities, and evolutionary correlations between different alcohol dehydrogenases and dehydrogenases in general, have been implicit in comparisons of amino acid sequences aligned on the basis of essential amino acid residues such as cysteine and other physical properties. The complete amino acid sequences of the horse liver enzyme, rat liver enzyme (except few uncertain positions), and partial sequences of proteins from other sources had been determined. Knowledge of the horse liver alcohol dehydrogenase (HLADH) tertiary structure as well, determined by X-ray diffraction had provided the firm ground for the elucidation of functional and evolutionary relationships (4, 11).

The liver alcohol dehydrogenase belongs to major pathway of alcohol metabolism and catalyzes the oxidation of various primary and secondary alcohols to the corresponding aldehydes and ketones (Scheme 1).

Scheme 1. Oxidation of alcohols by LADH

In addition to its normal dehydrogenase activity, horse liver alcohol dehydrogenase (HLADH) exhibits multifunctional activities (12, 13). It catalyzes irreversible dismutation of aldehydes to the corresponding carboxylic acids in presence of NAD^+ . While optimal primary alcohol oxidation occurs with n-butanol for this enzyme, longer chained aldehydes are preferred for dismutation (14) with optimal activity occurring at octanal.

The enzyme also catalyzes an NAD^+ -independent hydrolysis of esters, namely the hydrolysis of p-nitrophenylesters (15). Three reactions catalyzed by HLADH are outlined in Scheme 2.

Scheme 2. Physiological role of HLADH

It has been established that the physiological role of HLADH is mainly described by its dehydrogenase activity. Its *in vivo* participation in aldehyde dismutation and esterolysis is negligible (16). Due to its wide ranging sociological as well as physiological ramifications, ethanol metabolism has been intensively studied. Alcohol dehydrogenase has been the focus of these studies at the biochemical level. The horse liver alcohol dehydrogenase

(HLADH) is an NAD⁺-dependent enzyme that provided a superb example for collaborative interactions between protein chemists and kineticists.

Three main isozymes of HLADH are formed by the dimeric combination of two different types of protein chains called E for "ethanol active" and S for "steroid active", based on a difference in substrate specificity and six amino acid residues. Commercially active enzyme form has a molecular weight 84,000 daltons and is a dimer of two identical subunits (the ethanol active form "EE"). The two subunits are joined by a homologous interactions within regions of the coenzyme binding domain (4). Each subunit consists of two domains: a coenzyme binding domain of 143 residues and a larger catalytic domain comprising 231 residues. The two domains are joined by a narrow neck region and separated by a deep active site cleft. This cleft accomodates the substrate and nicotinamide moiety of the coenzyme (17). The catalytic domain firmly binds two zinc atoms; one of these ions plays a structural role. The other, called "active site zinc" plays a role in the oxidation-reduction reactions as a Lewis acid.

The sequence of the 374 amino acids in the single polypeptide chain of one subunit of the E isozyme has been determined by Jornvall (18). The X-ray structure of the native enzyme (EE isozyme), in the open form has been determined by Eklund et al. to 0.24nm resolution (19).

Coenzyme binding induces a conformational change of the enzyme from open to closed form (20). The structures of the enzyme complexes with NADH and dimethylsulfoxide (21), NAD⁺ and pyrazole (22), NAD⁺ and bromobenzyl alcohol (23), and tetrahydro-NAD⁺ and dimethylamino-cinnamaldehyde (24), are all in the closed form has been determined to 0.29nm resolution. Furthermore, the binding of a number of coenzyme analogues and inhibitors have been studied in isomorphous complexes of this structure (25 and refernces therein). These crystals are orthorhombic, on the other hand, ternary complexes with coenzyme substrate or most inhibitors are either monoclinic or triclinic (21).

The structure of dimeric HLADH molecule is presented in Figure 1.1. The coenzyme binding domain of the subunit consists of the residues 176 to 318. Within this domain, the amount of secondary structure is considerable: 45% of the residues are helical, mainly α -helices, 32% are in pleated-sheet structure and 13% in reverse bends. Residues that have no regular secondary structure constitute only about 10%, and no continuous stretch of irregular structure is longer than four residues (4, 19).

The basic element in the coenzyme binding domain is the six parallel stands β A- β f surrounded by the helices α A- α E and 3¹⁰S consecutively from the amino end in a regular pattern. The physical sequence of strands from top to bottom of the sheet is C-B-A-D-E-F (4, 11, 28). A schematic diagram

Figure 1.1. Schematic diagram of the polypeptide fold of HLADH dimer designed by Bo Furugen. One subunit is shaded. The central part of molecule is the two coenzyme-binding domains bound together across a two fold axis perpendicular to the plane of the paper. The two catalytic domains are separated from this central part by two clefts. Shaded circles are the four zinc atoms (22).

of the fold of polypeptide chain in the domain is shown in Figure 1.2. Unique folding of the nucleotide binding domain creates a specific crevice for binding a dinucleotide molecule. Coenzyme molecule binds in this crevice with the AMP part in the region above helix αE and the NMN part in the region below αB (3).

The structures at high resolution, where individual polypeptide chains and side groups can be accurately positioned, are available for few dehydrogenases: dogfish lactate dehydrogenase LDH (29-31), pig soluble malate dehydrogenase s-MDH (32, 33), horse LADH (28, 34) and lobster glyceraldehyde-3-phosphate dehydrogenase GAPDH (35, 36). They revealed remarkable similarity in the structures of those parts of the polypeptide chain whose function is to bind the NAD^+ coenzyme, although the complete subunit conformations are not alike. The same general polypeptide fold built by arrangement of strands has been reported in the ATP-binding domain of muscle phosphoglycerate kinase (35, 37). Although the coenzyme-binding domains of HLADH, LDH, MDH and GAPDH exhibit fundamental similarities in their substructures as well as the conformation, orientation, and position of the bound coenzyme on the protein, catalytic domains on the other hand which bind different substrate molecules, have different three-dimensional structures.

Figure 1.2. Schematic diagram illustrating the fold of polypeptide chain within the coenzyme binding domain of HLADH and the nomenclature of the elements of secondary structure (4).

The HLADH binds one coenzyme molecule (NAD^+) per subunit. Occupancies of coenzymes at the two sites, the conformations of the two coenzyme molecules, and their interactions with the protein subunits are very similar (21). The coenzyme molecule remains anchored to the protein when the nicotinamide ribose moiety is tightly bound between the two domains of the subunit by specific hydrogen bonding. In HLADH, the coenzyme molecule has a number of specific interactions that give hydrogen bonds from oxygen atoms of the phosphate and the two ribose moieties to the side chains of Asp-223, Arg-47, Ser-48, His-51, Arg-369, and many other main-chain atoms of the enzyme (17, 19, 38). These interactions are shown schematically in Figure 1.3. The catalytic and coenzyme binding domains of a subunit in HLADH as mentioned previously are separated by a cleft containing a deep pocket lined almost entirely by hydrophobic residues (4). The catalytic zinc is bound at the bottom of this pocket to three protein ligands, two sulphur atoms from Cys-46 and Cys-174 and one nitrogen atom from His-67. The three protein ligands are arranged in three corners of a distorted tetrahedral arrangement. The fourth corner of this distorted tetrahedron points into the substrate-binding pocket (17). A water molecule or hydroxyl ion depending on pH or, if present, a bound inhibitor or substrate completes the tetrahedral coordination in both open and closed forms of the enzyme (19, 28). A water molecule is involved in a

Figure 1.3. Schematic representation of interactions from the coenzyme (NAD⁺) and substrate to the enzyme molecule HLADH.

system of hydrogen bonds which include Ser-48 and His-51 (Figure 1.3). These hydrogen bonds are important for proton release upon NAD⁺ binding (39) and for substrate polarization in the catalytic mechanism (21, 34). The X-ray crystallographic studies indicate that the residue His-51 participate in a proton relay system during enzymatic catalysis (23, 40, 41).

The preliminary X-ray structures of lactate, malate, alcohol, and glyceraldehyde-3-phosphate dehydrogenases provided vital information as to the general nature of NAD⁺-enzyme interactions (11, 42). These proteins have certain important invariant residues (20, 21). Other specific interactions also show a number of similar features seen in Table 1.1. One intriguing similarity is the structurally equivalent aspartic acid residue (Asp-223 in HLADH) that is also chemically identical in the coenzyme binding domains of the above known dehydrogenases. This residue hydrogen bonds to O-2' hydroxyl of the adenosine ribose (21). The conservation of this particular residue in HLADH, yeast alcohol dehydrogenase (YADH) and fourteen other alcohol dehydrogenases as well as one sorbitol dehydrogenase demonstrates the importance of this residue (4, 43). It has been suggested that a high degree of NAD⁺ specificity in HLADH, YADH, LDH, GAPDH, MDH and other dehydrogenases is a consequence of the preservation of such groups as Asp-223 (11).

Unlike the electrostatic attraction exhibited by the adenosine ribose moiety of NAD^+ and the carboxyl oxygen of Asp-223, the repulsive interactions of such acidic groups with the 2'-phosphate of NADP^+ provide an explanation as to the NAD^+ specificity of above enzymes. Matthews et al. have identified an arginine residue in the active site of dihydrofolate reductase. This arginine species provides an attractive interaction with the 2'-phosphate of NADPH (44) and provides an explanation for the unique specificity of this enzyme for NADPH rather than NADH .

The mammalian alcohol dehydrogenase from horse liver (HLADH), remains to be the only alcohol dehydrogenase that has been analyzed crystallographically (19), and studies included a number of complexes, both in the presence and absence of NAD^+ . Later the structural studies of other alcohol dehydrogenases were extended in different directions. The amino acid sequence of baker's yeast enzyme (YADH) was shown to be distantly related to HLADH (45, 46), as well as differences in their quaternary structure and enzymatic efficiency. However, HLADH and YADH have clearly (though distantly) related three dimensional structure (43, 47). Furthermore, alcohol dehydrogenases have been found to be related to polyol dehydrogenases (48, 50). Jörnvall et al. in their comparison (43) aligned sixteen characterized alcohol dehydrogenases and one sorbitol dehydrogenase (SDH). The proteins used represent three different types of

Table 1.1

Enzyme-coenzyme interactions in LDH, LADH and G3PDH. Underlined residues on the same line are structurally equivalent according to the alignment by Rossmann *et al.*, (11).

Coenzyme moiety	LDH	Residues LADH	GAPDH	Type of contact
Adenine	<u>Val 27</u>	<u>Phe 198</u>	<u>Asn 6</u>	Interior of pocket
	<u>Val 52</u>	<u>Val 222</u>	<u>Asn 31</u>	---
	<u>Asp 53</u>	<u>Asp 223</u>	<u>Asp 32</u>	H-bond to ring N
	<u>Val 54</u>	<u>Ile 224</u>	<u>Phe 34</u>	Hydrophobic
	<u>Tyr 85</u>			H-bond to ring N
	<u>Ile 96</u>			Hydrophobic
	<u>Ala 98</u>	<u>Ile 269</u>	<u>Thr 96</u>	---
	<u>Ile 119</u>		<u>Phe 99</u>	---
	<u>Ile 123</u>			---
		<u>Arg 271</u>		H-bond to N6
Adenosine ribose	<u>Gly 28</u>	<u>Gly 199</u>	<u>Gly 7</u>	
	<u>Asp 53</u>	<u>Asp 223</u>	<u>Asp 32</u>	H-bond to O-2'
	<u>Lys 58</u>	<u>Lys 228</u>	<u>C=O 8</u>	H-bond to O-3'
	<u>Asp 30</u>			H-bond to O-3'
	<u>Met 55</u>	<u>Ile 200</u>		Hydrophobic
			<u>Leu 187</u> <u>Pro 188</u>	Hydrophobic from adjacent subunit
Pyrophosphate	<u>N 31</u>	<u>N 202</u>	<u>N 10</u>	H-bond to main chain
	<u>N 32</u>	<u>N 203</u>	<u>N 11</u>	---
	<u>Arg 101</u>	<u>Arg 47</u>	<u>Asn 180</u>	Charged or polar
	<u>Lys 58</u>	<u>Arg 369</u>		---
			<u>Gly 99</u> <u>Ala 31</u>	
Nicotinamide ribose	<u>C=O 98</u>	<u>C=O 269</u>	<u>C=O 96</u>	H-bond to O-3'
	<u>N 140</u>	<u>N 294</u>	<u>anion</u>	H-bond to O-2'
		<u>His 51</u>	<u>Ser 119</u>	H-bond to O-3'
	<u>Gly 99</u>	<u>Gly 270</u>	<u>Gly 97</u>	
	<u>Val 32</u>	<u>Val 203</u>		Hydrophobic
	<u>Thr 97</u>			
	<u>Pro 139</u>	<u>Gly 293</u>	<u>Ala 120</u>	---
		<u>Val 294</u>		---
Nicotinamide	<u>Val 32</u>	<u>Val 203</u>	<u>Ile 11</u>	Hydrophobic
	<u>Val 138</u>	<u>Val 292</u>		---
	<u>Asn 140</u>	<u>Val 294</u>		
	<u>Leu 167</u>	<u>Thr 178</u>	<u>Tyr 317</u>	
	<u>Thr 246</u>			
	<u>Ile 250</u>			
			<u>Cys 149</u>	Charge transfer
Carboxamide	<u>C=O 139</u>	<u>C=O 292</u>		H-bond
	<u>Ser 163</u>	<u>N 319</u>	<u>Asn 313</u>	---

molecules (dimeric ADH, tetrameric ADH, tetrameric SDH), metalloproteins with different zinc content (1 or 2 atoms per subunit), and polypeptide chains from different kingdoms and orders (mammals, higher plants, fungus and yeasts). The structures included in their alignments were the following (43 and references therein); (a) horse liver ADH, E-type subunit, from protein data, verified by X-ray crystallography (4); (b) human liver class I alcohol dehydrogenase, α , β , and γ subunits; (c) human liver class II alcohol dehydrogenase, π subunit; (d) mouse liver alcohol dehydrogenase; (e) rat liver alcohol dehydrogenase; (f) maize alcohol dehydrogenase, isozymes 1 and 2, as well as pea alcohol dehydrogenase and alcohol dehydrogenase from *Arabidopsis thaliana*; (g) *Aspergillus nidulans* alcohol dehydrogenase; (h) yeast cytosolic alcohol dehydrogenase from *Saccharomyces cerevisiae*, isozyme 1 and 2; (i) mitochondrial alcohol dehydrogenase from *Saccharomyces cerevisiae*; (j) yeast alcohol dehydrogenase from *Schizosaccharomyces pombe*; (k) sheep liver sorbitol dehydrogenase (49). The comparisons revealed extensive variations in alcohol dehydrogenase, but with evolutionary changes that are of the same order in different branches and at different times. They emphasize the general importance of particular residues, suggesting related overall functional constraints in the molecules. Only 22 residues were found strictly to be conserved; half of these are glycine (43).

Most of the remaining ones are proline or acidic residues, especially those acidic residues that have defined binding interactions such as Asp-223, Asp-49 and Glu-68. All these separate comparisons rely heavily on the one crystallographically analyzed structure of an alcohol dehydrogenase, that is HLADH, and span quite extensive differences from about 25% to over 90% residue identity (51).

In spite of many comparisons and conclusions, overall comparisons of all presently known zinc-containing alcohol dehydrogenases are still lacking. Nevertheless, the structural features of HLADH are considered to be common amongst ADHs from different sources, and evolutionary relationship has been inferred (43, 45, 52). While the present model is sufficient to establish the overall relationships, and may explain many, however not all of the effects on enzymic and other properties (48). To substantiate such generalization, additional data from other alcohol dehydrogenases are required.

Apart from the presently characterized and reported enzyme structures of the above mentioned enzymes (43), only partial characterizations have been made on ADHs from other sources (4-10), partly due to the unavailability of efficient purification procedures. The rat liver alcohol dehydrogenase (RLADH) has been purified (53-57) and three of its isozymes were identified quite recently (58-60). The

purification procedure of this enzyme in this study was developed independently (61). The RLADH is known to be an NAD^+ specific, however, in this study it was observed that NADP^+ may also serve though inefficiently as electron acceptor. Nevertheless, RLADH uses NADP^+ as a coenzyme more efficiently than HLADH. This aspect of coenzyme preference is particularly interesting as both NAD^+ and NADP^+ can function as redox cofactors of dehydrogenases and has not been investigated previously. Such study will help gain an insight into the structure-function relationship of alcohol dehydrogenases.

The alcohol dehydrogenase has been shown to be present in both liver and intestinal caeca of a salmonid fish (5) and in rainbow trout (61). The ADH from pickerel fish has not been purified or characterized. In this study, initial investigation of the enzyme-coenzyme specificity, revealed this enzyme to use NAD(P)^+ almost equally. The NADP^+ -linked alcohol dehydrogenase isolated from thermophilic bacteria by Lamed et al. (10) has been purified and characterized partially. The NAD^+ -linked activity of this bacterial enzyme was not investigated. In this work attempts to purify alcohol dehydrogenases from rat liver, pickerel fish liver and thermophilic bacteria, *Thermoanaerobium brockii* are to emphasize on the $\text{NAD}^+/\text{NADP}^+$ specificity of these enzymes. That is to elucidate the implication of coenzyme specificity to the structural features of the enzyme to accomodate one

or both coenzyme, as well as enzyme mechanism. When it is necessary, comparisons are made with reference to the well characterized alcohol dehydrogenase from horse liver (HLADH) and/or yeast alcohol dehydrogenase (YADH).

The study of enzyme mechanisms can follow several different approaches. One of them is to analyze the kinetics of reaction catalyzed by the enzyme (62). By this, one can learn about the order in which substrates bind and product leaves the enzyme, and the velocity at which these processes take place. These kinetic analyses are still among the most informative means for investigation of mechanism. Kinetic analysis alone does not elucidate the detailed molecular mechanism by which these transformations occur and how the structural elements (amino acid residues) contribute to them. To learn about the catalytic mechanism by which an enzyme operates with either coenzyme, it is therefore essential to study the structural elements of the active site (amino acid side chains) and the three dimensional structure of the active site.

The most powerful technique currently available for such structural studies is X-ray diffraction (63). If a diffraction pattern of sufficient resolution can be obtained, one can get a very accurate picture of the three dimensional structure of the enzyme and thus of its active site. However, due to the many limitations of X-ray diffraction, of which a few to mention are: the need to

obtain enzyme in crystalline form adequate for analysis, and the question of how valid are the results obtained in crystalline state when extrapolated to aqueous solution (63). A host of other analytical techniques, individually less powerful than X-ray diffraction, have been developed through the years to address the structure question. Quite often these are the only ways of probing active-site structure and function. Of specific importance in probing active-site structure is the chemical modification.

Chemical modification studies with group-specific reagents have provided much information regarding the functional role of individual amino acid residues in a large number of proteins (64). Modifications of amino acid residues also take place in vivo subsequent to the translational process in protein synthesis (65). Such modifications cause desirable changes in the properties and/or activities of proteins without deleterious structural effects. Current methodologies of chemical modification of active sites residues on the other hand, mostly lead to an inactivation of enzymes.

Chemical modification remains as one of the feasible, direct, and most frequently used approach in studying the chemical basis of enzyme function, in spite of some of its obvious limitations (66).

A variety of active-site directed chemical modifying reagents have been used to investigate the structure and

mechanism of horse liver alcohol dehydrogenase (HLADH). Many of those amino acids that function intimately in catalysis were identified and extensive informations were obtained by the use of those reagents available for chemical modification. An enormous literature exist on modification of different residues in HLADH. No attempt to review all literature on experimental work related to this issue will be made. Nevertheless, a few examples of some recent papers will be reviewed to illustrate the power of this chemical approach to the unraveling of reaction mechanism of HLADH and other enzymes in general.

The halo acids iodoacetate and 3-bromopropionate have been shown to selectively alkylate Cys-46 and Cys-174 residues respectively, both are active-site zinc ligands (18, 67-70) in HLADH. Enzyme alkylated with iodoacetate retains less than 5% of the original catalytic activity (71). The kinetics of inactivation by these reagents indicate a high-affinity, reversible enzyme-inhibitor Michaelis-Menten complex is formed prior to covalent modification (70, 72). On the basis of X-ray crystallographic studies of the Cys-46 carboxymethylated enzyme (75), and chemical modification studies of arginine residues by Lange et al. (74, 75), it has been suggested that iodoacetate binds to Arg-47 prior to alkylation of Cys-46 to form the reversible Michaelis-Menten complex.

A number of different modifications that affect the ϵ -amino groups of lysine at the active site of HLADH have been studied (4). One such modification, is the reaction of HLADH with methyl picolinimide by Plapp (76, 77), which modifies 50 of the 60 lysine residue causing the overall enhancement in enzymic activity.

By differential acetimidylation, one of the lysine residues involved was identified as Lys-228 (78). This lysine hydrogen bonds through its ϵ -amino to a carboxyl oxygen of Asp-223, which is involved in the binding of adenosine ribose as mentioned previously (11). Other modifications of lysine residues include the reaction of HLADH with pyridoxal phosphate (79). This modification inactivates the enzyme and is attributed to a change in the charge of the lysine and modify 11 residues. Both picolinimidylation and phosphopyridoxylation modify an identical lysine residue which interacts with nicotinamide coenzymes (78). Different lysine residue(s) on the other hand are modified by methylation (80, 81) resulting in an enhancement in enzyme activity. Two other modifications of lysine that change enzymic activity, are the acetylation inactivation and glycosylation (82, 83). Acetylation reacted with approximately two-thirds of the total lysines (41 ± 9) to induce the greatest structural changes in HLADH. Glycosylation modified only one lysine residue (Lys-315) selectively with indiscernible structural changes. From

these different modifications of lysine residues, it is obvious that some of the lysines modified are implicated as essential to the activity of HLADH.

Histidine residues in HLADH have been modified by carbethoxylation with diethylpyrocarbonate, resulting in a rapid activation followed by slower inactivation. None of the four histidine residues per subunit which were modified, are at the active centre, but one or more may be important for maintaining the enzyme native conformation (84). Further investigation with diethylpyrocarbonate revealed this reagent modifies lysine residues as well, thus complicating the results due to counteracting effects of activation and inactivation. Hennecke and Plapp (40) used HLADH that was first acetimidylated on all accessible lysine residues, then modified with diethylpyrocarbonate to overcome above problem. Two histidine residues were modified during inactivation, one is probably His-51 (40) that participate in a proton relay system during enzymatic catalysis (23, 40). Dye sensitized photo-oxidation carried out under mild conditions, allows modification of restricted number and types of amino acid residues. Histidine residues in HLADH may be preferentially destroyed by photo-oxidation (85, 86). The recent study of Tsai et al. (87) on photo-oxidation of HLADH by efficient sensitizers (methylene blue and rose bengal) indicated that seven histidine and one tyrosine

residues were affected per subunit, though the conversion of histidines into aspartate was not quantitative (87).

The role of arginine residues in a wide variety of enzymes and other biologically active protein molecules has been obtained from chemical modification studies with α -dicarbonyl reagents (74, 88-93). In general, it seems that arginyl residues have important roles in the binding of anionic substrates and coenzymes in wide variety of enzymes (90). In horse liver alcohol dehydrogenase it has been suggested that the role of arginine is to participate in NADH binding. Arginine interaction with the coenzyme's pyrophosphate bridge (74) agrees with crystallographic results (4, 21).

Apart from the above-mentioned amino acid residues which have been modified, and their roles in catalysis in HLADH have been identified, very few detailed studies on the modification of acidic amino acids have been reported. The presence of a carboxyl group (Asp-223) is of particular interest. Its involvement in hydrogen bonding to the adenosine ribose moiety is responsible for the NAD⁺ specificity exhibited by HLADH and other dehydrogenases (11, 21, 94) as mentioned previously. Modification of this residue in HLADH has not been reported. With the development of a selective chemical modification procedure using water-soluble carbodiimides and an amine (95, 96), it is possible to use this approach to determine the role of carboxylic

group in proteins. Therefore the effect of such modification of carboxylic residues on proteins that have the ability to use both NAD^+ and NADP^+ , could provide an explanation of the nature of the active site.

Study of protein-ligand interactions has proved to be one of the most fruitful application of high-resolution nuclear magnetic resonance (NMR) to biological problems. The NMR can provide a variety of information on both structural and dynamic aspects to the recognition of small molecules by proteins in solution state (97). Examples are the use of ^{31}P NMR in providing an understanding of the nature of of coenzymes binding to the active site of isocitrate dehydrogenase (98) and dihydrofolate reductase (99, 100).

Dihydrofolate reductase catalyzes the NADPH-linked reduction of dihydrofolate to tetrahydrofolate. This enzyme is so far one of the NAD(P)H binding enzymes for which both high resolution ^{31}P NMR spectroscopy and detailed X-ray structure for the binary complex with the reduced coenzyme are available (98, 100).

The ^{31}P NMR spectrum of NADP^+ consists of two absorption bands in the intensity ratio 1:2. The low field signal arises from the 2'-phosphate group, and that at high field arises from the two phosphorus nuclei in the pyrophosphate group, which have the same shift. In the binary complex of NADP^+ with dihydrofolate reductase, a markedly different spectrum is observed. A significant

downfield shift of the 2'-phosphate resonance is observed. The resonances of the two phosphorus nuclei are both shifted upfield. It was strongly suggested the dianionic 2'-phosphate group is involved in a strong electrostatic interaction with cationic site on the enzyme (100). The X-ray data of the enzyme given by Matthews et al. (44), identified this cationic specie as an arginine. The shifts in resonances of the two pyrophosphate were assigned to specific conformational change in the 5'-pyrophosphate when it binds to dihydrofolate reductase (100).

In order to determine the contribution of each of the interactions between active site species and the coenzymes, it is necessary to make modifications either to the ligand, or to the protein. Modification is designed to block one specific interaction. A crucial step in this process is to compare the native and modified enzyme complexes, in order to establish that only the one desired interaction has been disrupted. The NMR can be very useful in making this comparison. The ^{31}P -NMR is a useful tool that can be used in the identification of interactions between active site species and coenzymes.

It is known that some dehydrogenases are strictly NAD^+ , and others are strictly NADP^+ linked. There are other dehydrogenases that display dual nucleotide specificity. Examples are animal mitochondrial glutamate dehydrogenase (101), homoserine dehydrogenase in plants (102), glucose-6-

phosphate dehydrogenase from *Leuconostoc mesenteroides* (103) and soluble malate dehydrogenase (s-MDH) located in mitochondria of rabbit heart, calf adrenal cortex, human skeletal muscle and other species (104, and references therein). In some cases it was revealed that NAD^+ and NADP^+ interact with different forms of the enzyme (105). Nevertheless, little is known about the dual nucleotide specificity of alcohol dehydrogenases and the mechanisms which control the utilization of NAD^+ and NADP^+ in such enzymes.

The objectives of the present study could be summarized as follows:

(i) Develop a purification procedure that could be well applied to all alcohol dehydrogenases from the different sources used in this study.

(ii) Compare properties, nucleotide specificity, mechanism, multifunctionality, and essential amino acid residues in active site of the well studied alcohol dehydrogenase from horse (HLADH) and to a lesser extent (YADH) to those isolated from pickerel fish liver (FLADH) and thermophilic bacteria; *Thermoanaerobium brockii* (TBADH) used in this study.

(iii) Examine the mechanisms of both NAD^+ -linked and NADP^+ -linked reactions catalyzed by FLADH as well as the TBADH. Variety in kinetic mechanism, if there is any, may

reflect differences in prevailing physiological conditions, necessitating different control mechanisms.

(iv) Employ chemical modifications to demonstrate its potentiality in exerting similar or dissimilar effects on NAD⁺-linked and NADP⁺-linked activities of the investigated enzymes.

(v) Based on the known structure of HLADH, those amino acid residues that are considered essential, are in active site or involved in binding of coenzymes will be modified chemically in FLADH and TBADH. The effect of chemical modification on these residues will be compared to HLADH. Differences in such modifications could indicate the implication of targeted residues in binding, active site or otherwise. This will be supported by the ³¹P-NMR analysis that may play a role in the identification of interactions between active site species and coenzymes.

(vi) In connection with such nucleotide duality, it is pertinent to ask what protein structural features underlie such dual nucleotide specificity and how enzymes that can utilize both coenzymes differ from those which are NAD⁺ or NADP⁺ specific.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Materials

2.1.1. Proteins and biological materials

Horse liver-alcohol dehydrogenase (HLADH) (EC.1.1.1.1), yeast alcohol dehydrogenase (YADH) (EC.1.1.1.1), *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH) (EC.1.1.1.2), egg white Lysozyme (EC.3.2.1.17), bovine liver catalase (EC.1.11.1.6), cytochrome C (horse heart, type III), and electrophoresis protein molecular weights standards (BSA, ovalbumin, trypsinogen, pepsin and β -lactoglobulin) were all obtained from Sigma Chemical Company.

Rat livers were obtained from Pel-Freeze Biologicals Inc. Pickerel fish livers were either provided by Lake Winnepig hatchery or fresh fishes were obtained from a local hatchery and livers were removed and stored frozen at -20°C until needed.

2.1.2. Biochemicals.

Thio-NAD⁺, Thio-NADP⁺, NADP⁺, NADPH, NADH, acPy-AD⁺, acPy-ADP⁺, and DTE were products of Sigma Chemical Company. Deamino-NAD⁺ and PyA-AD⁺ were obtained from United States Biochemicals Corporation. NAD⁺ and 2-mercaptoethanol were products of Boehringer-Mannheim and J.T.Baker Chemical

Company, respectively. Dithiothreitol (DTT) was purchased from Calbiochem-Behring and Bio-gel p-6, was obtained from Bio-Rad Laboratories.

Agarose-Hexane-NAD⁺ gels: AG-NAD⁺ type 3, AG-NAD⁺ type 1, and AG-NADP⁺ type 3 were all purchased from Pharmacia P.L. Biochemicals. Sephadex G-25, Sephadex G-100, Sephadex G-200, and DEAE-A50 Sephadex were products of Pharmacia Fine Chemicals. DEAE-A50 cellulose was purchased from Whatman Incorporation. Fractogel TSK DEAE 650 S, and Fractogel TSK HW 55F were products of EM Science.

2.1.3. Modifications reagents.

The following reagents were obtained from Sigma Chemical Company: iodoacetate, iodoacetamide, and ethylenediamine. Fisher Scientific supplied: D-glucose, ammonium chloride, rose bengal, methylene blue, formaldehyde solution and methylamine. Methylacetimidate hydrochloride and 1-ethyl-3-(3'-dimethylamino propyl)carbodiimide (EDC) were products of Pierce Chemical Company. Phenylgloxal monohydrate and taurine were products of Aldrich Chemical Company and Mann Research Laboratories Incorporation respectively. Methylamine (51.8mCi/mmol) was a product of New England Nuclear (NEN).

2.1.4. Miscellaneous chemicals.

The reminder of the chemicals used are listed below: Fisher Scientific supplied. ScintiVerse E, phenol reagent, sodium pyrophosphate, ammonium persulfate, cupric chloride, 1,4-dioxane, iso-butanol, propan-1-ol, pentan-1-ol, cyclohexanol, octanol, and benzylalcohol. The following were obtained from Sigma Chemical Company: tris-buffer salts, acrylamide, TEMED, and the p-nitrophenyl esters of acetate, butyrate, caproate, caprylate, caprate and laurate. Anachemia supplied: sodium phosphate salts, sodium borate salts, pH buffers, acetaldehyde, isopropyl alcohol and ethylamine. Sodium borohydride, TCA, and propan-2-ol were products of J.T. Baker Chemical Company. Ammonium sulfate and sodium acetate came from Analar. Potassium dichromate, sodium chloride and dodecyl sulfate de sodium (SDS) were obtained from BDH. 1,4-butanediol was purchased from K.&K. Laboratories Inc. Octanic acid and propen-1-ol were supplied by Eastman Organic Chemicals. Silver nitrate and potassium sodium tartarate were products of Mallinckrodt Chemical works. Calbiochem-Behring Corporation supplied 2-(N-morpholino)-ethanesulfonic acid (MES) .

Absolute ethanol was supplied by Consolidated Alcohol LTD. Tetranitromethane (TNM), p-nitro blue tetrazolium chloride (NBT) and phenazine methosulfate (PMS) came from Nutritional Biochemicals Corporation. Deuterium chloride and sodium deuterioxide were obtained from Stohler Isotope

Chemicals (SIC). Deuterium oxide was supplied by MSD Isotopes.

2.2. Methods

2.2.1. Preparation of buffers

Protic buffers were prepared from the appropriate weights of basic and acidic components. Solutions were prepared using ultrapure water obtained from a Millipore Milli-Q™ water purification system, at a measured resistivity of 10-18 MΩcm. The pH determinations were made using a "Fisher Accumet model 825 MP pH meter standardized with Anachemia standard buffers and read to ±0.01 pH units with temperature corrections if necessary. To prepare buffer solutions in D₂O, the original weight ratio of protic buffer were dissolved in D₂O and lyophilized. This cycle was repeated at least twice. The required volume of D₂O was then added. Apparent pH readings in D₂O were converted to pD according to :

$$pD = pH + 0.4$$

2.2.2. Enzymes purifications

2.2.2.1. Purification of Pickerel fish liver alcohol dehydrogenase (FLADH)

All procedures were performed at 0-4°C. Centrifugations were performed in refrigerated Sorvall RC-2B, using SS-34

rotor at 31,000 X g for 20 minutes, unless otherwise indicated.

Fish livers (40-60 gm) were chopped and homogenized with 3 volumes of ice cold 10 mM tris/HCl buffer, pH 7.5 containing 1.0 mM DTT, (referred to as tris/HCl buffer). Homogenization was carried out on ice using Sorvall-Omi mixer at speed 6 in three 30 seconds bursts, spaced 1 minute apart. Homogenate was centrifuged at 31,000 X g for 20 minutes. Pellet was discarded. Extra care was practiced to avoid withdrawing of a yellow fat layer along with enzyme extract. Enzyme extract was made up to 30% saturation in ammonium sulfate (176 g/litre). The pH was maintained at pH 7.5 by dropwise addition of concentrated ammonium hydroxide. Extracts were left to stand additional 20 minutes and then centrifuged. Ammonium sulfate concentration of supernatant was increased to 60% saturation (164 g/litre). The pH was maintained at pH 7.5 again and solution left to stand for 20 minutes. The pellet precipitated between 30-60% ammonium sulfate saturation was collected by centrifugation. Pellet was dissolved in minimum volume of tris/HCl buffer and dialyzed for 15 hours against 2 L of same buffer with at least two buffer changes. The dialyzed fraction was clarified by centrifugation in Beckman L8-70M ultracentrifuge (fixed angel, rotor type 60 Ti) at 143,380 X g for 60 minutes. About 10 ml of this ultrasupernatant was applied to a DEAE-A50 Sephadex column (38.0 X 2.5 cm)

equilibrated with tris/HCl buffer. DEAE-A50 was replaced later by Fractogel TSK DEAE-650S. This fractogel TSK DEAE-650S demonstrated higher sample capacity, better resolution and sharper chromatographic peaks. Enzyme was eluted isocratically. The peak fractions, which were located by measurment of absorbancy at 280 nm, and activity assays, were pooled. The dehydrogenase active fractions were either concentrated by Amicon Centricon microconcentrators or lyophilized using Virtis Research Equipment freeze dryer. Lyophilized enzyme was dissolved in minimum volume of 10 mM tris/HCl and delivered to a Sephadex G-100 column (37.0 X 2.5 cm). Enzyme was eluted with same buffer. Small fractions were collected (35 drops/fraction). Dehydrogenase active fractions (located by assay and absorbancy at 280 nm) were pooled. Sephadex G-100 was replaced by Fractogel TSK HW 55F for same reasons mentioned earlier for replacing DEAE-A50. The G-100 pool was either concentrated by a Millipore CX-10 Ultrafiltration Units, or applied directly on to an AG-NAD⁺ type 3 column (8.0 X 0.9 cm). The column was washed extensively with tris/HCl buffer until no more U.V. absorbing material was detected, and A₂₈₀ of elute dropped to that of buffer. The enzyme was eluted off the column immediately on addition of tris/HCl buffer that contained 10 mM NAD⁺, and dehydrogenase active fractions were combined. Coenzyme was then removed by 24 hours dialysis against three changes of 1 L tris/HCl buffer (free of ethanol) or pooled

fractions were applied to Bio-gel P-6 column (27.0 x 0.9 cm) equilibrated with same buffer but free of ethanol .

2.2.2.2. Purification of rat-liver alcohol dehydrogenase (RLADH)

Purification procedure is similar to the fish liver enzyme, with the changes indicated below:

- a- Rat livers (20-40 g) were used in each prepration .
- b- tris/HCl buffer used through out the procedure contained 5% ethanol, DTE concentration was 0.5 mM and pH 8.4.
- c- Centrifugation was in Sorvall-Rc 2B but at 27,000 X g for 20 minutes.
- d- Enzyme extract was made up to 35 % saturation in ammonium sulfate (209 g/litre).
- e- The anion exchanger used was Sephadex DEAE A-50 and had lower capacity for this enzyme compared to FLADH.
- f- The DEAE-A50 pooled active fractions were precipitated by ammonium sulfate at 70% (472 g/liter) saturation. The pH was maintained at 8.4 and pellet collected by centrifugation at 39,000 X g for 30 minutes. The pellet was suspended in minimum volume of 10 mM tris/HCl buffer, 0.5 mM DTE and 5% ethanol and delivered on to next column.

**2.2.2.3. Purification of *Thermoanaerobium brockii*
alcohol dehydrogenase (TBADH)**

Thermoanaerobium brockii alcohol dehydrogenase (TBADH) was obtained from Sigma Chemical Company. SDS-gel electrophoresis of this sample revealed its non-homogeneity. The enzyme could not be used as it is in most of the experiments, and necessitated its purification. The enzyme obtained from Sigma had been at least heat treated following the method of Lamed and Zeikus (10).

Twenty milligrams of Sigma enzyme was dissolved in minimum volume of 10 mM tris/HCl buffer, pH 7.5, 0.5 mM DTT. Enzyme solution (2-3 ml) was layered on to a Whatman DEAE-52 Cellulose column (13.0 X 0.9 cm) previously equilibrated in above buffer. The column was washed with the tris/HCl buffer and eluted by a linear 0-1 M sodium chloride gradient in the same buffer. Active fractions were assayed for absorbancy at 280 nm, activity assay, and conductivity using Bach-Simpson Limited Conductivity meter type CDM 2d. The Dehydrogenase (DHase) active fractions were pooled and applied to a Sephadex G-200 column (38.0 X 2.5 cm) equilibrated and eluted with tris/HCl buffer. The DHase pooled active fractions were applied to an affinity column AG-NADP⁺ type 3. The AG-NADP⁺ column (0.9X9.7 cm) previously equilibrated with tris/HCl buffer was washed extensively until absorbancy of elute were similar to that of buffer. Enzyme was eluted off the column immediately upon addition of same buffer but

contained 10 mM NADP⁺. The DHase active fractions were located by activity assay only and pooled. The NADP⁺ was removed by either dialysis of pooled fraction for 24 hours versus three X 1 L of buffer, or by filtration of enzyme on Bio-gel P-6 column.

2.2.2.4. Purification of yeast alcohol dehydrogenase (YADH)

The Sigma YADH as supplied, was not homogenous enough. It was evidenced by the presence of large number of bands obtained when sample was subjected to SDS-gel electrophoresis. Purification of enzyme was achieved by single step, using affinity chromatography gel AG-NAD⁺ type 1. About 30 mg of Sigma enzyme in 5.0 ml of 10 mM tris/HCl buffer pH 7.5, 0.5 mM DTT was applied to (8.2 X 0.9 cm) column previously equilibrated with above buffer. Column was washed extensively until very low absorbancy at 280 nm was observed. Enzyme was eluted by addition of 10 mM NAD⁺ to buffer. Enzyme active fractions located by assay were pooled. Coenzyme was removed by either dialysis for 24 hours against 3 changes of 1 L tris/HCl buffer or enzyme pool was applied to Bio-gel P-6 column.

2.2.3. Protein determinations

Protein concentrations were determined following the method of Lowry et al., (106) as modified by Miller (107). A

solution labelled as mixture I was freshly prepared by mixing in the specified order: 1.0 ml of 2% (w/v) sodium potassium tartarate, 1.0 ml of 1% (w/v) cupric sulfate pentahydrate were made up to 100 ml by addition of 2% (w/v) sodium carbonate in 0.1 N NaOH. A 5.0 ml of mixture I was added to 1.0 ml of enzyme preparation from any particular step (with appropriate dilution which ranged from 20-500 fold), or stock BSA standard in range of 5-100 $\mu\text{g/ml}$. This solution was left for 10 minutes then 0.5 ml of freshly prepared phenol (108) reagent (diluted 1:1 with water) was added. The solutions were mixed well and left to stand 30 minutes. Absorbances were measured against blank solution at 750 nm. Protein concentrations were extrapolated from constructed BSA standard curve.

2.2.4. Assay for alcohol dehydrogenase activity.

Initial velocities were measured in assay mixtures 1.0 ml containing 0.5 ml of 0.10 M sodium pyrophosphate buffer (pH 9.0) for RLADH or 0.10 M tris/HCl, pH 9.0 for fish, yeast and *Thermoanaerobium brockii* ADH's, 1.0 mM NAD(P)⁺ and 10 mM ethanol for rat, fish and yeast ADH'S or 10 mM propan-2-ol for TBADH. The reaction was initiated by addition of 50 μl enzyme. The NADP(H) formation was monitored spectrophotometrically in Helma quartz cells at 340 nm ($\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ for NADH and $\epsilon_{340} = 6.20 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for NADPH) using a Beckman model 25

spectrophotometer. One unit (U) of dehydrogenase activity is that the amount of enzyme needed to produce 1 μmol of NAD(P)H per minute at room temperature.

2.2.5. Assay for dismutase activity

Initial activity was measured as for dehydrogenase activity except in 0.10 M sodium phosphate buffer, pH 7.4 with 1.0 mM octanal replacing ethanol. One unit is as defined as that amount of enzyme needed to produce 1 μmol of NADH per minute at room temperature.

2.2.6. Assay for esterase activity

Esterase activity was monitored spectrophotometrically at 25°C by the release of p-nitrophenol at 400 nm ($\epsilon_{400} = 16.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The assay mixture (1.0 ml) contained 0.10 M sodium borate buffer pH 8.2, 0.1 mM p-nitrophenyl caprylate (NPO) in dioxane and 50 μl of enzyme. Reaction was initiated by addition of NPO. A stock solution of 5 mM NPO was prepared freshly in 1,4-dioxane to minimize spontaneous hydrolysis. All reaction rates were corrected for the spontaneous hydrolysis by running a blank with enzyme being omitted. One unit of esterase activity is that amount of enzyme needed to produce 1 μmol of p-nitrophenol per minute at 25°C.

2.2.7. SDS-discontinuous gel electrophoresis.

SDS-discontinuous gel electrophoresis with a 10% separating gel and 5% stacking gel was prepared according to the anionic system tris/glycine method of Davis (109) and modified by Laemmli (110). A 1mm slab gel apparatus was obtained from Bethesda Research Laboratories.

The gels were prepared from the following stock solutions:

(A) Separating gel buffer: 0.4% SDS, 1.5 M, tris/HCl, pH 8.2.

(B) Stacking gel buffer: 0.2% SDS, 0.25 M tris/HCl, pH 6.8.

(C) Acrylamide/Bis: 30.0% Acrylamide, 0.8% Bis.

(D) Reservoir buffer: 0.125 M tris/HCl, 0.96 M glycine, 0.5% SDS, pH 8.3.

(E) Sample buffer: 2.5% SDS, 0.062 M tris/HCl and 10% glycerol.

For 10% separating gel; 12.5 ml of A, 16.7 ml of C and 25 μ l TEMED were mixed separately and added to 20.8 ml water that contained 50.0 mg ammonium persulfate. This was poured immediately into the gel casting space. Water was layered gently on top and gel was allowed to polymerize for 30 minutes.

After polymerization was completed, the water layer was removed and the stacking gel solution was added.

Stacking gel (5%) was prepared by mixing 5.0 ml of B, 1.7 ml C and 5 μ l TEMED. To this, 3.3 ml water containing 10.0 mg ammonium persulfate was added, mixed and poured into the casting area. The sample well forming comb was inserted and water was layered. After polymerization (10-15 minutes), the comb was removed and the gel unit was assembled into the reservoir box. The electrode reservoirs were filled with 1:4 dilution with water of solution D.

Enzyme fractions and molecular weight standards (1 mg/ml) were dissolved in sample buffer (E) and heated for 1-2 minutes in boiling water bath.

The samples ranging 20-40 μ l were layered on top of the stacking gel by means of a Hamilton syringe. Electrophoresis was run for 5-6 hours at 200 volts, at 0-2°C using an Ortec 4100 power supply.

Proteins were stained using one of the methods described in the following sections.

2.2.7.1. Coomassie brilliant blue dye

Procedure of Blakesly and Boezi(111) was adapted. Gel was immersed in a tray of staining solution (0.25% coomassie brilliant blue G250 in 50% methanol and 10% acetic acid) for overnight. The gel was then destained in 40% methanol and 50% acetic acid solution for 1-2 days.

2.2.7.2. Silver stain

Following method of Nielson and Brown(112), the gel was fixed for 30 minutes in 10% (W/V) TCA, washed out (3 washes) with 10% (V/V) ethanol in 5% (V/V) acetic acid, each wash for 10 minutes, and oxidized with 3.4 mM potassium dichromate in 3.2 mM nitric acid for 6 minutes. The oxidant solution was discarded and gel was washed 5 minutes thrice with deionized water. The gel was then placed in 12.0 mM silver nitrate solution for 20 minutes. Silver nitrate solution was replaced with two deionized water washings, each lasting about 1 minute.

The steps above were performed at 30°C with gentle shaking. The gel was incubated for 30 seconds at 40°C in a developer solution consisting of 0.28 M sodium carbonate and 0.0185% formaldehyde solution which was added just before adding the developer solution to the gel. This was replaced with another fresh warm developer solution and incubated for 5 minutes or until black-brown protein bands were visualized. The staining was stopped by replacement of the developer solution with 5-10% acetic acid solution.

The position of purified enzymes bands and standards (BSA, ovalbumin, trypsinogen, β -lactoglobulin and lysozyme) were measured. Subunit molecular weight were extrapolated from a constructed plot of log molecular weight against mobility of the standards (113).

2.2.8. Non-SDS-discontinuous gel electrophoresis

This was performed in the same way as the SDS-discontinuous gel electrophoresis except that SDS was excluded from all the buffers. Separating gel (7%) was prepared by mixing 12.5 ml of A, 11.7 ml of C and 25 μ l TEMED. Deionized water 25.8 ml containing 40 mg ammonium persulfate was added to the above, mixed and poured immediately in gel casting area. A 4% stacking gel was prepared as mentioned previously in section 2.2.7.

Samples poured must be enzymatically active and were suspended in tris/HCl buffer with few drops of glycerol added. When electrophoresis run (5-6 hours) was over, gel was removed and placed in the dehydrogenase activity stain solution instantly. Staining system was prepared as outlined in (114). The gel was placed in a solution (175.0 ml) of 0.1 M tris/HCl pH 8.7 which contained 30.0 mg NBT, 12.0 mg PMS, 125.0 mg NAD(P)⁺ and 150.0 μ l of absolute ethanol or propan-2-ol. The gel was kept in this staining solution in the dark until desired staining is attained. Staining solution was replaced with deionized water.

Gel was scanned using Gel Scanning Densitometer GS 300, Hoefer Scientific Instruments, with Fisher Recordall Series 5000.

2.2.9. Density gradient centrifugation

Sucrose gradients (15-30%) in 10 mM tris/HCl, pH 7.5 were formed using a Dekastaltic pump. Linear gradients were prepared in Beckman polyallomer tubes (1/2"X2") with pump $\text{Rate}_{\text{in}}/\text{Rate}_{\text{out}}=0.5$. Gradients were allowed to chill for 2-3 hours at 4°C. Sample proteins and/or standards were suspended in tris/HCl buffer, pH 7.5 containing 14% sucrose (w/v). By a syringe, 200 μ l samples were layered on top of gradient and centrifuged in Beckman L8-70 M ultracentrifuge using swinging bucket rotor type SW55 Ti. Centrifugation was allowed for 17.4 hours at 100,000 X g and 3-5°C accumulating a centrifugal effect = 7.59×10^{11} rad²/sec. The tubes were punctured at the bottom with a needle and 10 drops fractions were collected through the needle. Fractions were diluted to 0.5 ml with buffer and protein bands were located by absorbancy at 280 nm and DHase activity assay. Lysozyme, catalase, YADH and HLADH were used as references (2.0 mg/ml each). Molecular weights for RLADH (2.0 mg/ml), FLADH (1.0 mg/ml) and TBADH (2.0 mg/ml) were extrapolated from constructed curve of log molecular weight against R_f (where R_f = elution volume/total volume), or calculated following Martin and Ames (115) method. The ratio R of the distances travelled by two species (d_1 and d_2) is inversely proportional to their elution volume ratio (V_2/V_1), which in turn is inversely proportional to the ratio of sedimentation coefficients (S_1/S_2). Sedimentation coefficients (S_1/S_2)

varies as the $2/3$ root of the molecular weight ratio as follows:

$$\begin{aligned} R &= d_1/d_2 \propto V_2/V_1 = S_1/S_2 \\ &= (MW_1/MW_2)^{2/3} \end{aligned}$$

2.2.10. Temperature dependence of enzyme activity

The thermal stability of purified enzyme was determined by immersing enzyme solution (2-3 ml) in a Gyrotory water bath shaker model G76 "New Brunswick Scientific Co. Inc.". Initial temperature was recorded and enzyme assayed for activities at this temperature. Temperature was increased gradually 2-5°C at a time. Aliquots (60 µl) of the enzyme were withdrawn, placed in a microfuge vial, chilled on ice and assayed for the indicated activities.

2.2.11. Stability of enzyme activity with different stages of purification

Enzymes fractions were assayed as soon as they were obtained from each step. The enzyme was stored under optimal conditions (0-4°C). Enzyme solutions were in 10 mM tris/HCl buffer, 0.5 mM DTT, pH 7.5, except RLADH. Buffer used for RLADH was as above but contained 5% ethanol in addition, and pH was 8.4. Enzymes were then assayed for the indicated activities periodically.

2.2.12. Effect of stabilizing reagents on RLADH stability

The RLADH obtained from G-100 Sephadex step in 10 mM tris/HCl pH 8.4, 0.5 mM DTT was used in this stability study. The effect of ethanol (5%), high concentration (5.0 mM) of thiol reagent (DTT) and 0.1 mM NAD⁺ on stability of both esterase and NAD⁺-linked activity were followed with respect to time. Enzyme solution (7-8 ml) of concentration (1.1 mg/ml) was divided into 4 portions. The above reagents were added individually, each to about 2.0 ml portion of the enzyme solution. The enzyme was assayed for its NAD⁺-linked and esterase activities over a period of time. All fractions were kept on ice.

2.2.13. Extinction coefficient

The purified enzyme was dialyzed against deionized water and lyophilized. The extinction coefficient was determined from a UV scan (240-320 nm) of the enzyme with concentration indicated in the legend. Enzyme was dissolved in the appropriate buffer and spectra was recorded at room temperature on a Beckman model 25 spectrophotometer.

2.2.14. Stokes radius

A calibrated gel filtration column was used to estimate Stokes radius for the enzymes. A Sephadex G-100 column (45.0 X 2.5 cm) equilibrated with 10 mM tris/HCl buffer, pH 7.5 or

8.4, 0.5 mM DTT was used to determine elution volumes of the purified enzymes (RLADH, FLADH, YLADH) and protein standards. A Sephadex G-200 column (2.5 X 38.0 cm) equilibrated with 10 mM tris/HCl, pH 7.5, 0.5 mM DTT was used to determine the elution volume of TBADH.

Parameters used to characterize the elution behavior of a protein molecule from the gel chromatography column are related by the following equation (116):

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$

where V_t refers to the total bed volume of the packed gel column; V_o designate the void volume, and V_e defines the elution volume of the migratory solute molecule. The partition coefficient K_{av} is indicative of the degree of interaction between a given gel and a solute molecule for a particular set of experimental conditions (116).

The frictional ratio, f/f_o was calculated from the effective hydrodynamic radius using the following equation:

$$f/f_o = r / (3\bar{v}M/4\pi N)^{1/3} \quad (117)$$

Where r is the Stokes radius, N is Avogadro number, M is the molecular weight and $\bar{v}$ is the partial specific volume of the protein which is in the range of 0.71-0.75 mlg⁻¹ for many proteins (118).

2.2.15. Chromatofocusing

The preparation of the gel and column packing were carried out as described in Chromatofocusing with

polybuffer™ and PBE™ handbook, Pharmacia Fine Chemicals, Inc..

A column (28.0 X 0.9 cm) and (53.7 X 0.9 cm) for RLADH and FLADH, respectively, were packed with poly buffer exchanger PBE 94 and equilibrated with starting buffer 0.025 M ethanolamine-CH₃COOH, pH 9.4. The sample protein was applied by first running on 5.0 ml of eluent (poly buffer 96-acetate, pH 6.0), followed by the protein solution, and then switching back to the eluent again. The pH, protein adsorption and DHase activity of the fractions were monitored.

2.2.16. Cofactor specificity

2.2.16.1. *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH)

All determinations were carried out in 50 mM tris/HCl buffer, pH 9.0 at 25°C. Stock solutions (10 mM) of coenzymes were prepared freshly. Assay mixtures contained fixed concentrations of the substrate propan-2-ol (0.1 M), variable amounts of coenzymes as indicated in corresponding figures, and made up to 1.0 ml with water. Reactions were initiated by addition of 50 µL of enzyme (5.0 mg/ml). Ability of enzyme to utilize NAD⁺, NADP⁺ and various analogues was investigated. Analogues used were 3-acetylpyridine adenine dinucleotide (acPy-AD⁺), 3-pyridine

aldehyde adenine dinucleotide (PyA-AD⁺), deamino adenine dinucleotide (Deamino-NAD⁺), thio-NAD(P)⁺, and 3-acetyl pyridine adenine dinucleotide phosphate (acPy-ADP⁺).

Velocities were calculated using the following extinction coefficients: acPy-AD(P)H, $\epsilon_{363nm} = 9.1 \times 10^3 M^{-1} cm^{-1}$; PyA-ADH, $\epsilon_{358nm} = 9.3 \times 10^3 M^{-1} cm^{-1}$; deamino-NADH, $\epsilon_{339nm} = 6.2 \times 10^3 M^{-1} cm^{-1}$; thio-NAD(P)H, $\epsilon_{395nm} = 11.3 \times 10^3 M^{-1} cm^{-1}$ (122). One unit (U) of dehydrogenase activity is that amount of enzyme needed to produce 1 μ mol of the reduced coenzyme per minute at room temperature.

2.2.16.2. Fish liver alcohol dehydrogenase (FLADH)

As with TBADH, FLADH was tested for its ability to utilize NAD⁺ and NADP⁺ and their analogues. Assay mixtures contained fixed concentration of ethanol replacing propan-2-ol and varied concentration of the coenzymes. Enzyme aliquots were 50 μ l per assay. Velocities were calculated as mentioned for the TBADH.

2.2.17. Substrate specificity

The ability of the enzymes, RLADH, FLADH and TBADH to oxidize various alcohols were tested. A wide range of aliphatic, aromatic, primary as well as secondary alcohols were used. All substrates concentration were 0.1 M, prepared in deionized water unless otherwise indicated. Assays were in 0.1 M tris/HCl buffer, pH 9.0 at room temperature, with

fixed concentrations (10.0 mM) of substrates. The NAD⁺ and NADP⁺-linked activities were followed with NAD(P)⁺ concentrations fixed at 0.5 or 1.0 mM.

For the enzymes FLADH and TBADH the effect of varying substrate concentration on the NAD⁺ or NADP⁺-linked activities were studied. Enzymes concentrations varied and are indicated in legends of corresponding figures.

2.2.18. Effect of pH on enzymes activities

2.2.18.1. Dehydrogenase (DHase) activity

dependence on pH

Cursory investigations for the effect of variation of pH on NADP⁺-linked activity of TBADH and the NADP⁺-linked activity of FLADH were performed. The pH profiles were generated from duplicate initial rate data determined at high substrate concentration over the pH range 5.0-11.0. This wide pH range necessitated use of different buffers. Sodium acetate was used at pH 5.0 and pH 5.5, sodium phosphate buffer for the pH range 6.0-8.0, sodium pyrophosphate buffer for the pH range 8.5-10.5 and finally sodium bicarbonate buffer for pH 11.0. Buffer concentrations were kept constant at 50 mM in assays.

The pH profile for NAD⁺ linked activity of FLADH was generated by measurement of initial rates against varied concentrations of NAD⁺ (0.25 mM-2.0 mM) at varied

concentration (0.50 mM-5.0 mM) of ethanol and pH variation corresponding to pH 7.0, 7.5, 8.0, 8.5, 9.0, 10.0, 10.5 and 11.0. At the limiting pH values of 5.0, 5.5, 6.0, and pH 6.5, initial velocities at high constant substrate, and coenzyme concentrations were measured.

2.2.18.2. Esterase activity dependence on pH

The effect of pH variation on esterase kinetics of FLADH was also investigated. Kinetics experiments were performed under assay conditions of varied pH. The substrate NPO concentration was fixed at 0.2 mM and initial rate data were corrected for the spontaneous hydrolysis at each pH. Buffers used were the same as those used for the DHase activities.

2.2.18.3. Dismutase activity dependence on pH

The pH profile of the FLADH dismutase activity was generated in a similar way to that of the esterase activity.

Buffers used were sodium acetate, sodium phosphate, sodium pyrophosphate and sodium bicarbonate for the pH ranges indicated above. Duplicated single assays were performed with octanal concentration fixed at 0.2 mM and NAD⁺ fixed at 1.0 mM at the varied pH.

2.2.19. Steady state kinetics

2.2.19.1. Dehydrogenase kinetics

All kinetics experiments were performed at 25°C in 0.1 M tris/HCl, pH 9.0 except for RLADH. It was performed in 0.1 M sodium pyrophosphate buffer, pH 9.0. For RLADH, reaction mixtures contained 0.5 ml buffer, varied concentration of NAD⁺ (0.1-2.0 mM), varied concentration of ethanol (0.5-10.0 mM) and 50µl of enzyme with protein concentration of 1.0 mg/ml. In FLADH, NAD⁺-linked reactions used lower enzymatic concentrations (5.0-6.0 mg/ml) than the NADP⁺-linked reactions. Reaction assay mixtures contained 0.1-20.0 mM NAD⁺ or 0.5-13.3 mM NADP⁺, and 0.5-10.0 mM ethanol. In contrast, in TBADH, the NAD⁺-linked reactions employed higher enzymatic concentrations (7.5 mg/ml) than NADP⁺-linked reactions (3.0-5.0 mg/ml). Assay mixtures contained varied concentrations of NAD⁺ or NADP⁺ and varied concentration of propan-2-ol or ethanol as substrate as indicated in figures legends and axes. In general NAD(P)⁺ concentrations ranged between (0.1-20.0 mM) and propan-2-ol or ethanol concentrations ranged between 0.4-20.0 mM.

2.2.19.2. Dehydrogenase product inhibition studies

The products of a reaction can be used to investigate the kinetic mechanism of an enzyme through inhibition studies. The experimental protocol for a multisubstrate enzyme involves holding one substrate or more constant at

nonsaturating level, and varying the other substrate in a concentration range around its Michaelis constant. The product (one at a time) is added at several fixed concentrations (including zero). Each substrate and product is evaluated in this manner. Kinetic experiments for TBADH were performed in the presence of either 2-propanone or reduced coenzyme (NADPH) by variation in either propan-2-ol or NADP⁺ concentration while holding the other at fixed concentration. For FLADH, the product inhibition studies were performed in presence of either acetaldehyde or reduced coenzymes NADH or NADPH and again by variation in either ethanol or NAD(P)⁺ concentration while holding the other at fixed concentration. Stock solutions of acetaldehyde or 2-propanone used were 10.0 mM. Reduced coenzyme stock solutions were 1.0 mM for FLADH product inhibition experiments. In TBADH product inhibition studies, higher concentration of reduced coenzyme solutions were required. Stock solution (10.0 mM) of reduced coenzyme NADPH was prepared. Concentrations used in the experiments are indicated in the corresponding figures legends.

Cross product inhibition studies by NADPH on NAD⁺-linked activity and vice versa, was performed for FLADH to investigate the duality of the active site. The kinetic assays were performed as described above, using stock solution of NADPH (1.0 mM) to inhibit NAD⁺-linked activity

and a stock solution of NADH (1.0 mM) to inhibit NADP⁺-linked activity.

2.2.19.3. Esterase kinetics of FLADH

The esterase kinetic studies of FLADH were carried out in 50 mM sodium borate buffer, pH 8.2 at 25°C.

The effect of variation in ester chain length on esterase kinetics was investigated. Stock solutions of p-nitrophenyl ester substrates (5.0 mM) were prepared freshly. Ester substrates were prepared in 5.0 mM 1,4-dioxane to limit spontaneous hydrolysis. The kinetic assays were performed using a series of p-nitrophenyl esters ranging from two to twelve carbons in length. The concentration were varied and specified in the figures legends.

2.2.19.4. Dismutase kinetics of FLADH

The dismutase kinetics of FLADH was performed in 50 mM sodium phosphate buffer, pH 7.4 at 25°C. The stock solutions of octanal (10.0 mM) and NAD⁺ (0.10 M) were prepared freshly. Reaction mixtures contained 0.5 ml of buffer, varied concentrations of octanal (0.015-0.40 mM), NAD⁺ ranging in concentration from 0.5 mM to 6.0 mM and 50 µl of enzyme solution. Enzyme concentration as determined by Lowry assay was on average 0.98 mg/ml.

2.2.20. Chemical modifications

The information concerning the chemical basis of enzyme function can be obtained through the application of protein modifications. One of several possibilities is the correlation of the change in enzymatic activity with the modification of active site residue(s). Purified alcohol dehydrogenase enzymes were each subjected to several chemical modifications using a number of reagents intended to target specific amino acid residues.

Modifications described below were intended for activity studies. Aliquots were removed at timed interval during the course of reaction. Unless otherwise indicated, aliquots removed were used directly for activity assay only when sufficient controls indicated no change in activity due to residual reaction over the assay period. Enzyme and modifying reagents concentrations employed varied from one enzyme to another and also with type of modification performed. The procedures described below are general ones.

2.2.20.1. Acetamidination

A stock solution of methylacetimidate hydrochloride was prepared in sodium phosphate, pH 7.5. Reaction was initiated by addition of 10-15 μ l of methylacetimidate stock solution (200 mg/ml) to 0.5-1.0 ml of purified enzyme preparation with mixing on ice (120).

Catalytic activity was measured by withdrawing 50 μ l aliquots at suitable time intervals. Activities assayed were followed up to 1 hour after initiation of reaction.

2.2.20.2. Amidation

Carboxyl groups of proteins can be converted to amides through reaction with one of several amines promoted by a water-soluble carbodiimide (WSC). Reaction mixtures consisted of EDC, alcohol dehydrogenase and in some cases NAD(P)⁺ in 0.1 M MES buffer pH 6.0 (95, 121). Reaction was initiated by addition of the amine with mixing at 0°C. Aliquots were removed at various times for activity measurements. Amidation was performed using various amine nucleophiles, bearing different charged groups. Nucleophiles used were methylamine, ethylenediamine, n-propylamine and taurine (aminoethylsulfonic acid). Controls were prepared in parallel experiments by omitting amine nucleophiles from the reaction mixtures.

2.2.20.3. Carboxymethylation

Fresh stock solutions of 0.20 M iodoacetate or iodoacetamide were prepared prior to their use in 0.1 M sodium phosphate buffer pH 7.5, since they are subjected to slow hydrolysis. To enzyme solution (0.5-1.0 ml) with varying concentration, 10-20 μ l of above alkylating stock solution was mixed at 0-4°C (122-124). Aliquots were removed

at specific times between 2 and 120 minutes, and both modified and unmodified control enzymes were assayed spectrophotometrically for NAD⁺ and NADP⁺-linked activities, dismutase and esterase activities.

The bacteria enzyme TBADH was carboxymethylated with iodoacetate or iodoacetamide in presence of 0.5 mM NADP⁺. Aliquots of enzyme solution were removed and desalted through Bio-gel P-6 column and assayed for DHase activities, and proteins concentration using Lowry procedure (106).

2.2.20.4. Dye-sensitized photo-oxidation

Purified enzyme solutions (2.0 μ M-32 μ M) in 0.1 M sodium phosphate buffer, pH 7.5-8.0 in the presence of a dye sensitizer (50 μ M-1.2 mM) was placed in a water jacketed cell (25°C) and illuminated from a distance of 25-28 cm with a 250 W tungston lamp (87, 125, 126). Samples (50 μ l) of reaction mixtures were withdrawn at time intervals for enzymic assays. Controls were treated as above with the dye sensitizer being excluded from reaction mixture. Among the commonly used dyes , two low energy sensitizers (methylene blue and rose bengal) and one higher energy sensitizer (riboflavin) were used. The RLADH was modified with these three sensitizers. The FLADH and TBADH modifications were performed using rose bengal only.

2.2.20.5. Glycosylation

Glycosylation was performed either in 10 mM tris/HCl buffer pH 7.5, or 0.1 M sodium phosphate buffer pH 7.5 (83). Purified enzyme solutions (0.2 ml- 0.6 ml) with protein concentration ranging from 0.5 mg/ml - 3.0 mg/ml were mixed with varied concentration of D-glucose (0.33 mg/ml- 42.0 mg/ml). Aliquots were withdrawn for NAD⁺ and NADP⁺-linked activities. Dismutase and esterase activity were assayed at 60 minutes after initiation of reaction and expressed with respect to a control. The enzyme, RLADH (0.2 mg-0.4 mg) was modified also by addition of 0.13 mg of D-fructose. Aliquots were used for NAD⁺-linked activity assays.

2.2.20.6. Glyoxalation

Cyclohexanedione and certain 1,2- diketones such as phenylglyoxal have been shown to react with arginine residues in proteins in aqueous solutions between pH 8 and 9 (74, 127). This modification was carried out with FLADH, RLADH and TBADH. The exact specifications are presented with the graphic results of each modification. For FLADH and TBADH the reactions were performed in 0.1 M borate buffer, pH 8.2 with phenylglyoxal in excess (4.2-50.6 mM). Enzymes concentration ranged between 0.78 mg/ml to 5.0 mg/ml. In protection experiments, enzyme was incubated with NAD(P)⁺ in the concentration indicated in figure legends before phenylglyoxal addition. Reactions were followed up to 60

minutes after initiation. Aliquots removed were used without further purification for NAD⁺ and NADP⁺-linked activities.

Rat liver alcohol dehydrogenase (RLADH) was modified with phenylglyoxal as well as 1,2-cyclohexanedione (128). Modifications were carried out in three different buffers which were; 0.10 M sodium bicarbonate buffer, pH 7.9; 0.10 M sodium borate buffer, pH 8.2 and 40 mM tris/HCl buffer, pH 8.2. Enzyme, phenylglyoxal and 1,2-cyclohexanedione concentrations were varied, with ratio of reagent to enzyme varied from a minimum molar excess of 100 to maximum molar excess of 220. Reactions were allowed to proceed for 1 hour and aliquots were withdrawn for enzymatic assays.

2 2.21. Radioactive incorporation of methylamine

The radioactive incorporation of [¹⁴C]-methylamine by FLADH and YADH were investigated. Purified YADH enzyme solution 0.5 ml (1.15 mg/ml) was mixed with 0.5 ml MES buffer, pH 6.0 which contained 5.0 mg EDC. To enzyme and EDC mixture 5 μ l of 0.4 mM ¹⁴CH₃NH₂ (specific activity = 51.8 mCi/mmol) was added. Control was treated same way but omitting EDC from the reaction mixture. In FLADH labelling experiment, 0.7 ml of purified enzyme solution (0.48 mg/ml) was mixed with 0.7 ml of 0.10 M MES buffer, pH 6.0 that contained 7.0 mg EDC. Reaction was initiated by addition of 7 μ l of 0.4 mM [¹⁴C]-methylamine (specific activity = 51.8 mCi/mmol). Control was prepared as for YADH. At time

intervals, two 50 μ L aliquots were removed from each sample, and absorbed onto two Whatman 3 MM paper disk mounted on straight pin. The disks were immediately placed in ice cold 10% (w/v) TCA bath (minimum volume 400 ml), and left in TCA bath for at least 30 minutes. The disks were placed on a large filter paper (Whatman No. 1, 12.5 cm diameter) in a Buchner funnel. The disks were rinsed 3 times with 200 ml of ice-cold 5% TCA, followed by 3 X 200 ml of ether-ethanol mixture (1:1). The disks were then air dried briefly to remove ether and placed in scintillation counter vials and covered with 10 ml of Scintiverse E cocktail and counted in Beckman LS 150 scintillation counter.

2.2.22. ^{31}P -NMR spectroscopy studies

Affinity purified enzymes, YADH, FLADH and TBADH were extensively dialyzed against 10 mM tris/HCl buffer, pH 7.5 or passed through Bio-gel P-6 column (13.6 X 2.5 cm) equilibrated with above buffer. Pooled enzymes were lyophilized. The freeze dried sample was dissolved in D_2O , then lyophilized and this cycle was repeated at least twice. The final lyophilized enzyme was stored at -20°C under vacuum until use. Enzyme was dissolved in 0.5-0.7 ml of deuterated 10 mM tris-DCI, pD 7.5. This buffer was prepared by adding required volume of D_2O to the appropriate amounts of deuterated tris buffer salts. The pD was adjusted by addition of DCI or NaOD and apparent pH in

D₂O were converted to pD as mentioned in section 2.2.1. Deuterated tris salts, NAD⁺ and NADP⁺ were prepared by repeated dissolution in D₂O and lyophilization (three cycles).

The spectra of the free 5.0 mM coenzymes NAD⁺ and NADP⁺ were obtained conveniently by monitoring the ³¹P chemical shift of the 2'-phosphate group in NADP⁺ and ³¹P chemical shift of the pyrophosphate in both NAD⁺ and NADP⁺. The various concentrations (1.0 mM-50.0 mM) of NAD(P)⁺ in the presence of enzymes were scanned similarly. This comparison study was performed for YADH, TBADH and FLADH. All spectra were taken at room temperature (20°C) in 10 mM tris/DCl pD 7.5. Enzyme concentration, however, varied from one enzyme to another; but was kept constant within the study for each specific enzyme. The ³¹P-Nuclear magnetic resonance spectra were recorded with a Varian XL-200 NMR spectrometer equipped with a Nicolet TT-100 data acquisition system. All spectra were taken in a 5 mm tube using 80% H₃PO₄ as a reference under same conditions. An average of 1500 transients were accumulated for each spectrum. The following represents the system parameters used for obtaining the ³¹P-NMR spectra.

Aquisition

Transmitter nucleus	31.000
Sweep width	4000.0 (HZ)
Aquisition time	0.500 (S)
Pulse width	10.0 (μS)

Delay	1.000 S
Transmitter offset	-1000 HZ

Decoupling

Decoupler nucleus	1.700
Decoupler mode	NNN

Processing

Line broadening	5.000 (HZ)
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Enzymes were assayed before and after ^{31}P -NMR scans to ensure no change in enzyme properties had occurred.

The association constant (K_a) between a ligand and an enzyme can be calculated according to equation below:

$$\frac{1}{\delta_{obs} - \delta_a} = \frac{1}{K_a E_t (\delta_{ea} - \delta_a)} + \frac{1}{E_t (\delta_{ea} - \delta_a)}, A_t$$

where δ_{obs} and δ_{ea} are observed chemical shifts and chemical shift of complexed ligand, respectively, and δ_a is the chemical shift of free ligand. The parameters A_t , E_t , and K_a refer to total ligand concentration, total enzyme concentration, and association constant respectively.

2.2.23. Ligands (NADP⁺) ³¹P-NMR spectra in presence of modified enzymes

Purified enzymes were subjected to a chemical modification targeted at a specific amino acid residue. The effect of chemical modifications on these amino acids and in their role in ligand binding to enzyme was monitored by ³¹P-NMR. Following modifications were carried out; amidation and phenylglyoxalation for FLADH, amidation and phenylglyoxalation for TBADH and finally, amidation for YADH.

To prepare the enzyme for ³¹P-NMR spectra, purified enzyme solution was subjected to the specific modification. The reactions were allowed to proceed until the desired change in activity had occurred. The reaction was stopped immediately by filtering through a Bio-gel P-6 column. Pooled fractions were assayed for enzymatic activity, protein content and lyophilized. Freeze dried sample was dissolved in deuterated buffer and lyophilized. This process was repeated twice and final sample was stored as before under vacuum at -20°C. Modified enzyme was then dissolved in deuterated 10 mM tris /DCl, pD 7.5 in the presence of 1.0 mM NAD(P)⁺. The spectra were obtained as before and compared to native enzyme under same conditions.

CHAPTER THREE

RESULTS

3.1. Purification of enzymes

3.1.1. Rat liver alcohol dehydrogenase (RLADH)

Alcohol dehydrogenase was purified to homogeneity from rat liver cytosol. In initial preparations, studies were performed to increase or at least maintain enzyme stability throughout the purification procedure. Sulfhydryl reagent such as DTE was required to maintain the stability. Enzyme precipitated from 35-60 % saturation with $(\text{NH}_4)_2\text{SO}_4$ was dialyzed in 10 mM sodium phosphate buffer at different pH, and stabilizing reagents for 15 hours. Table 3.1 summarizes the results. High NAD^+ -linked activity was obtained in 10 mM sodium phosphate buffer at pH 8.4. Both 1% glycerol and PEG ($M_w = 20K$) enhanced enzyme activity more than 5% ethanol, but reduced flow rate through columns in the subsequent chromatographic steps. The highest dehydrogenase activity was obtained in 10 mM tris/HCl pH 8.4 containing 0.5 mM DTE and 5% ethanol.

The purification procedure consisted of ammonium sulfate fractionation as a first step. Fractionation range of 35-60% gave a good yield of RLADH. Other steps included anion exchange, gel filtration, affinity chromatography as the final step. Table 3.2 summarizes the purification

Table 3.1

Summary of NAD⁺-linked activity of RLADH in different buffers^a

Buffer	Activity (Units/ml) $\times 10^3$		
	5% ethanol	1% glycerol ^b	1% PEG ^b
NAPB pH 7.0	37.1 $\pm$ 4.2	---	---
NAPB pH 7.6	84.7 $\pm$ 5.3	---	---
NAPB pH 8.4	107 $\pm$ 21.2	244 $\pm$ 12.2	145 $\pm$ 16.7
tris/HCl pH 8.4	301 $\pm$ 39.8	---	---

a Buffer concentration are 10 mM, all contain 0.5 mM DTE.*b* Corrected enzyme activity.

Table 3.2Detailed protocol for the purification of NAD⁺ - linked activity of RLADH^a

Step	Total Volume (ml)	Protein mg/ml	Specific Activity (Units/mg) × 10 ³	Purification	Yield (%)
Crude	80.0 ± 52.6	100 ± 7	2.27 ± 0.9	1.0	100
35-60% (NH ₄) ₂ SO ₄ Fraction	30.5 ± 13.4	60.3 ± 3.9	5.0 ± 0.8	2.20 ± 1.5	50.3 ± 32.6
Ultra super- natant	33.3 ± 7.9	40.7 ± 9.8	8.15 ± 1.9	3.58 ± 1.7	60.5 ± 44.1
DEAE-A50 pool	77.0 ± 34.2	1.15 ± 0.65	53.6 ± 26.7	23.6 ± 10.3	25.8 ± 8.1
G-100 pool	15.4 ± 4.6	0.28 ± 0.4	176 ± 83	77.6 ± 42.6	4.2 ± 0.6
AG-NAD ⁺ pool	6.5 ± 1.4	0.25 ± 0.4	285 ± 36	125 ± 25	2.5 ± 1.1

a Average data of four experiments.

procedure with respect to NAD⁺-linked activity and represents an average data of four independent trials using 16-40 g of liver per experiment.

Earlier preparations of enzyme involved use of the cationic exchanger CM-52 Cellulose with a salt gradient of 5.0-50 mM tris/HCl buffer, pH 8.4. Enzyme was eluted in the washings at the lower salt concentration (Figure 3.1). Enzyme recovered as high UV absorbing band with low specific activity and purification. Thus, CM-52 Cellulose chromatographic media was rejected on this basis. The anion-exchange DEAE-A50 Sephadex at pH 8.4 likewise resulted in nonbinding of the enzyme. However when the enzyme was applied onto this column, a UV absorbing, unretarded colorless protein band which passed through column, was pooled (Figure 3.2). Bulk of red-colored pigments were detained on exchanger, though they could be partially eluted with continued washings. Therefore, it was necessary to use a column with sufficient bed height. Mass loading on anion exchanger was increased proportionately to the increased volume of the larger columns. Though enzyme was not bound to ion exchanger, conditions such as choice of buffer salt, pH were adjusted to facilitate free passage of enzyme. Higher flow rates, high specific activity and significant purification fold led to the selection of this anion exchanger as the first chromatographic step.

Figure 3.1. CM-Cellulose chromatographic profile of RLADH. Protein was eluted with a linear 5-50 mM NaCl gradient in 10 mM tris/HCl, pH 8.4. (O) is NAD⁺-linked activity.

Figure 3.2. DEAE-A50 Sephadex chromatographic profile of RLADH in tris/HCl, pH 8.4, DTE and 5% ethanol. NAD⁺ (O) and NADP⁺ (Δ) activities were determined.

Gel filtration using Sephadex G-100 afforded at most 3-fold purification. Small volume fractions had to be collected in order to obtain good resolution, and separate the high UV absorbing inactive band from DHase activity band (Figure 3.3). This step was necessary to produce a purified homogenous RLADH. Trials, in which this step was omitted, yielded a non homogenous enzyme preparation with large number of bands revealed by SDS-discontinuous gel electrophoresis (Plate 1).

Affinity chromatography as final step in the purification sequence gave rise to a final 125-fold increase in specific activity. Both AG-NAD⁺ type 1 and type 3 were examined at this point. Affinity chromatography with AG-NAD⁺ type 1 consistently gave the elution profile shown in Figure 3.4. Enzyme activity was eluted with low concentration of the coenzyme NAD⁺ (129). Purification from AG-NAD type 1 was only about 2.0-fold with 2.5% recovery. This step was necessary to obtain a final homogenous preparation as revealed by SDS-discontinuous electrophoresis (Plate 2). Other biospecific adsorbents such as Amicon Matrex Gel Blue A and matrex Gel Red A were assessed also. The blue dye (Cibacron blue F3G-A) and red dye (Procion Red HE3B) have the capability of binding strongly to many proteins including kinases, dehydrogenases and most other enzymes requiring adenylyl-derivatives (e.g. NAD⁺) (130-133). Blue and red dye affinity gels also bind several non-enzymic

Figure 3.3. Gel filtration of RLADH on G-100 Sephadex in tris/HCl, pH 8.4, 0.5 mM DTE and 5% ethanol. Fractions were 1.5 ml each, where (O) is NAD⁺-linked activity.

Figure 3.4. Elution profile of ALADH from AG-NAD⁺ type 1 affinity column. Bound enzyme was released on addition of 10 mM NAD⁺ in buffer as indicated by the arrow. NAD⁺-linked activity is (O).

2

MICROD

1 2 3 4

Plate 1

Plate 2

proteins (134). These two dyes were used as well to purify RLADH. Such trials were characterized by low activity recovered caused by the irreversible binding of enzyme to those dyes. High salt or high coenzyme concentration failed to elute RLADH. Since relatively high specific activity and purification was obtained with AG-NAD⁺ type 1, this affinity absorbant (AG-NAD type 1) was employed in the final step of purification.

The presence of 5% ethanol in all buffers, masked the dimutase activity. Both dismutase and esterase activity were not followed during the different purification steps. They were assayed in the final NAD⁺ and ethanol free affinity pool. The NADP⁺-linked activity was very low to be followed, but its elution behavior from anion exchanger DEAE-A50 (Figure 3.2) was very similar to that of NAD⁺-linked activity.

3.1.2. Fish liver alcohol dehydrogenase (FLADH)

The procedure developed for RLADH was common and could be well applied to the purification of ADHs from other mammals, plants (61) and bacteria. The enzyme required DTE or DTT but not ethanol for stability. Table 3.3 summarizes the purification of NAD⁺ and NADP⁺-linked activities throughout the different steps. Table 3.4 shows the purification of dismutase and esterase activities of FLADH. All activities coeluted in same peak from both the

Table 3.3
Summary of the purification procedure for NAD⁺ and NADP⁺-linked activities of FLADH^a

Step	Total Vol. (ml)	Protein (mg/ml)	Specific Activity ^b		Purification		Yield (%)	
			NAD ⁺	NADP ⁺ × 10 ³	NAD ⁺	NADP ⁺	NAD ⁺	NADP ⁺
Crude	168 ± 109	46.3 ± 9.1	(3.17 ± 2.69) × 10 ⁻³	1.32 ± 1.01	1.0	1.0	2.41	100
30-60% (NH ₄) ₂ SO ₄	36.6 ± 10.9	26.5 ± 13.8	(21.4 ± 7.9) × 10 ⁻³	2.67 ± 0.67	6.76 ± 3.6	1.98 ± 1.41	8.2	84.5 ± 42.4
Ultra super- natant	29.7 ± 15.0	12.6 ± 5.1	(47.1 ± 28.3) × 10 ⁻³	2.70 ± 1.43	14.9 ± 6.2	2.05 ± 1.20	17.0	71.9 ± 46.4
DEAE-A50 (Dilute)	106 ± 32.5	0.32 ± 0.1	0.266 ± 0.180	50.1 ± 22.0	83.8 ± 36.6	37.9 ± 13.0	5.3	61.3 ± 42.6
DEAE-A50 (Concentrated)	19.7 ± 5.14	1.72 ± 0.30	0.223 ± 0.137	50.9 ± 11.7	70.2 ± 59.2	38.6 ± 4.1	4.4	30.6 ± 14.1
G-100 pool	26.5 ± 1.3	0.70 ± 0.52	0.298 ± 0.193	31.8 ± 25.6	94.1 ± 20.3	24.1 ± 16.5	9.4	22.4 ± 12.7
AG-NAD ⁺ pool	10.5 ± 3.4	0.25 ± 0.18	0.429 ± 0.021	81.5 ± 14.3	136 ± 55	64.1 ± 29.1	5.07	4.58 ± 0.73
								2.18 ± 1.64

^a Average data of four experiments.

^b Specific activity is in Units/mg.

Table 3.4

Summary of the purification of dismutase and esterase activities of FLADH^a.

Step	Total vol. (ml)	Protein (mg/ml)	Specific Activity ^b		Purification		Yield (%)	
			Dmase	Esase	Dmase	Esase	Dmase	Esase
Crude	80.0	33.8	2.00	3.22	1.00	1.00	100	100
30-60% (NH ₄) ₂ SO ₄ supernatant	21.0	13.2	1.17	64.9	0.58	20.2	6.0	207
Ultra super- natant	20.0	11.6	4.43	196	2.22	60.9	19.0	523
DEAE-A50 pool (dilute)	90.0	0.35	4.59	523	2.30	162	2.7	190
G-100 Sephadex pool	22.7	0.88	3.94	51.7	1.97	16.0	1.46	12.0
AG-NAD ⁺ pool	5.0	0.20	9.65	179	4.8	55.7	0.18	2.1

*a Average of two experiments.**b Specific activity in Units/mg $\times 10^3$.*

anionic and gel filtration chromatographic steps (Figures 3.5, and 3.6). Affinity chromatography was the final step employing AG-NAD⁺ type 3 as the absorbant. Activities followed were the esterase and NAD⁺-linked activity. Elution profile (Figure 3.7) shows enzyme eluted with 10 mM NAD⁺ in one peak with high NAD⁺-linked activity and low esterase. High esterase activity was eluted in the adjacent peak.

Co-elution of all activities throughout the various steps and electrophoresis of the final isolated enzyme (Plate 3) demonstrated chromatographic and electrophoretic homogeneity. The NAD⁺/NADP⁺ activity ratio could not be maintained at a relatively constant value during the different steps. The ratio ranged between 2.0 to 17.0 (Table 3.3); this will be discussed later in section 3.2. The DEAE-A50 Sephadex anionic exchanger and G-100 Sephadex were replaced by fractogel TSK DEAE-650 S and fractogel TSK HW 55, respectively. Elution profiles clearly showed sharper peaks and better resolution (Figure 3.5 inset, Figure 3.8).

3.1.3. *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH)

This enzyme had been obtained by Lamed and Zekius as a 44 fold purified preparation that gave one band after SDS-polyacrylamide gel electrophoresis (10). The enzyme used in this study was supplied by Sigma. The enzyme obtained had been partially purified by a heat treatment step (10)

Figure 3.5. Elution profile of multifunctional activities of FLADH from DEAE-A50 Sephadex in 10 mM Tris/HCl, pH 7.5 buffer. Activities are NAD⁺ (O), NADP⁺ (Δ), dismutase (□) and esterase (●). Inset shows elution profile of NAD⁺-linked activity (O) from Fractogel TSK DEAE 650 S.

Figure 3.6. Gel filtration of multifunctional activities of FLADH on G-100 Sephadex in 10 mM tris/HCl, pH 7.5. Activities followed are NAD⁺ (○), NADP⁺ (Δ), dismutase (□) and esterase (●).

Figure 3.7. Elution profile of NAD⁺-linked and esterase activities from AG-NAD⁺ type 3 affinity gel. Activities were eluted by 10 mM tris/HCl, pH 7.5 containing 10 mM NAD⁺. Activities are: NAD⁺-linked activity (O), and esterase (●).

1 2 3 4 5

Plate 3

Figure 3.8. Elution profile of FLADH on Fractogel TSK HW 55, in 10 mM tris/HCl, pH 7.5, 0.5 mM DTT, where (O) is NAD⁺-linked activity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated the presence of many bands (Plate 4). The procedure developed for purification of this enzyme is similar to RLADH and FLADH purification procedures. The anion exchanger DEAE-A52 Cellulose required a sodium chloride gradient to desorb the enzyme off column. The enzyme is more active toward secondary alcohols than other alcohols (10). Activity of TBADH was followed using propan-2-ol as a substrate with either NAD⁺ or NADP⁺ as a coenzyme. Enzymic activity of TBADH with primary alcohol propan-1-ol was assayed as well using NADP⁺ as a coenzyme. All activities were followed through DEAE-A52 Cellulose and G-200 Sephadex, elution profiles are represented in Figures 3.9 and 3.10. Activities coeluted in the same peak from both anion exchange and gel filtration chromatographic steps.

The final step was an affinity chromatography. This type of adsorption chromatography is dependent on a specific reversible adsorption of the enzyme to the ligand immobilized on an insoluble support. Such type of specific adsorption was not obtained when Sigma AG-NADP⁺ affinity gel was used. Enzymic activity was recovered in the washing and nonhomogenous TBADH preparation was obtained (Plate 4, line 3). Binding of TBADH was achieved when P.L. Biochemicals AG-NADP⁺ type 3 was used. Elution of enzyme commenced after addition of 10 mM NADP⁺ in equilibrating buffer (Figure 3.11). Enzyme preparation was pure as seen by the one band

Figure 3.9. Elution profile of TBADH activities from DEAE-A52 Cellulose in 10 mM tris/HCl, pH 7.5, 0.5 mM DTT. Activities followed were (O) NAD⁺-linked with propan-2-ol and (Δ) NADP⁺-linked with propan-2-ol and (▲) NADP⁺-linked with propan-1-ol.

Figure 3.10. The G-200 Sephadex chromatographic profile of TBADH, where (O) NAD⁺-linked activity with propan-2-ol, (Δ) NADP⁺-linked activity with propan-2-ol and (▲) NADP⁺-linked activity with propan-1-ol.

obtained by SDS-gel electrophoresis (Plate 5). Some of the enzymic activity was recovered in the washing, but constituted a low percentage (11.2%) of total units loaded on the column. The total purification was low (c.f. Table 3.5) though it could not be compared to the crude extract.

3.1.4. Yeast alcohol dehydrogenase (YADH)

The purification of this commercial crude enzyme (Plate 6) was achieved by a single step. Both AG-NAD⁺ type 1 and type 3 were examined. The enzyme did not exhibit any binding for AG-NAD⁺ type 3, therefore does not yield satisfactory results in terms of purification. Enzyme exhibited some binding to AG-NAD⁺ type 1. Enzymic activity was recovered in the buffer washings, as well as, in NAD⁺ pulse (Figure 3.12). This resulted in low total activity recovery and purification fold. Such behaviour could be due to saturation of the affinity column with respect to this enzyme. In Plate 6, gel electrophoresis of the purified enzyme obtained by NAD⁺ pulse revealed only one band.

3.2. Characterization and some physical properties of the enzymes

The enzymes purified were subjected to electrophoresis under denaturing conditions. The gels were stained using silver stain, that are 100 times more sensitive for proteins than the commonly used Coomassie blue. Plates 2, 3, 5 and 6

Figure 3.11. Affinity chromatography of TBADH on AG-NADP⁺ type 3 in 10 mM tris·HCl, pH 7.5 and 0.5 mM DTT. Arrow indicates addition of 10 mM NADP⁺. Activity followed is (Δ) NADP⁺-linked activity with propan-2-ol.

i

1 2 3
Plate 4

1 2 3 4 5
Plate 5

Table 3.5

Summary of the purification of NAD⁺ and NADP⁺ -linked activities of TBADH^a.

Step	Total vol. Protein (ml)		Specific Activity ^b NAD ⁺ × 10 ³ NADP ⁺		Purification NAD ⁺ NADP ⁺		Yield (%) NAD ⁺ NADP ⁺	
Crude	3.35 ± 0.21	10.7 ± 0.5	7.33 ± 1.22	0.217 ± 0.092	1.00	1.00	29.7	100
DEAE-A52 pool	16.5 ± 11.2	0.990 ± 0.370	2.76 ± 2.07	0.294 ± 0.009	0.38 ± 0.21	1.36 ± 0.35	107	17.2 ± 9.7
G-200 Sephadex pool	39.1 ± 14.1	0.113 ± 0.088	16.5 ± 5.32	0.820 ± 0.065	2.25 ± 1.64	3.78 ± 0.21	49.8	27.8 ± 15.6
AG-NADP ⁺ (type 3) pool	8.55 ± 0.64	0.093 ± 0.054	97.1 ± 67.0	1.70 ± 0.46	13.3 ± 5.6	7.84 ± 3.42	17.6	29.5 ± 4.4
								17.5 ± 8.1

^a Activities were measured with propan-2-ol as substrate.^b Specific activity are in Units/mg.^c Average data of four experiments.

Figure 3.12. Elution profile of YADH on AG-NAD⁺ type 1 in 10 mM tris/HCl, pH 7.5, 0.5 mM DTT. Arrow indicates addition of 10 mM NAD⁺, and (O) is NAD⁺-linked activity.

1 2 3 4

Plate 6

indicated the purity of these enzymes prepared by the purification procedures described in the experimental section. The relative mobility (R_f) of the SDS-treated enzymes were compared to a linear regressed standard curve prepared from reference proteins that were run under the same conditions. The apparent subunit molecular weights of sample proteins were then determined (Figure 3.13).

Molecular weights of native enzymes were obtained by zonal sedimentation velocity method. The proteins were sedimented as a zone through a performed sucrose density gradient. The ratio (D_1/D_2) is ratio of the distance that the unknown enzyme sediments to the distance that standard protein sediments from the meniscus.

Molecular weights of the enzymes were calculated according to the equation in section 2.9., as

$$\begin{aligned} d_1/d_2 &\propto v_2/v_1 = s_1/s_2 \\ &= (MW_1/MW_2)^{2/3} \end{aligned}$$

Molecular weights obtained are listed in Table (3.6). The effective hydrodynamic radius (Stokes radius) were determined by comparative elution of purified enzymes and reference proteins from a calibrated G-100 Sephadex. Results are an average of 4 trials, obtained from a plot of $(-\log K_{av})^{1/2}$ versus Stokes radii for the standards (Figure 3.14) according to (116). The TBADH effective hydrodynamic radius was obtained similarly, however from a G-200 Sephadex column. A similar plot of relative partition coefficient (-

Figure 3.13. The subunit molecular weights of alcohol dehydrogenases from SDS-discontinuous gel electrophoresis. Standards were (a) BSA, (b) ovalbumin, (c) HLADH, (d) trypsinogen, (e) β -lactoglobulin, (f) Lysozyme and enzymes were 1. FLADH, 2. TBADH, 3. RLADH, 4. YADH.

Table 3.6

Physical properties of different alcohol dehydrogenases

parameter	HLADH ^a	RLADH	FLADH	YADH ^a	TBADH
MW of Native enzyme (Daltons)	8.40×10^4	$8.15 \times 10^4 \pm 0.160$	$8.33 \times 10^4 \pm 0.413$	1.51×10^5	$1.60 \times 10^5 \pm 0.0406$
MW of Subunit (Daltons) $\times 10^4$	4.20	4.00	4.15	3.78	4.04
Proposed Structure (Native)	Dimer	Dimer	Dimer	Tetramer	Tetramer
Stokes Radius (Å)	32.3	30.8 ± 0.8	34.2 ± 1.4	45.0	42.0
f/f_0	1.23	1.08	1.19	1.27	1.17
PI	^b EE 8.7 ES 9.3 SS > 10	8.8, 8.6	8.05, 6.51	5.4-5.8	
$\epsilon_{280} \text{ cm}^2 \cdot \text{mol}^{-1}$	3.54×10^4			1.89×10^5	2.3×10^7

^a Data taken from (3).^b EE is ethanol active isozyme, while SS is steroid active isozyme.

$\log K_{av})^{1/2}$ against known Stokes radii of reference proteins was prepared for TBADH. Results are listed in Table 3.6.

Chromatofocusing of RLADH revealed three peaks detected by A_{280} absorbance. A low UV absorbing protein band with high activity at pH 8.8, and a high UV absorbing doublet but very low single peak activity at pH 8.65-8.62 (c.f. Figure 3.15a). The 8.8 isoenzyme of RLADH represent the well-studied cathodic form of RLADH and has been the subject of most of the previous work on RLADH (59). Julia et al. have reported in their study on tissue distribution of rat alcohol dehydrogenase that in rat liver two isoenzymes are present. They were named as ADH-2 and ADH-3 with isoelectric points of 5.95-6.3 and 8.25-8.4, respectively. Mezey and potter (58) isolated four ADH isoenzymes from rat liver by CM-cellulose chromatography. The isoenzymes were numbered in order of decreasing mobility towards the cathode. The isoenzyme having the highest mobility numbered as one. The distribution of total enzyme activity of the isoenzymes eluted from CM-cellulose column was as follows: isoenzyme 1, 7.5%; isoenzyme 2, 75.2%; isoenzyme 3, 13.5%, and isoenzyme 4, 3.8%. The isoelectric points for isoenzyme 1 through 4 were 8.8, 8.7, 8.0 and 6.9, respectively. Distribution of total enzyme activity eluted from the chromatofocusing column in this study, was 94.8% for isoenzyme with pI 8.8 and 5.2% for the isoenzyme with pI of 8.65-8.62.

Figure 3.14. Stokes radii calibration plot of proteins on G-100 (I) and G-200 Sephadex (II). Proteins were (a) YADH, (b) BSA, (c) HLADH, (d) ovalbumin, (e) chymotrypsinogen, (f) cytochrome c, (g) catalase, and enzymes were 1. FLADh, 2. RLADH, 3. TBADH.

Figure 3.15a. Chromatofocusing of RLADH on PBE 94 in the pH range 9-6. Enzyme (5.5 μ M) was applied. (O) is NAD⁺-linked activity.

The chromatofocusing of crude extract of FLADH (ammonium sulfate fraction) on PBE 94 exchanger in the pH range 9-6 (Figure 3.15b), revealed several protein bands (7 bands). Two dehydrogenase activity bands were detected. The peak with highest absorbance and pI 8.05 displayed both NAD⁺ and NADP⁺-linked activities. Second activity peak with pI 6.5 was characterized by low absorbance, lower NAD⁺-activity and no NADP⁺-linked activity was detected in this peak. The ratio of NAD⁺/NADP⁺ in the first isoenzyme was 4.4, with 23% and 90% recovery for NAD⁺-linked and NADP⁺-linked activities respectively. The NAD⁺-linked activity recovered in the second isoenzyme was only 4%, and total NAD⁺-activity recovered from chromatofocusing was about 27%. The low activity recovered was the reason for not including the chromatofocusing as a purification step.

An ultraviolet absorption spectrum (Figure 3.16), was obtained for TBADH. Extinction coefficient was calculated at the wavelength of maximum absorption (272 nm) and was found to be $2.30 \times 10^7 \text{ cm}^2 \cdot \text{mol}^{-1}$ (Table 3.6).

A detailed stability study (at 0-4 °C) was carried out for RLADH. The stability of fractions from different steps were tested over a period of time. The RLADH became more labile as purification progressed (Figure 3.17a). In the fraction from DEAE-A50, about 80% of the activity remained after five days, but in pure fraction from AG-NAD⁺, approximately 50%

Figure 3.15b. Chromatofocusing of FLADH on PBE 94 in the pH range 9-6. Crude (ammonium sulfate fraction) was applied, where (O) is NAD⁺-linked activity and (Δ) NADP⁺-linked activity.

Figure 3.16. Ultraviolet absorption spectrum of TBADH on a Beckman model 25. The spectrum was measured at 25°C for 1 cm light path in 10 mM tris/HCl, pH 7.5. Enzyme concentration is 13.9 μ M.

activity remained after same period. In purest fraction (P-6 fraction), only 10% activity was retained after two days.

Therefore, the effect of some stabilizing reagents on the stability of purified RLADH was evaluated (Figure 3.18). The effect of these same stabilizing reagents were followed for the hydrolytic (esterase activity), and compared to the dehydrogenase activity. Addition of ethanol and 0.5 mM DTT stabilizes the dehydrogenase activity (Figure 3.18a). Increasing DTT concentration to 5.0 mM activated the enzyme temporarily followed by rapid decrease in activity (activity dropped to 20% as compared to 48% with 0.5 mM DDT). The activation effect of NAD^+ (0.1 mM) continued over the assayed period. The effect of 5% and 0.5 mM DTT on esterase activity was similar to that of the dehydrogenase. In presence of ethanol, retained activity was 50% as compared to 30% in absence of ethanol after 18 days. Esterase activity was inhibited by 0.1 mM NAD^+ and 5.0 mM DTT caused an activation for up to 10 days followed by rapid deactivation.

The stability of FLADH and RLADH was compared (Figure 3.17a, b). The NAD^+ -linked activity of FLADH is much more stable than RLADH under similar conditions.

The thermostability of RLADH (NAD^+ -linked activity) is shown in Figure 3.19. The Arrhenius plot indicated that the temperature effect on velocities is nonlinear. The

Figure 3.17. Stability of enzyme fractions (at 0-4°C) from different purification steps for (a) RLADH, (b) FLADH where for both (●) ammonium sulfate fraction (O) DEAE-pool (Δ) G-100 pool (□) AG-NAD⁺ pool and (○) P-6 pool.

Figure 3.18. Effect of different stability reagents on RLADH in 10 mM tris/HCl, pH 8.3. Activities were (a) NAD⁺-linked activity, (b) esterase activity. Reagents were (O, ●) 0.5 mM DTT; (Δ, ▲) 0.5 mM DTT and 5% ETOH; (□, ■) 5.0 mM DTT and 5% ETOH; and (○, ●) 5.0 mM DTT and 0.1 mM NAD⁺.

Figure 3.19. Thermostability of NAD^+ -linked activity of RLADH. Enzyme concentration was 1.06 mg/ml in 10 mM tris/HCl, pH 8.3. Inset: Arrhenius plot of the data.

denaturation was biphasic with slow denaturation up to 50°C. At 55°C there was a rapid deactivation of the enzyme.

It is well established that horse liver alcohol dehydrogenase (HLADH) catalyzes dehydrogenation, dismutation and esterolysis (13). On this basis, the purified ADH's preparations from various species were assayed for these multifunctional activities. Table 3.7 shows animal enzymes exhibit dismutation and esterolysis activities in addition to the dehydrogenation reaction. Such activities were not found in yeast, plants (61) or bacteria ADHs. Both rat and fish ADH's displayed dismutase activity that was lower than what is observed in HLADH. Comparable esterase activities were observed in both the HLADH and RLADH. Esterase activity of FLADH was about two order of magnitude higher than that of mammalian enzymes.

Enzymes used in this study were either NAD⁺ or NADP⁺ specific. All these enzymes utilized the other cofactor to a varying degree (Table 3.8). For example, the pickerel FLADH is NAD⁺-specific but could use NADP⁺ as a coenzyme. The NADP⁺-linked activities of HLADH and RLADH were comparable, but lower than that observed in FLADH. No activity with NADP⁺ as coenzyme could be detected with YADH, unless very high concentration of enzyme was employed, or when the YADH was amidated (this will be discussed further in a following section). On the other hand, TBADH displayed preference for NADP⁺ as a coenzyme compared to NAD⁺.

Table 3.7

Multifunctional activities of alcohol dehydrogenases

Dehydrogenases	Specific activity (Units/mg)		
	DHase ^b	Dismutase	Esterase
Horse liver ^a	1.78	13.4×10^{-3} (133) ^b	3.56×10^{-3} (500) ^c
Purified yeast	2.57	0	0
Rat liver	0.247	8.2×10^{-3} (30)	13.7×10^{-3} (18)
Pickereel liver	0.429	9.65×10^{-3} (44)	1.8×10^{-3} (2.4)
Ta. brockii	0.0971	0	0

a Data taken from 61 for comparison.

b Dehydrogenase activity (DHase) in presence of NAD^+ as coenzyme.

c Number in parenthesis refer to the ratio of dehydrogenase to respective activity.

d Average of at least two determinations.

Table 3.8

Nicotinamide coenzyme specificity of alcohol dehydrogenases

ADH	Specific activity ^b (Units/mg)		
	NAD ⁺	NADP ⁺	NAD ⁺ /NADP ⁺
Horse liver	1.78	0.0206	86.4
Pickarel liver	0.429	0.0850	5.07
Rat liver	0.232	0.0135	17.2
Purified yeast	360	---	---
Ta. brockii	0.0971	1.70	5.71×10^{-2} (17.5) ^a

*a For this enzyme number in parenthesis refer to NADP⁺/NAD⁺ ratio.**b Average of three determinations.*

The activities of FLADH and TBADH for both coenzymes were followed through out the different steps. The elution peaks for the NAD⁺ and NADP⁺-linked activities were superimposed from two adsorbants whose selectivity was based on different molecular parameters. This may indicate the association of these two activities with the same protein. It was not possible to assay for both NAD⁺ and NADP⁺ linked activities from any of the affinity columns, but pooled fractions were assayed for the above activities after dialysis or filtration on a Bio gel P-6 column. Dialyzed fractions when subjected to SDS-discontinuous electrophoresis indicated the purity of enzymes. The ratios of the NAD⁺ to NADP⁺ linked activities during the different step were not constant for FLADH nor for TBADH. The purified enzymes were subjected to non-SDS electrophoresis, and stained for enzymic activity based on the specific reaction catalyzed by ADH. The enzymes, FLADH and YADH were stained for their activity using ethanol and either NAD⁺ or NADP⁺ as a coenzyme, while TBADH was stained for its activity using propan-2-ol as a substrate with either NAD⁺ or NADP⁺ as a coenzyme.

Scheme 3 shows the electron transfer dye staining system for dehydrogenases.

Scheme 3. Electron transfer dye staining system for dehydrogenases (114).

Scans of the stained gels are illustrated in Figures 3.20-3.22. It is important to realize that under most circumstances, electrophoresis gives a qualitative rather than a quantitative assessment of these activities. It is possible though, to obtain comparative information on the $\text{NAD}^+/\text{NADP}^+$ activities at any one substrate concentration. From the tracings it is obvious that for each enzyme the absorption peaks corresponding to the activity bands are superimposed but differ in their intensity indicating again the association of these two activities with the same protein.

Figure 3.20. Detection of FLADH dehydrogenase activities after non-SDS discontinuous gel electrophoresis. Staining dye system adapted from (114). (a) Densitogram of a gel after incubation with a solution of NAD⁺ and ethanol, (b) Densitogram of a gel after incubation with a solution of NADP⁺ and ethanol.

Figure 3.21. Detection of TBADH dehydrogenase activities after non-SDS discontinuous gel electrophoresis
 (a) Densitogram of a gel after incubation with a solution of NAD^+ and propan-2-ol; (b) Densitogram of a gel after incubation with a solution of NADP^+ and propan-2-ol.

Figure 3.22. Detection of YADH dehydrogenase activities after non-SDS discontinuous gel electrophoresis. Staining dye system adapted from (114). (a) Densitogram of a gel after incubation with a solution NADP^+ and ethanol, (b) Densitogram of a gel after incubation with a solution of NAD^+ and ethanol.

3.3. Steady state kinetic studies

3.3.1. General NAD⁺ and NADP⁺ dehydrogenase kinetics

Kinetic characterization of enzyme mechanisms frequently involves the use of initial velocity experiments. By inspection of initial rate plots one can distinguish between ping-pong and sequential mechanisms. In sequential class of bisubstrate enzyme-catalyzed reactions, substrates must bind in compulsory order prior to catalysis. For converging reciprocal plots (sequential mechanism), the general expression for the initial velocity dependence is shown in equation 1:

$$v = \frac{V_{max} [A] [B]}{[A][B] + K_b [A] + K_a [B] + K_{ia} K_b} \quad \text{.....1}$$

Where A and B are substrate concentrations; V_{max} is the maximum velocity; K_a , K_b , and K_{ia} are Michaelis constants for coenzyme (A), second substrate (B), and the inhibition constant for the coenzyme, respectively, according to Cleland's nomenclature (135). There are four basic bisubstrate mechanism that lead to the above steady state rate equation 1. These are the Ordered with one ternary complex, Ordered with two ternary complexes, Theorell-chance mechanism and the Rapid equilibrium random

mechanism. However, they have different product inhibition patterns.

Initial velocities in the asymptotic region which follow Michaelian kinetics were analyzed according to equation 1. Explanation of the kinetic parameters determination are provided in Appendix I.

A series of initial velocities were measured at different substrate concentrations for the NAD⁺ and NADP⁺-linked activities for each enzyme. Initial rate data were analyzed graphically by Lineweaver-Burk or double-reciprocal plots. Secondary plots were used to obtain the kinetic parameters. Kinetic parameters obtained for the NAD⁺ and NADP⁺-linked activities of different alcohol dehydrogenases are listed in Tables 3.9, and 3.10.

3.3.1.1. NAD⁺ kinetics of RLADH

Initial velocity data were analyzed with Lineweaver-Burk double-reciprocal plots (Figures.3.23a & b). An increase in velocity at higher NAD⁺ concentrations occurred at all ethanol concentrations. No substrate inhibition was observed in the concentration range used. Michaelis constant for both ethanol and NAD⁺ were in the millimolar range, as well as the dissociation constant of NAD⁺ (0.668 mM) which is considerably high. Lad and Leffert (56) in their study of RLADH from rat Fischer-344 obtained nonlinear double reciprocal plots at high ethanol concentration. The

Table 3.9

Kinetic parameters for the NAD⁺-linked activity of alcohol dehydrogenases^a

ADH	$V_{\max}$ (mM.min ⁻¹)	K_a (mM)	K_b (mM)	K_{ia} (mM)	$V_{\max}/E_t$ (min ⁻¹)	$[E_t]$ (μ M)
Horse liver ^b	0.0813	0.335	0.0687	0.263	1.87×10^3	0.0436
Fish liver	0.192	0.164	0.565	0.190	57.8	3.33
Rat liver	0.0491	0.117	0.204	0.668	86.2	0.569
Ta. Brockii ^a	1.67	46.8	1.10	92.1	714	2.34

a Parameters are obtained at pH 9.0 with ethanol, except in Ta. Brockii where substrate was propan-2-ol.

b Data are taken from (13).

c Average errors on the constants are calculated from correlation coefficients of linearly regressed data and are as follows:

$V_{\max} \pm 13.2\%$, $K_a \pm 29.8\%$, $K_b \pm 16.7\%$, $K_{ia} \pm 29.4\%$ and $V_{\max}/E_t \pm 8.7\%$.

Table 3.10

Kinetic parameters for the NADP⁺-linked activity of alcohol dehydrogenases^a

ADH	$V_{\max}$ (mM.min ⁻¹)	K_a (mM)	K_b (mM)	K_{ia} (mM)	$V_{\max}/E_t$ (min ⁻¹)	$[E_t]$ (μM)
Horse liver ^b	0.00179	0.714	6.73	1.02	0.681	2.63
Fish liver	0.136	0.121	0.469	0.691	5.09	26.7
Ta. brockii (EtOH)	0.0781	0.0656	6.27	0.178	50.1	1.56
Ta. brockii (propan-2-ol)	1.053	0.0763	0.337	0.0525	1.13×10^3	0.935
Ta. brockii (butan-2-ol)	2.17	--	0.557	--	1.39×10^3	1.56

a All parameters are obtained in tris/HCl, pH 9.0 with ethanol as a substrate unless otherwise stated.

b Data from C.S. Tsai (personal communication).

c Average errors on kinetic parameters are as follow:

$V_{\max} \pm 3.5\%$, $K_a \pm 18.6\%$, $K_b \pm 14.4\%$, $K_{ia} \pm 26.4\%$ and $V_{\max}/E_t \pm 4.8\%$.

comparison study of Lad and Leffert (57) of RLADH from Fischer-344 and Sprague-Dawley showed the same kinetic properties. They obtained identical Lineweaver-Burk plots, when activity was assayed in 0.5 M tris/HCl buffer, pH 7.2 and 8.3 for ADH from both rat types. For both enzymes the K_m for ethanol was 0.4 mM (at pH 7.2), 0.1 mM at pH 8.3. The K_m for ethanol obtained in our study is 0.204 mM in 0.1 M pyrophosphate buffer, pH 9.0. All four kinetic constants for the forward reaction (Table 3.9) were obtained by replotting the slopes (apparent K_m /apparent V_{max}) and intercepts ($1/\text{apparent } V_{max}$) from Figure 3.23a against $1/[\text{Ethanol}]$ as in Figure 3.23b.

3.3.1.2. Pickerel FLADH

The ability of this enzyme to use both NAD^+ and NADP^+ was further investigated kinetically. Curves for the velocity dependence on varied coenzyme concentrations at fixed ethanol concentration (10 mM) were prepared for both NAD^+ and NADP^+ (Figure 3.24).

The NAD^+ velocity plot followed a normal hyperbolic pattern. Maximum activity was obtained at NAD^+ concentration equal to 0.5 mM. The activity plateau was maintained up to NAD^+ concentration equal to 13.3 mM, with 5% inhibition in the activity. An increase in NAD^+ concentration to 20.0 mM caused 23% inhibition. The NADP^+ velocity plot, differed markedly from that of NAD^+ linked-activity. The maximum

Figure 3.23a, b. Double reciprocal plot of NAD^+ -linked activity of RLADH. Enzyme ($0.5 \mu M$) in $0.1 M$ pyrophosphate, pH 9.0. (a) Primary Lineweaver-Burk; each line represents variation in $1/v$ plotted against $1/NAD^+$ at the indicated ethanol concentration, (b) Secondary plots of intercepts ($\bullet$) and slopes ($\blacktriangle$).

activity was attained at a much lower concentration (1.33 mM). Inhibition occurred at only 1.7 mM. In comparison to the NAD⁺-linked activity, NADP⁺-linked activity was 75% inhibited at 13.3 mM NADP⁺ concentration.

The catalysis by FLADH was studied over a broad range of substrate and coenzyme NAD(P)⁺ concentrations. Double reciprocal plots for the NAD⁺ and NADP⁺-linked activities are represented by Figures 3.25a and 3.26a respectively. The most dominant kinetic characteristic is the nonlinearity in both plots. The reciprocal plot for the NADP⁺ passed through a minimum (Figure 3.26a), and curved up as it approaches the 1/v axis. The plot approached a straight line asymptote only at high values of 1/[NADP⁺]. Extrapolation of the asymptote yielded apparent values for the kinetic parameters. As concentration of ethanol was increased, the minimum moved closer to the 1/[NADP⁺] axis. The reciprocal plot for NAD⁺ resembled the NADP⁺ plot with much less curvature. It is of interest to note the substantial decrease in enzymatic sensitivity to inhibition by NAD⁺ as ethanol concentration was increased. Secondary plots (Appendix III) of the data (Figures. 3.25b, and 3.26b) yielded the apparent Michaelis constants listed in Tables 3.9 and 3.10.

Inspection of kinetic parameters obtained for NAD⁺ and NADP⁺ indicated that combination of greater turnover number (V/E_t) and smaller dissociation constant (K_{1a}) values may be responsible for the enhanced affinity of enzyme to NAD⁺. It

Figure 3.24. Saturation plots of NAD⁺ and NADP⁺-linked activities of FLADH. Enzyme concentration was 3.4-27 μM, ethanol concentration fixed at 10 mM and assays were at pH 9.0. Activities were, NAD⁺ (O), NADP⁺ (Δ).

Figure 3.25a. Double reciprocal plot of NAD^+ -linked activity of FLADH. Enzyme concentration was $3.4 \mu\text{M}$ in tris/HCl, pH 9.0. Initial rates were measured at varied NAD^+ concentrations at fixed ethanol concentrations. Ethanol concentration in mM are represented by number on each line.

Figure 3.26a. Double reciprocal plot of NADP⁺-linked activity in tris/HCl, pH 9.0. Numbers indicate ethanol concentrations in mM. Enzyme concentration was 27.0 μ M.

explains the increased rate of catalysis by NAD^+ as compared to the NADP^+ reaction. Increased rate by NAD^+ was partially offset by the K_a and K_b values which were smaller for the NADP^+ linked activity.

Nonlinearity in the double reciprocal plots of NAD^+ and NADP^+ may suggests substrate inhibition of the enzyme by high concentration of NAD^+ and NADP^+ , respectively. The mode of inhibition in NAD^+ -linked activity was slightly relieved by higher ethanol concentration. Inhibition was 23% at 10.0 mM ethanol, 20.0 mM NAD^+ , compared to 35% inhibition at the same concentration of NAD^+ but 0.5 mM ethanol. On the other hand, 72% inhibition was observed in the NADP^+ -linked activity at 10.0 mM ethanol and 13.3 mM NADP^+ . In comparison, 75% inhibition was observed at 13.3 mM NADP^+ and 0.75 mM ethanol. Thus, even the highest concentration of coenzyme, at least in NAD^+ -linked activity, resulted in finite rate, and partial inhibition. This indicated that high coenzyme concentrations were probably causing an alteration in the reaction pathway leading to some randomness in the mechanism. Nonlinearity in Lineweaver-Burk plots, indicated that rate equation contains reactant concentration terms raised to power greater than one. The rate equation in these cases takes the general form of a rational polynomial as in equation 2;

$$v = \frac{\sum_{i=1}^n \alpha_i S^i}{\sum_{i=1}^n \beta_i S^i} \quad \dots\dots\dots 2$$

where S is NAD(P)^+ concentration, α_i and β_i are kinetic coefficients related to rate constants and alcohol concentration for NAD(P)^+ of the i th degree in numerator and denominator respectively. Their rate equation was analyzed for the $n:m$ function graphically according to diagnostic plots of Bardsley and Child's (136). The n and m depend on the maximum number of enzyme species reacting with the substrate, and therefore represent the highest degree of substrate terms in the numerator and denominator, respectively. Plotting $v[\text{NAD(P)}^+]^\lambda$ ($\lambda = \text{integer}$) versus $[\text{NAD(P)}^+]$, and identifying the plot with a plateau, where $\lambda = m-n$ revealed a difference between NAD^+ and NADP^+ -linked activities (Figures. 3.27a and b). Figure 3.27a indicated that for NAD^+ , $\lambda=0$ ($m=n > 1$) and

$$\lim_{S \rightarrow \infty} (V_{\infty} - n) = \text{constant}$$

The plateau was observed at $\lambda=1$ ($m-n = 1$) for NADP^+ activity (Figure 3.27b), in another word $m > n$ due to a dead-end inhibition by substrate. Reciprocal rate reached infinity as the reciprocal NADP^+ concentration approaches zero and satisfied:

$$\lim_{1/S \rightarrow 0} 1/v = \infty$$

To further investigate the nonlinearity, the initial rates for both NAD^+ and NADP^+ were plotted versus $1/[\text{Ethanol}]$. The evaluated slopes and intercepts were replotted versus $1/[\text{coenzyme}]$. The slope and intercept

replots for NADP⁺ were both nonlinear and tended to infinity as $1/[\text{NADP}^+]$ approached zero. The slope and intercept replots for NAD⁺ were nonlinear but reached a finite value as $1/[\text{NAD}^+]$ approached zero (c.f. Figures. 3.28a, b).

These replots and the diagnostic plots of Bardsley and Childs could indicate a randomness in FLADH mechanism with a dead-end inhibition for the NADP⁺ mechanism. This will be discussed further in section 3.3.6.2.

3.3.1.3. *Thermoanaerobium brockii*-ADH

Detailed kinetic studies are required for this enzyme. Differences observed in enzyme activity with primary to secondary alcohols may be a reflection of a change in rate limiting steps or mechanism for each class of the substrates. Kinetics of the enzyme with a secondary alcohol (propan-2-ol) with NADP⁺ and NAD⁺ are presented in Figures 3.29a and 3.30a. A difference in kinetic behaviour of TBADH with primary alcohol (ethanol) and NADP⁺ as coenzyme was observed (c.f. Figure 3.31a). Linear reciprocal plot was obtained for the NADP⁺-linked activity with propan-2-ol. Nonlinear reciprocal plot was obtained with the same substrate, but with NAD⁺ as coenzyme. Plot was nonlinear at high $1/[\text{NAD}^+]$ and linearized as $1/[\text{NAD}^+]$ approached zero. Initial rate data at high NAD⁺ concentration (low $1/[\text{NAD}^+]$) was replotted (inset of Figure 3.30a), and parameters are listed in Table (3.9). No inhibition was observed with the

Figure 3.27a, b: Diagnostic plots of NAD^+ and NADP^+ -linked activities of FLADH. (a) $v[\text{NAD}^+]^\lambda$ versus $[\text{NAD}^+]$, (b) $v[\text{NADP}^+]^\lambda$ versus $[\text{NADP}^+]$, where $\lambda=0$ (O), 1 (Δ), and 2 ($\square$) for $[\text{ETOH}]=10 \text{ mM}$.

Figure 3.28a, b. Secondary plots for (a) NAD⁺-linked and (b) NADP⁺-linked activity of FLADH at pH 9.0. The primary intercepts (O) and slopes (Δ) obtained from $1/v$ versus $1/[\text{ethanol}]$ are replotted versus $1/[\text{NAD(P)}^+]$. For other conditions see Figures 3.25a and 3.26b.

NAD⁺-linked activity with propan-2-ol as substrate even when as high as 20.0 mM of NAD⁺ was used. Curvature observed at high NAD⁺ concentration indicates a high degree of the rate dependence on NAD⁺ concentration. The dependence of initial rate on NAD⁺ concentration in presence of low fixed propan-2-ol is sigmoidal (Figure 3.30c). The Hill coefficient value of 1.18 was obtained (inset of Figure 3.30c) at 0.8 mM propan-2-ol.

Reciprocal plot of TBADH initial rate with primary alcohol (ethanol), and NADP⁺ exhibited substrate inhibition at high NADP⁺ concentration. Lineweaver-Burk plot passed through a minimum and curved up as it approached the 1/v axis. Extrapolation of the asymptote at high 1/[NADP⁺] yielded kinetic parameters in Table 3.10. Comparison between the NADP⁺- and NAD⁺-linked activities (when propan-2-ol is the substrate) indicated NADP⁺ preference for the enzyme. The kinetic constant of NAD⁺ is 610 fold larger than K_a for NADP⁺ (Tables 3.9 and 3.10). The same can be said concerning the dissociation constant K_{1a} . In NAD⁺-linked activity, K_{1a} is 1.8×10^3 larger than that for NADP⁺. Thus, poor enzyme affinity for NAD⁺, slow dissociation of NAD⁺, as well as smaller turnover number dictate the enzyme preference for NADP⁺ rather than NAD⁺. Inspection of kinetic parameter for NADP⁺ linked reaction with ethanol as a substrate explains the specificity of TBADH to secondary alcohols.

Figure 3.29a. Double reciprocal plot of NADP⁺-linked activity of TBADH in tris/HCl, pH 9.0. Initial rates measured at varied NADP⁺ concentration at fixed concentration of propan-2-ol. Numbers on each line represent propan-2-ol concentration in mM. Enzyme concentration was 0.94 μM .

Figure 3.30a. Double reciprocal plots of NAD^+ -linked activity with propan-2-ol of TBADH. All assays were at 25°C , pH 9.0 with enzyme concentration $2.3 \mu\text{M}$. Numbers on each line indicate propan-2-ol concentration in mM. Inset shows initial rate data at high NAD^+ concentration.

Figure 3.30c. Dependence of reaction velocity of TBADH on concentration of NAD^+ . Initial rates measured at 25°C at pH 9.0, propan-2-ol concentration 0.8 mM, and enzyme concentration $2.3 \mu\text{M}$. Inset shows Hill's plot of reaction velocity dependence on NAD^+ concentration.

Figure 3.31a. Double reciprocal plot of NADP⁺-linked activity of TBADH with primary alcohol (ethanol). All assays were at 25°C, pH 9.0 with enzyme concentration 1.6 μ M. The ethanol concentration varied as 0.8, 1.0, 1.25, 2.0, 2.5, 5.0, 10 and 20 mM from top to bottom.

The Michealis constant for ethanol was 19 fold larger than K_b for propan-2-ol. In addition, the turnover number for the enzyme with secondary alcohols is 21 fold higher than with primary alcohols. The use of Bardsley and Childs diagnostic plots (136) revealed the difference in enzyme kinetic behavior (if there is any with respect to the different alcohols as well as NAD^+ versus $NADP^+$). Plots of $v[NAD(P)^+]\lambda$ versus $[NAD(P)^+]$ at 2.0 mM concentration of the alcohols, ethanol and propan-2-ol are given in Figures 3.32a, b and c. In the diagnostic plots for the NAD^+ and $NADP^+$ -linked activity with propan-2-ol as substrate, a positive horizontal asymptote as $[NAD(P^+)] \longrightarrow \infty$, could not be distinguished indicating an $n:m$ function of 1:1. Diagnostic plot of $v[NADP^+]\lambda$ versus $[NADP^+]$ with ethanol as substrate, the horizontal asymptote was attained at $\lambda=1$, which means ($m-n=1$), indicating a dead-end inhibition by substrate. Unlike FLADH, all diagnostic plots for the TBADH showed a different trend as alcohol concentration was increased. This may indicate that random mechanism is not involved in this enzyme, though a dead-end inhibition is operative for the $NADP^+$ linked activity with primary alcohol (ethanol).

3.3.2. Kinetics of FLADH dismutase activity

In view of the interest in the multifunctionality of FLADH and its similarity to HLADH in this aspect (14), the

Figure 3.32a, b, c. Diagnostic plots of TBADH activities in presence of 2.0 mM propan-2-ol for (a), (b) and 2.0 mM ethanol for (c). In (a) $v[\text{NAD}^+]^\lambda$ versus $[\text{NAD}^+]$, (b) $v[\text{NADP}^+]^\lambda$ versus $[\text{NADP}^+]$ and (c) $v[\text{NADP}^+]^\lambda$ versus $[\text{NADP}^+]$. $\lambda = 0$ (O), 1 (Δ), and 2 ($\square$).

kinetic of dismutase activity was investigated. Kinetic study was performed using an aldehyde of medium chain length (octanal). Initial velocities of FLADH catalyzed dismutation gave nonlinear reciprocal plots with respect to NAD^+ (Figure 3.33a). Extrapolation of the linear region at high $1/[\text{NAD}^+]$, provided the apparent kinetic parameters listed in Table 3.11. Both secondary plots of intercepts and slopes versus $1/[\text{octanal}]$ were linear in the range studied, though a slight curvature could be seen at low $1/[\text{octanal}]$ concentration (Figure 3.33b in Appendix III). The initial velocities data were plotted against $1/[\text{octanal}]$ to further investigate the nonlinearity behaviour. The double reciprocal plot of initial rate against octanal, was nonlinear. Both secondary plots of intercepts and slopes versus $1/[\text{NAD}^+]$ were nonlinear, and bend up as approaching the intercepts or slopes axes (Figure 3.34). This indicate a complexity in the kinetic mechanism of FLADH catalyzed dismutation, and a possible inhibition of the enzyme by high concentration of both NAD^+ and octanal.

The diagnostic plot of the dismutase activity, when plotted as $v[\text{NAD}^+]^\lambda$ against $[\text{NAD}^+]$ at lowest and highest concentration of octanal showed the same trend. As in TBADH diagnostic plots, no positive horizontal asymptote could be distinguished. On the other hand, the diagnostic plot of $v[\text{octanal}]^\lambda$ versus $[\text{octanal}]$ for dismutase reaction, was dependent on NAD^+ concentration (Figure 3.35a, b and c). As

Figure 3.33a. Double reciprocal plot of the dismutase activity of FLADH. All assays were in phosphate buffer, pH 7.4 at 25°C. Enzyme concentration was 0.59 μ M. The octanal concentration varied, from top to bottom as follows: 0.015, 0.03, 0.06, 0.2 and 0.1 mM.

NAD⁺ concentration increased, initial rate decreased indicating inhibition of the enzyme. In conclusion, from diagnostic plots (Figures 3.35a, b) an enzyme mechanism for dismutase activity is difficult to be redrawn. Unlike the FLADH dismutase activity, the initial rate of HLADH catalyzed dismutation reaction gave linear reciprocal plots with respect to NAD⁺ but nonlinear with respect to octanal (14). Substrate inhibition by octanal was partially relieved by NAD⁺ as well as higher temperature. The simplest mechanistic explanation for the observed results was a steady-state random mechanism with formation of the ternary E. NADH octanal complex at low temperature. At high temperatures however, this ternary complex does not contribute to the rates (14). The nonlinear reciprocal plots of FLADH dismutation reaction with respect to both NAD⁺ and octanal indicate an operation of a complexed mechanism. Comparison of the kinetic parameters for this activity (Table 3.11), shows a high turnover number and lower K_b for the FLADH dismutase activity. However, K_a for this activity for both enzyme is comparable, though K_{ia} of FLADH is about 4 fold larger than K_{ia} of HLADH.

3.3.3. Esterase kinetics of FLADH

It was mentioned previously in section 3.2., that FLADH as well as RLADH, are able to catalyze esterolysis, dismutation, and dehydrogenation, just as HLADH. The

Figure 3.34. Secondary plot of dismutase activity of FLADH at pH 7.4. The primary intercepts (O) and slopes (Δ) obtained from $1/v$ versus $1/[\text{octanal}]$ are plotted against $1/[\text{NAD}^+]$. For more details see Figure 3.33a.

Figure 3.35a, b, c. Diagnostic plots of dismutase activity of FLADH. (a) Plot of $v[\text{NAD}^+]^\lambda$ versus $[\text{NAD}^+]$ at $[\text{Octanal}] = 0.015 \text{ mM}$, (b) and (c) plot of $v[\text{Octanal}]^\lambda$ versus $[\text{Octanal}]$ at $[\text{NAD}^+] = 0.5 \text{ mM}$ and 6.0 mM respectively. $\lambda = 0$ (O), 1 (Δ), and 2 ($\square$).

esterolytic reaction catalyzed by HLADH does not require nicotinamide coenzyme and proceeds via a Uni Bi mechanism (13). Both Zn^{+2} and sulfhydryl group are required for the multifunctional activities of HLADH (13).

The esterase kinetics of FLADH with p-nitrophenyl esters with variable chain length ranging from C_2 (p-nitrophenyl acetate) to C_{12} (p-nitrophenyl laurate), are presented in Figure 3.36 a-f. Double reciprocal plots of initial rates over a wide range of concentration for each ester were obtained by averaging duplicate or triplicate measurements at each concentration. The kinetic parameters obtained from these plots are listed in Table 3.12. From the table, it is noted that there is no systematic change in either the turnover number or the dissociation constant. The p-nitrophenyl caprylate (C_8) has the highest affinity for the enzyme and is the only ester with a Michaelis constant in the micromolar range unlike the other esters. Highest turnover number was observed with p-nitrophenyl caproate (C_6), while its Michaelis constant is 16 fold larger than for p-nitrophenyl caprylate (C_8). At high concentration inhibition was observed with p-nitrophenyl acetate (Figure 3.36a). In the concentration range studied, enzyme showed an activation at high concentration of p-nitrophenyl caprylate (Figure 3.36c). The linear region at low p-nitrophenyl caprylate concentrations was extrapolated to estimate (apparent) kinetic parameters.

Table 3.11

Kinetic parameters for multifunctional reactions catalyzed by HLADH and FLADH

Kinetic parameter	HLADH ^a		FLADH	
	Dismutase ^b	Esterase ^c	Dismutase ^b	Esterase ^c
V _{max} (mM min ⁻¹)	0.0161	1.22×10 ⁻³	0.0230 ±3.8% ^d	0.0580 ±10.6%
K _a (mM)	1.15		1.46 ±17.2%	
K _b (mM)	0.0415	0.0259	4.34×10 ⁻³ ±28.8%	7.5×10 ⁻³ ±24.3%
K _{ia} (mM)	0.961		4.04±31.6%	
V/E _i (min ⁻¹)	13.4	0.248	39.0 ±5.1%	39.6 ±11.9%
[ADH] (μm)	1.20	5.00	0.590	1.50

a Data are those reported in (13).

b Dismutase parameter are obtained in 0.10 M NaPB, pH 7.4 with octanal as substrate.

c Esterase kinetic parameters are for *p*-nitrophenyl caprylate (NPO) at pH 8.2.

d Errors are calculated from correlation coefficients of linearly regressed data.

e Results of FLADH are average of two experiments.

Figure 3.36 a-f. Double reciprocal plots for substrate specificity of FLADH, esterase activity. All assays were at 25°C in borate buffer, pH 8.2. Enzyme concentrations were 1.7 μM for substrates C₂-C₆ and 1.5 μM for C₈-C₁₂. The p-nitrophenyl-ester substrates were as follows: (a) acetate, (b) caprate, (c) caprylate, (d) laurate, (e) butyrate and (f) caproate.

Table 3.12

Substrate specificity of esterase activity of FLADH

p-Nitrophenyl ester	C_n^a	V_{max} (mM min ⁻¹)	K_m (mM)	V_{max}/E_t^b (min ⁻¹)
Acetate	C_2	0.141	0.038	83.6
Butyrate	C_4	0.106	0.065	62.5
Caproate	C_6	0.313	0.122	185
Caprylate	C_8	0.058	7.5×10^{-3}	39.6
Caprate	C_{10}	0.236	0.113	163
Laurate	C_{12}	0.124	0.0950	51.7

Kinetic data were obtained from an average of duplicate experiments. Errors in kinetic parameters were determined by regression analysis of rate data. Average errors are as follow:

$$V_{max} = 17.1\%$$

$$K_m = 29.0\%$$

$$V_{max} / E_t = 18.8\%$$

a: C_n refer to carbon chain length of ester.

b: Enzyme concentration $[E_t]$ for $C_2 - C_6$ was $1.7 \mu M$ and for $C_8 - C_{12}$ was $1.5 \mu M$.

Previously (Table 3.7), it was shown from a single assay that the FLADH esterase activity is higher than in HLADH. Kinetic parameters of p-nitrophenyl caprylate obtained for the FLADH were compared to that of HLADH (Table 3.11) to justify above conclusion. Higher esterase activity of FLADH is a consequence of the higher turnover number and larger affinity of enzyme to p-nitrophenyl caprylate.

3.3.4. Substrate specificity for the dehydrogenase activity

Liver alcohol dehydrogenases in general possess a very broad specificity for their alcohol substrates. Table 3.13 shows that the liver enzymes (fish, horse and rat) react with a variety of primary and secondary alcohols, aromatic as well as with aliphatic alcohols. The rate of oxidation of methanol by FLADH is very low. Rate of oxidation increased as carbon chain length increased, with maximum activity observed for FLADH with both ethanol and propan-1-ol. The rate of oxidation decreased then progressively with further increase in carbon chain length. In HLADH, highest rate of oxidation was with butan-1-ol. That both YADH and FLADH can oxidize unsaturated alcohol, such as 2-propen-1-ol is particularly surprising, in view of the fact that the rate of oxidation of this alkene is much higher than for the primary alcohol by FLADH. It is known that yeast and plant-ADH have stringent substrate specificity (61). In YADH it is explained by differences between yeast and liver enzyme's residues

Table 3.13

Substrate specificity of NAD⁺-linked activity of alcohol dehydrogenases^a

Substrate	Relative activity (% of ethanol)				
	HLADH	FLADH	RLADH	YADH	TBADH ^b
Methanol	-	4	-	-	0
Ethanol	100	100	100	100	100
Propan-1-ol	102	105	83	64	50
Butan-1-ol	131	93	83	41	0
Pentan-1-ol	-	39	-	-	0
Hexanol	-	12	-	-	-
Propan-2-ol	7	20	43	0.8	584
2-Propen-1-ol	92	143	92	129	0
Butan-2-ol	36	15	74	-	865
Iso-butanol	-	54	102	-	0
Iso-pentanol	-	29	90	-	0
Cyclohexanol	41	9	73	0.1	366
Benzylalcohol	14	67	-	0	0

a All assays are in 0.10M tris/HCl, pH 9.0 with 10mM substrate in assay mixture, NAD⁺ concentration per assay mixture for all was 1.0mM except in TBADH.

b NAD⁺ concentration was 0.5mM per assay mixture.

c Data are average of at least duplicate assays, enzymes concentration was in the range 0.48-2.1 μ M.

which are in the substrate binding pocket (4). The main differences in this region, are the changes from Phe-93 and Ser-48 in HLADH to tryptophane and threonine, respectively in YADH. This results in a smaller substrate binding pocket in YADH as judged from the HLADH structure. Also, in the active site pocket, a change from Val-58 to tryptophan narrows the pocket in YADH. The two prolines which are inserted on both sides of residue 58 in YADH could diminish the pocket even further.

The rate of oxidation of secondary alcohols by bacteria enzyme is about 5-8 fold higher than with that of primary alcohols, with NAD^+ and NADP^+ as a coenzyme (Table 3.14).

In Table 3.13, the activity of TBADH with different substrates is relative to ethanol, in order to have a complete picture of substrate specificity among these different ADHs. As the TBADH is NADP^+ specific enzyme, the substrate specificity with this coenzyme is given in Table 3.14 for a comparison. This bacterial enzyme has the ability to oxidize cyclic alcohol with both NAD^+ and NADP^+ .

Substrate specificity of FLADH with NADP^+ as a coenzyme is given in Table 3.14. The data for this enzyme with NAD^+ are included for comparative purposes. There is a difference in the substrate specificity of FLADH with these two coenzyme. The FLADH in the presence of NAD^+ has the highest activity with unsaturated alcohol, while in the presence of NADP^+

Table 3.14

Substrate specificity of the NAD^+ - and NADP^+ -linked activities of FLADH and TBADH

Substrate	Relative activity (% of ethanol)			
	FLADH ^a		TBADH ^b	
	NAD^+	NADP^+	NAD^+	NADP^+
Methanol	4	18	0	4
Ethanol	100	100	100	100
Propan-1-ol	105	95	50	67
Butan-1-ol	93	79	0	67
Pentan-1-ol	39	108	0	84
Hexanol	12	22	-	32
Propan-2-ol	20	26	584	59×10^2
2-Propen-1-ol	143	68	0	155
Butan-2-ol	15	53	865	64×10^2
Iso-butanol	54	55	0	73
Iso-pentanol	29	82	0	25
Cyclohexanol	9	95	366	796
Benzylalcohol	67	13	0	0

a The NAD(P)^+ concentration were 1.0 mM in assay mixture with 10 mM of substrate in 0.10 M tris/HCl, pH 9.0.

b Same conditions as above except NAD(P)^+ concentration, were 0.5 mM.

c Data are average of at least duplicate assays, enzymes concentration was in the range of 1.6–2.1 μM .

highest activity was observed with primary saturated alcohol pentan-1-ol. Activity of FLADH with cyclohexanol in the presence of NADP⁺, is higher than in the presence of NAD⁺, keeping in mind these activities are % relative to ethanol.

Kinetics of FLADH with methanol, propan-1-ol, butan-1-ol, and pentan-1-ol are presented in Figure 3.37a-d. These kinetic plots were prepared at fixed NAD⁺ concentration of 1.0 mM. The kinetic plot of ethanol and NAD⁺ is presented in Figure 3.25a. The kinetic parameters obtained for above substrates are listed in Table 3.15. It was not possible to obtain kinetic parameters for pentan-1-ol due to the drastic inhibition exhibited by FLADH for this substrate. An inhibition was also observed with methanol, but only at very high concentration. The double reciprocal plot bend up as $1/[\text{methanol}]$ approaches zero. No inhibition of FLADH was observed with either propan-1-ol or butan-1-ol in the concentration range studied.

The saturation plots of TBADH (Figure 3.38a) were prepared for ethanol as primary alcohol and both propan-2-ol and butan-2-ol. Kinetic parameters for both ethanol and propan-2-ol were obtained from the double reciprocal plots (Figures. 3.29a, 3.31a) and listed in Table 3.10. Double reciprocal plot (Figure 3.38b) for butan-2-ol, represent data collected at fixed concentration of NADP⁺ (0.5 mM). The kinetic parameters listed in Table 3.10 indicate that the TBADH has comparable affinity for both propan-2-ol and

Figure 3.37 a-d. Initial rate plots for the substrate specificity of NAD⁺-linked activity of FLADH. All assays were at 25°C and pH 9.0. Enzyme concentration was 2.1 μM and NAD⁺ concentration was fixed at 1.0 mM. Substrates were as follows: (a) methanol, (b) propan-1-ol, (c) butan-1-ol and (d) pentan-1-ol.

Table 3.15

Summary of kinetic parameters for the different substrates of NAD⁺-linked activity of FLADH

Substrate	$V_{\max}$ (mM min ⁻¹)	K_b (mM)	$V_{\max} / E_t^b$ (min ⁻¹)	$[E_t]$ (μM)
Methanol	0.0115	0.343	5.45	2.11
Ethanol ^a	0.192	0.565	57.8	3.33
Propan-1-ol	0.114	0.577	54.0	2.11
Butan-1-ol	0.0943	0.825	44.7	2.11

a Data are from Table 3.9 and listed here for comparison.

b Average errors calculated from correlation coefficient of linearly regressed data are:

$$V_{\max} = 4.24\%$$

$$K_b = 27.9\%$$

$$V_{\max} / E_t = 5.51\%$$

c Kinetic data obtained at high fixed NAD⁺ concentration (1.0mM), data are average of duplicate.

butan-2-ol. The turnover number for the latter is slightly higher than for propan-2-ol. This was previously observed with single point assay in Table 3.14.

3.3.5. Coenzyme specificity

The behavior of NAD(P)⁺ analogues toward TBADH and FLADH was investigated. The structures of the various analogues are presented in Figure 3.39a-h. The analogues, 3-acetylpyridine adenine dinucleotide (phosphate) (acPy-AD(P)⁺); 3-Pyridine aldehyde adenine dinucleotide (PyA-AD⁺), and thio-NAD(P)⁺ are all modified at the nicotinamide moiety (Figure 3.39c-g). Deamino-NAD⁺ analogue (Fig. 3.39h) has the adenine amine (C₆) replaced by a hydroxyl group.

The values of K_m and V_{max} (Table 3.16) were determined at a constant saturated concentration of the alcohol for each analogue (Figures. 3.40 and 3.41) for TBADH and FLADH, respectively. Kinetic parameters for both NAD⁺ and NADP⁺ are included for comparison. From the Table, an interesting observation was made with respect to TBADH behavior with NAD⁺ analogues. A change in the nicotinamide moiety drastically reduced the turnover number, (roughly 100-1000 fold decrease). A modification in the adenine part however resulted in 7 fold decrease in turnover number, with concomitant slight increase in binding affinity of TBADH to Deamino-NAD⁺ analogue. From the NADP⁺ analogues available for this study, the above conclusion for NAD⁺ analogues does not seem to apply for the TBADH behavior toward NADP⁺.

Figure 3.38 a,b. Substrate specificity saturation plot (a) of NADP⁺-linked activity of TBADH. All assays were at 25°C, pH 9.0, NADP⁺ (0.5 mM), enzyme concentration (15.6 μM) and activity is expressed as % of maximum for the substrates; ethanol (○), propan-2-ol (Δ), and butan-2-ol (□). (b) double reciprocal plot for the butan-2-ol data.

(a) NAD^+ (b) NADP^+ (c) thio- NAD^+ (d) thio- NADP^+ (e) acPy- AD^+ (f) acPy- ADP^+ (g) PyA- AD^+ (h) deamino- NAD^+

Figure 3.39a-h. Structures of $\text{NAD(P}^+)$ analogues. Analogues are: (a) NAD^+ , (b) NADP^+ , (c) thio- NAD^+ , (d) thio- NADP^+ , (e) acPy- AD^+ , (f) acPy- ADP^+ , (g) PyA- AD^+ , and (h) Deamino NAD^+ .

analogues. A change in pyridine amide moiety of NADP⁺ to an acetyl group (acPy-ADP⁺) decreased turnover number by 7-fold and increased Michaelis constant by 3-fold. Thio-NADP⁺ experienced a slight decrease in turnover number (3 fold), and Michaelis constant was virtually the same.

The above kinetic values can be partly correlated to structural information. It seems that an introduction of groups which have a different polarities than NH₂ at C-3, exert an affect on the affinity of TBADH for these analogues.

The differential behaviour of TBADH toward NAD⁺ and NADP⁺ analogues could be due to the different modes of NAD⁺ and NADP⁺ binding. The TBADH enzymic reaction, catalyzed by acPy-AD⁺ analogue, exhibit an activation at high concentration (Figure 3.40d). Activity of TBADH with thio-NAD⁺ and PyA-AD⁺ analogues (Figure 3.40a and f, respectively) indicated substrate inhibition at high concentration. On the other hand, NADP⁺ analogues used followed a hyperbolic relationship between the rate and substrate concentration, and gave a linear double reciprocal plot (Figure 3.40b and c).

The FLADH kinetics with NAD⁺ and NADP⁺ analogues are given in Figure 3.41a-f. All double reciprocal plots are linear in the concentration range studied, except in PyA-AD⁺ plot there was slight curvature at high coenzyme concentration (Figure 3.41b). Kinetic parameters obtained

Figure 3.40 a-f. Double reciprocal plots of TBADH coenzyme specificity. Assays were at 25°C, pH 9.0, propan-2-ol (10 mM) and enzyme was 1.6 μM . Analogues were: (a) Thio- NAD^+ , (b) Thio- NADP^+ , (c) acPy- ADP^+ , (d) acPy- AD^+ , (e) Deamino- NAD^+ , and (f) PyA- AD^+ .

Figure 3.41 a-f. Double reciprocal plots of FLADH coenzyme specificity. Assays were at 25°C, pH 9.0, ethanol (10 mM) and enzyme concentrations were 1.2 μM for acPy-AD⁺, PyA-AD⁺ and 18.6 μM for the rest. Analogues were: (a) acPy-AD⁺; (b) PyA-AD⁺; (c) Deamino-NAD⁺; (d) acPy-ADP⁺; (e) Thio-NAD⁺ and (f) Thio-NADP⁺.

Table 3.16

Kinetic parameters for NAD^+ and NADP^+ analogues of FLADH and TBADH

Analogues	FLADH				TBADH			
	$V_{\max}$ (mM.min ⁻¹)	K_a (mM)	$V_{\max}/E_t$ (min ⁻¹)	$[E_t]$ (μM)	$V_{\max}$ (mM.min ⁻¹)	K_a (mM)	$V_{\max}/E_t$ (min ⁻¹)	$[E_t]$ (μM)
NAD^{+a}	0.192	0.164	57.8	3.33	1.670	46.8	714	2.34
acPy- AD^+	2.84	0.653	2.45×10^3	1.16	1.15×10^{-3}	0.0414	0.718	1.60
PyA- AD^+	0.282	0.254	243	1.16	3.23×10^{-3}	1.06	2.02	1.60
Deamino- NAD^+	0.578	0.308	31.1	18.6	0.168	44.9	105	1.60
Thio- NAD^+	0.0550	0.0720	2.96	18.6	9.47×10^{-3}	2.08	5.92	1.60
NADP^{+a}	0.136	0.121	5.09	26.7	1.05	0.0763	1.13×10^3	0.935
acPy- ADP^+	0.0394	0.125	2.12	18.6	0.255	0.225	159	1.60
Thio- NADP^+	4.06×10^{-3}	0.0869	0.218	18.6	0.571	0.0655	357	1.60

a Data for NAD^+ and NADP^+ are obtained from Table 3.9 and 3.10 in this chapter and included for comparison.

b Average errors calculated from correlation coefficients of linearly regressed data are $V_{\max} \pm 11.9\%$, $K_a \pm 19.3\%$, and $V_{\max}/E_t \pm 13.2\%$.

Kinetic parameters are obtained at fixed saturated (10 mM) concentration of ethanol or propan-2-ol for FLADH and TBADH respectively.

are listed in Table 3.16 with NAD(P)⁺ parameters included for comparison. Both acPy-AD⁺ and PyA-AD⁺ analogues experienced an increase in their turnover number to a varying degree. There was a 42 fold increase in turnover number of the acPy-AD⁺ analogue with concomitant 4 fold decrease in binding affinity. The PyA-AD⁺ analogue increase in turnover number was 4 fold, with 2 fold decrease in its binding affinity (Table 3.16). The other two NAD⁺ analogues Deamino NAD⁺ and Thio-NAD⁺ had a lower turnover number than NAD⁺. There was no consistent increase or decrease in the K_m for the different NAD⁺ analogues. Both NADP⁺ analogues experienced a decrease in their turnover number with K_m for Thio-NADP⁺ decreasing as well (Table 3.16).

3.3.6. Product inhibition

The original formulation of product inhibition was done by Alberty (137) to differentiate between various two-substrate mechanisms. The rate equations describing product inhibition for the four basic bisubstrate mechanisms can be found in Rudolph (138).

There are three general types of inhibitions observed with product inhibitors. The inhibition patterns are defined by the effect on the slope, intercept or both on double reciprocal plots of the variable substrate in the presence of different fixed concentration of a product. This leads to competitive, uncompetitive, or noncompetitive inhibition,

respectively, described by the equations in Appendix (II). The inhibition patterns predicted by rate equations of two-substrate systems are given in Table 3.17. A choice between the different sequential mechanisms can be readily made if the products act as indicated in the table. The formation of ternary-abortive complexes (dead-end complexes) cause changes in the actual observed inhibition patterns for a given mechanism. This may complicate the utility of the product inhibition, however information about the mechanism can be still obtained from such experiments (138).

In this section, product inhibition results presented will be focused on NADP⁺-linked activity for the TBADH, and both NAD⁺ and NADP⁺-linked activity of FLADH.

3.3.6.1. *Thermoanaerobium brockii*-ADH (TBADH)

The product inhibition patterns obtained for NADP⁺-linked activity of above enzyme is consistent with an Ordered BiBi mechanism with NADP⁺ taken as the leading substrate (Table 3.17), and then propan-2-ol.

The double reciprocal form of bisubstrate rate equation 1 can be rewritten to include the products as inhibitors. Different forms of the equation can be obtained depending on the variable substrate, fixed substrate and the product that is acting as an inhibitor (139, 140). The rate equation in presence of product P, [Q]=0, variable A concentration, and fixed B concentration is presented by equation 3:

Table 3.17

Product inhibition patterns for Bireactant Mechanisms

Mechanism	Product	Varied Substrate			
		A		B	
		Unsaturated	Saturated	Unsaturated	Saturated
		with B	with B	with A	with A
Ordered BiBi	P	N(N) ^{a,b,c}	U	N(N) ^d	N
	Q	C(C)	C	N(N)	-
Theorell-Chance BiBi	P	N(N) ^c	-	C(C) ^d	C
	Q	C(C)	C	N(N)	-
Ping Pong Tetra Uni	P	N(N) ^d	-	C(NL) ^e	C
	Q	C(C)	C	N(NL) ^e	-
Rapid Equilibrium	P	-(U)	-	-(C)	-(C)
Ordered BiBi	Q	C(C)	-(C)	C(N)	-
Rapid Equilibrium	P	C(N)	-	C(C)	-(C)
Random BiBi	Q	C(C)	-(C)	C(N)	-
Random BiBi	P	N ^f	N ^g	N ^f	N ^g
	Q	N ^f	N ^g	N ^f	N ^g

Table adapted from Rudolph (138) and Segel (140)

a The abbreviations are C, competitive; N, noncompetitive; U, uncompetitive; and NL, nonlinear. A dash indicates that no inhibition is observed.

b Intercept replots versus inhibitor concentration are parabolic.

c The patterns in parentheses indicate effect of abortive complex formation.

d Slope replots versus inhibitor concentration are parabolic.

e Hyperbolic concave up when binary abortive complexes form.

f Reciprocal plots are theoretically nonlinear although the curvature may be difficult to see. The intercept replots are hyperbolic functions of product inhibitor concentration, while the slopes are more complex functions of inhibitor concentration.

g Reciprocal plots are linear. Slope and intercept replots are hyperbolic functions of product inhibitor concentration.

$$\frac{1}{v} = \frac{K_a}{V_{max}} \left[1 + \frac{K_{ia}K_b}{K_a[B]} \left(1 + \frac{K_q[P]}{K_{iq}K_p} \right) \right] \frac{1}{[A]} + \frac{1}{V_{max}} \left[1 + \frac{K_b}{[B]} \left(1 + \frac{K_q[P]}{K_{iq}K_p} \right) + \frac{[P]}{K_{ip}} \right] \dots 3$$

Thus, at non-saturating concentration of B, the inhibition is non-competitive as both slope and intercept are increased by the factor $(1 + K_q[P]/K_pK_{iq})$. Saturating [B] reduces the slope factor to unity. The $1/v$ -axis intercept factor retains the $[P]/K_{ip}$ term, leading to P acting as an uncompetitive inhibitor with respect to A at saturating concentration of B (140).

When [B] is varied, and A is fixed, the equation becomes as in equation 4:

$$\frac{1}{v} = \frac{K_b}{V_{max}} \left[\left(1 + \frac{K_{ia}}{[A]} \right) \left(1 + \frac{K_q[P]}{K_{iq}K_p} \right) \right] \frac{1}{[B]} + \frac{1}{V_{max}} \left(1 + \frac{K_a}{[A]} + \frac{[P]}{K_{ip}} \right) \dots \dots \dots 4$$

Here P behaves as a non-competitive inhibitor with respect to B at all concentrations of A, as saturating [A] leaves [P] terms in the slope and intercept factors.

The velocity equation with Q as a product with respect to varied [A], [P]=0 and fixed B concentration becomes:

$$\frac{1}{v} = \frac{K_a}{V_{max}} \left[\left(1 + \frac{[Q]}{K_{iq}} \right) \left(1 + \frac{K_{ia}K_b}{K_a[B]} \right) \right] \frac{1}{[A]} + \frac{1}{V_{max}} \left(1 + \frac{K_b}{[B]} \right) \dots \dots \dots 5$$

From equation 5, Q acts as a competitive inhibitor with respect to A at all B concentration. However, when [B] is

the varied substrate and [A] is fixed, the equation becomes as in equation 6:

$$\frac{1}{v} = \frac{K_b}{V_{max}} \left[1 + \frac{K_{1a}}{[A]} \left(1 + \frac{[Q]}{K_{1q}} \right) \right] \frac{1}{[B]} + \frac{1}{V_{max}} \left[1 + \frac{K_a}{[A]} \left(1 + \frac{[Q]}{K_{1q}} \right) \right] \dots\dots\dots 6$$

Both intercept and slope terms are affected and Q acts as noncompetitive inhibitor with respect to B at unsaturating A concentrations. Saturation with A overcomes the inhibition, however [A] must be large enough compared to $K_{1a}[Q]/K_{1q}$, and $K_a[Q]/K_{1q}$ for these [Q] terms to disappear from the slope and intercept factors.

The product inhibition patterns as summarized in Table 3.17 would permit the establishment of the order of substrate addition and product release.

Product inhibition studies that were performed for the NADP⁺-linked activity of TBADH are presented in Figures 3.42a-3.45a. Details concerning concentration of the fixed substrate, varied substrate, and product under investigation are indicated in the corresponding figure legends. Secondary plots of the above figures could be found in Appendix III. Inhibition constants obtained are listed in Table 3.18.

Linear competitive inhibition was observed by NADPH with respect to NADP⁺ (Figure 3.42a) and NADPH was a linear noncompetitive inhibitor with respect to propan-2-ol (Figure 3.43a). Linear noncompetitive inhibition was observed by 2-propanone, when either NADP⁺ or propan-2-ol was the varied

Figure 3.42 a. Double reciprocal plot for product inhibition of $NADP^+$ -linked activity of TBADH. $NADP^+$ was varied in presence of NADPH as inhibitor at fixed ethanol concentration (0.5 mM). The number on each line represent the fixed NADPH concentration in mM. All assays were at 25°C , pH 9.0 and enzyme concentration was $0.94 \mu\text{M}$.

Figure 3.43 a. TBADH product inhibition at constant unsaturated $NADP^+$ (0.12 mM). Concentration of propan-2-ol was varied in presence of NADPH as inhibitor. NADPH (mM) varied as indicated by the number on each line. All assays were at 25°C, pH 9.0 and enzyme concentration was 0.94 μM .

substrate as can be seen in Figures 3.44a and 3.45a. The product inhibition patterns observed agreed with the Ordered-BiBi mechanism (Table 3.17). The information obtained here at unsaturating levels of fixed substrate concentration were sufficient to allow the distinctions between the mechanisms.

3.3.6.2. Pickerel fish alcohol dehydrogenase (FLADH)

It was suggested previously in section 3.3.2.1. that the nonlinearity in the Lineweaver-Burk plots of both NAD^+ and NADP^+ -linked activities of FLADH could indicate a randomness in the NAD^+ -linked activity and a random mechanism with dead-end inhibition for the NADP^+ -linked activity. Results of the product inhibition studies to be presented here, support the proposed mechanism of a Rapid Equilibrium Random BiBi with a dead end inhibition for NADP^+ -linked activity. As for the NAD^+ , the product inhibition patterns obtained support favourably a steady State Random BiBi mechanism.

The former mechanism (Rapid Equilibrium Random BiBi) with the possible abortive ternary complexes is presented in Scheme 4:

Figure 3.44 a. Double reciprocal plot of NADP⁺-linked activity product inhibition of TBADH at fixed unsaturated (0.5 mM) of propan-2-ol. The NADP⁺ concentration was varied in the presence of 2-propanone as inhibitor. Number on each line represent fixed 2-propanone concentration in mM. Other conditions are the same as in Figure 3.43a.

Figure 3.45 a. Product inhibition double reciprocal plot of NADP⁺-linked activity of TBADH at constant unsaturated concentration of NADP⁺ (0.12 mM). propan-2-ol concentrations were varied in presence of 2-propanone as inhibitor. Concentration of 2-propanone in mM was varied as indicated by the number on each line. Other conditions are as indicated in Figure 3.43a.

Table 3.18

Product inhibition constants of NADP⁺-linked activity of TBADH

Product	Varied substrate			
	NADP ⁺ ^a		Propan-2-ol ^b	
	K _{ii}	K _{is}	K _{ii}	K _{is}
2-Propanone	0.0662	0.0377	0.450	0.0204
NADPH	--	0.0060	0.241	0.0442

Inhibition constants given are in mM. When both constants are obtained a noncompetitive inhibition is observed. If K_{ii} only is obtained then uncompetitive inhibition. A competitive is observed if only K_{is} is obtained. Average error calculated from correlation coefficients of linearly regressed data are $K_{ii} \pm 21\%$ and $K_{is} \pm 28\%$.

- a At varied NADP⁺ concentration, propan-2-ol concentration was fixed at 0.50 mM.*
- b When propan-2-ol was the varied substrate, NADP⁺ concentration was fixed at 0.12 mM.*
- No value obtained.*

Enzyme concentration in all experiments was 0.94 μ m. Products concentration were, for NADPH 0-0.15 mM, and 2-Propanone 0-0.2 mM.

Scheme 4. Random BiBi mechanism with abortive ternary complexes.

The rate equations taking into consideration the presence of products are given by equations 7 and 8. Equations are different from those of the same system but with no abortive complexes (138), and lead to different inhibition patterns (Table 3.17). The rate equation in the presence of product P is

$$\frac{1}{v} = \frac{1}{V_{\max}} + \frac{K_a}{V_{\max}[A]} + \frac{K_b}{V_{\max}[B]} \left(1 + \frac{[P]}{K_{ip}}\right) + \frac{K_{ia}K_b}{V_{\max}[A][B]} \left(1 + \frac{[P]}{K_{ip}}\right) \dots\dots\dots 7$$

The analogous equation 8 is obtained in the presence of

$$\frac{1}{v} = \frac{1}{V_{\max}} + \frac{K_a}{V_{\max}[A]} \left(1 + \frac{[Q]}{K_{iq}}\right) + \frac{K_b}{V_{\max}[B]} + \frac{K_{ia}K_b}{V_{\max}[A][B]} \left(1 + \frac{[Q]}{K_{iq}}\right) \dots\dots\dots 8$$

The above equations predict that P will be competitive with B and noncompetitive with A, while Q will be noncompetitive with B, and competitive with A, when the nonvaried substrate concentration is unsaturated. With saturated concentration of varied substrate, P will be competitive with B and no inhibition by P will be observed when A is varied at saturating concentration of B.

No inhibition will be observed with Q when B concentration is varied at saturating constant A concentration, but Q will be competitive with A at saturated concentration of B.

The inhibition studies by any of the products at saturating levels of the nonvaried substrates could not be performed. Such levels gave rise to substrate inhibition effects that precluded the analysis.

The reduced coenzyme NADPH was a competitive inhibitor of NADP^+ in presence of fixed unsaturated concentration of ethanol (0.9mM). This competitive inhibition is depicted in Figure 3.46 by the series of converging lines that met on the ordinate axis. The inhibition constant K_i obtained from secondary plot (3.46b, Appendix III) is listed in Table 3.19. Double reciprocal plot obtained at fixed unsaturated concentration of NADP^+ while varying ethanol in the presence of NADPH as an inhibitor, clearly indicated a noncompetitive inhibition (Figure 3.47a). Acetaldehyde as an inhibitor exhibits a noncompetitive inhibition with respect to NADP^+

Figure 3.46 a. Double reciprocal plot for the product (NADPH) inhibition of $NADP^+$ -linked activity of FLADH at fixed unsaturated ethanol (0.9 mM). Concentration of $NADP^+$ was varied in presence of NADPH as inhibitor. NADPH concentration in (μM) is represented by the number on each line. All assays were at $25^\circ C$, pH 9.0 and enzyme concentration was $20.6 \mu M$.

Figure 3.47 a. Product inhibition double reciprocal plot of NADP^+ -linked activity of FLADH at unsaturated NADP^+ (0.22 mM). Ethanol was varied in presence of NADPH as inhibitor. The NADPH concentration in (μM) is indicated by the number on each line. Other conditions are the same as in Figure 3.46a.

in presence of fixed unsaturated levels of ethanol (Figure 3.48a), and a competitive inhibition with respect to ethanol (Figure 3.49a) at unsaturated levels of NADP⁺.

In the NAD⁺-linked activity of FLADH, nonlinear Lineweaver-Burk plot was obtained. Results obtained with Bardsley and Childs diagnostic plot (Figure 3.27a) suggested that NAD⁺ activity might operate through a random mechanism. The Random BiBi system yield a very complicated velocity equations with 37 new terms in the denominator, in addition to those of the ordered BiBi equation. The reciprocal plots are nonlinear unless one substrate is saturating (140). In product inhibition, P and Q yield noncompetitive inhibition kinetics. At a fixed unsaturating cosubstrate concentration, the intercept replots are hyperbolic, while the slope replots are more complex functions of the product inhibitor concentrations (ibid). At saturating concentration of the fixed substrate, both slope and intercept replots are hyperbolic. Product inhibition studies for NAD⁺-linked activity of FLADH by any of the products at high concentration of the nonvaried substrate were precluded for the same reason that precluded such analysis for NADP⁺-linked activity.

The results obtained for the product inhibition studies performed at non-saturating levels of nonvaried substrates are represented in Figures 3.50a-3.53a. Inhibition of the NAD⁺-linked by NADH was noncompetitive with respect to both

Figure 3.48 a. Product inhibition of NADP^+ -linked activity of FLADH in presence of unsaturated (0.9 mM) ethanol. The NADP^+ concentration was varied in presence of acetaldehyde as inhibitor. Acetaldehyde concentration was varied as follows from top to bottom: 0.1, 0.075, 0.05, 0.015 and 0 mM. For other conditions see Figure 3.46a.

Figure 3.49 a. Product inhibition of NADP^+ -linked activity of FLADH at unsaturated NADP^+ (0.22 mM). Ethanol was varied in presence of acetaldehyde as inhibitor. Acetaldehyde in (mM) was varied as indicated by the number on each line. For other conditions see Figure 3.46a.

Table 3.19

Product inhibition constants of NADP⁺-linked activity of FLADH

Product	Varied substrate			
	NADP ⁺ ^a		Ethanol ^b	
	K _{ii}	K _{is}	K _{ii}	K _{is}
Acetaldehyde	0.315	0.270	--	0.125
NADPH	—	0.0419	0.0689	0.0367

Inhibition constants given are in mM. For explanation of constants, see Table 3.18. Average error calculated from correlation coefficients of linearly regressed data are $K_{ii} \pm 14\%$ and $K_{is} \pm 20\%$.

- a When NADP⁺ was the varied substrate, ethanol concentration was fixed at 0.9 mM.*
- b NADP⁺ concentration was fixed at 0.22 mM when ethanol was the varied substrate.*
- No value obtained.*

Enzyme concentration in all was 20.6 μ m. Products concentration were, for NADPH 0-33.3 mM, and acetaldehyde 0-0.1 mM.

Figure 3.50 a. Double reciprocal of product (NADH) inhibition of NAD^+ -linked activity of FLADH, at unsaturated ethanol (0.8 mM). NAD^+ concentration was varied in presence of NADH as an inhibitor. The number on each line represents NADH concentration in (μM). Assays were at 25°C , pH 9.0 and enzyme concentration was $18.6 \mu\text{M}$.

Figure 3.51 a. Product inhibition of NAD^+ -linked activity of FLADH in presence of unsaturated NAD^+ (0.075 mM). Ethanol was varied in presence of NADH as inhibitor. The NADH concentration in μM was varied as indicated by the number on each line. For other conditions see Figure 3.50a.

Figure 3.52 a. Product inhibition of NAD^+ -linked activity of FLADH in presence of unsaturated ethanol (0.8 mM). Concentration of NAD^+ was varied in presence of acetaldehyde as inhibitor. Acetaldehyde concentration in mM is varied as indicated by the number on each line. All assays were at 25°C , pH 9.0 and enzyme concentration was $1.8 \mu\text{M}$.

Figure 3.53 a. Double reciprocal plot of product (acetaldehyde) inhibition of NAD^+ -linked activity of FLADH in presence of unsaturated fixed NAD^+ (0.075 mM) and varied ethanol concentration. Each line corresponds to a different acetaldehyde concentration and these are (shown top to bottom): 0.1, 0.075, 0.05, 0.025, 0.015 and 0 mM. Enzyme concentration was $1.8 \mu\text{M}$, assays at pH 9.0 and 25°C .

and ethanol. Acetaldehyde as a product was a noncompetitive inhibitor as well, with respect to both NAD^+ and ethanol. The four noncompetitive double reciprocal plots were linear, but slopes and intercepts replots were hyperbolic (Figures 3.50b-3.53b, Appendix III). Such type of inhibition is designated as "slope-hyperbolic, intercept-hyperbolic noncompetitive inhibition" (141), and represented by the following schematic diagram 5:

Scheme 5. Hyperbolic non-competitive inhibition where α and β are factors which influence binding and reaction rate, respectively. The degree of inhibition observed will depend on the relative sizes of α and β .

A plot of $1/\Delta_{\text{slope}}$ versus $1/[I]$ and $1/\Delta_{\text{intercept}}$ versus $1/[I]$ converts the relationship to straight line (141). The Δ_{slope} is defined as follows:

$$\Delta_{\text{slope}} = \text{slope}_I - \text{slope}_0$$

where slope_I is the slope at inhibitor concentration I , and slope_0 is the slope when $I=0$, and the same for the intercept. By using Δ_{slope} and $\Delta_{\text{intercept}}$, we are in fact

raising the horizontal axis so that curves become hyperbolae that start at the origin and is given by the equation 9:

$$1/\Delta_{\text{intercept}} = \frac{\alpha K_i V_{\text{max}}}{(1-\beta)} - \frac{1}{[I]} + \frac{\beta V_{\text{max}}}{(1-\beta)} \dots\dots\dots 9$$

The plot is linear with slope of $\alpha K_i V_{\text{max}}/(1-\beta)$ and an intercept on $1/\Delta_{\text{intercept}}$ - axis of $\beta V_{\text{max}}/(1-\beta)$.

Similarly, $1/\Delta_{\text{slope}}$ is given by equation 10:

$$1/\Delta_{\text{slope}} = \frac{\alpha K_i' V_{\text{max}}}{K_s (\alpha-\beta)} - \frac{1}{[I]} + \frac{\beta V_{\text{max}}}{K_s (\alpha-\beta)} \dots\dots\dots 10$$

Again the plot is linear with a slope of $\alpha K_i V_{\text{max}}/K_s(\alpha-\beta)$ and an intercept on the $1/\Delta_{\text{slope}}$ axis of $\beta V_{\text{max}}/K_s(\alpha-\beta)$. These $1/\Delta_{\text{slope}}$ and $1/\Delta_{\text{intercept}}$ replots for the four noncompetitive plots obtained are represented by Figures 3.50c to 3.53c (in Appendix III). With α , β determined, K_i from $1/\Delta_{\text{intercept}}$ and K_i' from $1/\Delta_{\text{slope}}$ are obtained and values are given in Table 3.20.

In the inhibition by NADH with respect to ethanol and by acetaldehyde with respect to NAD^+ , α value were less than unity ($\alpha < 1$). Such a value is obtained when an inhibitor converts the enzyme to an EI form with an increased affinity for the substrate, but the resulting ESI forms product slower than ES. In contrast, the inhibition by NADH with respect to NAD^+ , and by acetaldehyde with respect to ethanol gave a value greater than unity ($\alpha > 1$). This indicate these products as inhibitors decrease the affinity of the enzyme

Table 3.20

Product inhibition constants of NAD⁺-linked activity of FLADH

Product	Varied substrate							
	NAD ⁺ ^a				Ethanol ^b			
	K _i	K _i '	α	β	K _i	K _i '	α	β
Acetaldehyde	34.5	15.2	0.629	0.483	0.0859	0.548	3.62	0.0113
NADH	0.810	1.88	5.92	0.261	3.51	4.43	0.563	0.127

Inhibition constants given are in μM . All gave intercept-hyperbolic, slope-hyperbolic non-competitive inhibition. K_i , K_i' , α and β were determined from $1/\Delta\text{intercept}$ and $1/\Delta\text{slope}$ plots (see Appendix III). Average error calculated from correlation coefficient of $1/\Delta\text{intercept}$ and $1/\Delta\text{slope}$ are: $K_i \pm 7.2\%$, $K_i' \pm 6.1\%$, $\alpha \pm 33\%$, and $\beta \pm 30\%$.

a Here NAD⁺ was varied, and ethanol concentration was fixed at 0.8 mM.

b Ethanol was the varied substrate. NAD⁺ was fixed at 0.075 mM.

Enzyme concentration ranged between 1.80–18.6 μM . Product concentrations were, for NADH 0–10.0 μM , acetaldehyde range was 0–0.1 mM.

for the substrate. The β value in all cases is less than unity ($0 < \beta < 1$), indicating that these products as inhibitors decreased the V_{max} of the reaction leading to $V_{maxi} < V_{max}$ (Table 3.20).

In cross product inhibition, the action of NADPH as a product inhibitor on NAD⁺-linked activity of FLADH was investigated. The fact that NADPH inhibited competitively with respect to NADP⁺ (Figure 3.46a) as well as NAD⁺ (Figure 3.54a) makes it reasonable to assume that the binding sites for NAD⁺ are largely coincident with NADP⁺ binding sites. The inhibition pattern observed, differed for NADP⁺-linked activity in the presence of NADH as a product (Figure 3.55a). The hyperbolic non-competitive inhibition somewhat complicated interpretation of the equivalency of NAD⁺ and NADP⁺ binding sites. The cross product inhibition constants and inhibition constants obtained for each reduced coenzyme acting as an inhibitor on its own respective activity are presented in Table 3.21. As mentioned previously NADPH inhibited competitively both NAD⁺ and NADP⁺-linked activities. Inhibition constant of NADPH for NADP⁺-linked activity was 4-folds larger than inhibition constant obtained for NAD⁺ activity. The inhibition constant (K_i , K_i') obtained from hyperbolic noncompetitive inhibition exhibited by NADH on both the NAD⁺ and NADP⁺-linked activities are about the same magnitude.

Figure 3.54a. Double reciprocal plot for cross product inhibition of FLADH. NAD^+ was varied in presence of NADPH as inhibitor and fixed unsaturated ethanol concentration (0.8 mM). The NADPH concentration (μM) was varied as indicated by the number on each line. Enzyme was $1.8 \mu M$ and assays were at pH 9.0 and $25^\circ C$.

Figure 3.55 a. Double reciprocal plot for cross product inhibition of FLADH. $NADP^+$ was varied in presence of NADH as inhibitor and fixed unsaturated ethanol concentration (0.9 mM). NADH was varied as follows from top to bottom: 33.3, 15.0, 10.0, 5.0, 2.5 and 0 μ M. Enzyme concentration was 20.6 μ M. Assays were at pH 9.0 and 25°C.

Table 3.21

Cross product inhibition constants of NAD^+ - and NADP^+ -linked activities of FLADH

Kinetic parameter	NAD^+ -linked activity		NADP^+ -linked activity	
	NADH^a	NADPH	NADH^a	NADPH
K_i (μM)	0.810		1.72	
K_i' (μM)	1.88		2.95	
α	5.92		2.69	
β	0.261		0.859	
K_{is} (μM) ^b		9.38		41.9

a All inhibition constants (K_i , K_i' , α , β) except K_{is} were determined from $1/\Delta_{\text{intercept}}$ and $1/\Delta_{\text{slope}}$ plots (Appendix III). Errors on above constants were determined as mentioned in Table 3.20 and average errors are: $K_i \pm 3.2\%$, $K_i' \pm 6.4\%$, $\alpha \pm 22\%$, and $\beta \pm 29\%$.

b Inhibition constants K_{is} indicate competitive inhibition, and is obtained from secondary plots in Appendix III. Errors calculated from correlation coefficients of linearly regressed data and average error is $K_{is} \pm 31\%$.

Ethanol concentration was fixed at 0.8 mM for the NAD^+ -linked activity and at 0.9 mM for the NADP^+ -linked activity. Product concentration were, NADH as 0–8 μM , NADPH range was 0–33.3 μM . Enzyme concentration was 1.8–20.6 μM .

3.3.7. pH dependence of enzyme activities

The effects of variations of the hydrogen ion concentration on activity of enzymes have close similarities to the effects of either an activator or an inhibitor, and could yield valuable information on the kinetic mechanism obeyed by the enzyme (142). It is not surprising that the active sites on enzymes are frequently composed of ionizable groups which must be in the proper ionic form to maintain the conformation of the active site, bind the substrate, or catalyze the reaction. The objective of the pH studies is to identify those specific groups that play important role(s) in the enzymic reaction (143). Such identification is usually approached by use of $\log V_{max}$ versus pH plots and extrapolation of the appropriate regions of the curve to the abscissae to identify the ionizing species.

The effect of pH on the dehydrogenase activities of two enzymes (FLADH and TBADH) were investigated in this study, as well as the pH effects on the multifunctional activities of FLADH. Studies were investigated over the pH range of 5.0-11.0.

3.3.7.1 Effect of pH on NAD(P)⁺-linked activities of FLADH

Kinetic assays of the NAD⁺-linked activity were performed by varying NAD⁺ concentration at varied fixed ethanol concentration as well as variation in the pH.

Figures 3.56a and 3.57a are the double reciprocal plots of initial rates plotted against NAD^+ concentration for varied fixed ethanol concentration with variation in pH corresponding to pH 7.0 and 7.5. Double reciprocal plots (Figures 3.58a-3.63a) for pH 8.0, 8.5, 9.0, 10.0, 10.5 and 11.0 respectively are given in Appendix IV. Kinetic constants determined from secondary plots of the slopes and intercepts (Figures 3.56b - 3.63b) in Appendix IV, are listed in Table 3.22.

There was no systematic increase or decrease in the constants K_a , K_b or K_{ia} with change in pH. However, there was a gradual continuous increase in V_{max} . The turnover number increased by 9.0 fold as pH increased from 7.0 to 11.0. Estimates of V_{max} at low pH values (5.0-6.5) were made by measurements of the initial rate at saturating ethanol and NAD^+ concentration. A biphasic pH plot represented by Figure 3.64 is obtained when V_{max} is plotted versus pH. Curve rose to approach V_{max} asymptotically, and then rose again to a limit of γV_{max} . Such a limiting nonzero plateau may indicate that more than one ionic form of the enzyme are catalytically active (143). Takahashi et al. (144) in their study on the pH- velocity profiles with the PI 5 isozyme of horse liver aldehyde dehydrogenase found that the pH-velocity profile in the absence of added Mg^{2+} ions cannot be fitted to a simple sigmoidal curve. It indicated that events are not governed by just one proton dissociation.

Figure 3.56 a. and Figure 3.57 a. Double reciprocal plots of NAD^+ -linked activity of FLADH in sodium phosphate buffer, pH 7.0 and 7.5 respectively. Ethanol concentration (mM) is varied as indicated by the number on each line. Enzyme concentration was $0.28 \mu M$.

Table 3.22

The pH dependence of kinetic constants of NAD⁺-linked activity of FLADH

pH ^a	V _{max} (mM min ⁻¹)	K _a (mM)	K _b (mM)	K _{ia} (mM)	V _{max} /E _t ^b (min ⁻¹)	log V _{max}
5.0	0.00322	--	--	--	--	-3.49
5.5	0.00875	--	--	--	--	-2.06
6.0	0.0105	--	--	--	--	-1.98
6.5	0.0244	--	--	--	--	-1.61
7.0	0.0435	0.174	1.43	0.203	159	-1.36
7.5	0.200	0.340	4.59	0.131	730	-0.699
8.0	0.133	0.0708	1.46	0.105	485	-0.676
8.5	0.200	0.0900	0.897	0.110	730	-0.699
9.0	0.233	0.107	0.497	0.117	850	-0.633
10.0	0.372	0.0743	0.551	0.0964	1357	-0.430
10.5	0.385	0.0913	0.695	0.0996	1404	-0.415
11.0 ^b	1.77	0.0885	1.45	0.855	1451	+0.248

a Buffers used were: sodium acetate for pH 5.0 and 5.5, sodium phosphate for pH 6.0-8.0, sodium pyrophosphate for pH 8.5-10.5, and sodium bicarbonate for pH 11.0.

b Enzyme concentration at pH 11.0 was 1.22 μ M. Enzyme concentration for kinetic assays at other pH values was 0.274 μ M. Errors were determined as mentioned in previous tables and were: V_{max} $\pm$ 11.6%, K_a $\pm$ 18.2%, K_b $\pm$ 20.5%, K_{ia} $\pm$ 30.7%, and V_{max}/E_t $\pm$ 12.9%.

-- Not determined

The pH velocity profile in the presence of a fixed concentration of Mg^{2+} ions revealed a more complex pattern which resemble the pH-velocity profile of the NAD^+ -linked activity obtained in this study. Takahashi et al (144) observed that an elevated pH caused an increase in velocity of the rate limiting step; i.e. hydrolysis of acyl-enzyme intermediate. This event was governed by the ionization of two-functional groups model. In the region of pH 5-8 a group with $pK_{a1}=7.2$ is associated, while at pH above 8.0, rate limiting step is governed by above event plus that from a group with $pK_{a2}=9.5$. A charge relay system may function at this nonphysiological alkaline region. The group involved in this relay system could be a tyrosine, analogous to tyrosine-311 in glyceraldehyde phosphate dehydrogenase (GAPDH). At physiological pHs, a general base (presumably a histidine analogous to histidine-176 in GAPDH) catalyzed reaction prevails for the hydrolysis of the acyl intermediate. Hydrolysis of this acyl intermediate at the physiological pH does not require the action of the charge relay system (144). The biphasic pH-velocity plot of NAD^+ -linked activity of FLADH obtained suggests the independent presence of more than one catalytically active enzyme form. As the curve rises in the second phase of pH plot (143), then γ has to be greater than unity ($\gamma > 1$). In another word, $E^{n-1}S$ is catalytically more active than E^nS , and this system is represented in Scheme 6:

Figure 3.64. The pH-velocity profile of NAD^+ -linked activity of FLADH. (O) is $\log V_{\max}$, and (Δ) is specific activity. Inset shows the pH response of $\log V_{\max}/K_a$.

Scheme 6. Diprotic model, where all forms of "E" bind S, but only E^nS and $E^{n-1}S$ yield products.

The $\log V_{max}$ versus pH plot is biphasic as well (Figure 3.64). The γ value is estimated to be approximately equal to 22.4. This γ value indicates there is an increase in rate of reaction accompanying deprotonation of the enzyme. In this situation, hydrogen ions appear to be as inhibitor in the range between the two plateaus of Figure 3.64.

In an enzymic reaction of one substrate and one product, the pH dependence of the velocity equation for a diprotic model (Scheme 6) is well characterized, however not yet for a two substrate systems. The complexity of velocity equation of a bisubstrate reaction with pH dependence

obscure the identification of the pK value. However an estimate of the pK_a of groups that may be involved is possible from the pH-velocity curve in Figure 3.64. Two ionizable species can be identified from the pH plot, one with pK7.2 and the other with pK 9.45.

A pH profile for NADP⁺-linked activity was generated from initial rate data determined over the same range of pH 5.0-11.0 at a single saturating substrates concentration. Figure 3.65 shows a double maximum. The primary maximum was at the acidic pH 5.5 and showed higher enzyme activity with a value of $3.47 \times 10^{-3} \text{ Umg}^{-1}$. The second peak at the alkaline pH 10.5 represented a lower enzyme activity of $0.886 \times 10^{-3} \text{ Umg}^{-1}$. A remarkable difference in pH-velocity profile of NADP⁺-linked activity is noted when compared to the pH profile of the NAD⁺-linked activity.

3.3.7.2 pH dependence of the esterase activity of FLADH

A pH dependence of the esterase activity of FLADH was obtained also by single point assays. Corrected initial rate data were obtained at 0.2 mM concentration of p-nitrophenylcaprylate. The pH dependence of the esterase activity (Figure 3.66) showed a maximum at pH 7.0 and enzyme activity of 0.415 Umg^{-1} . There was another maximum at pH 8.0 with activity of 0.234 Umg^{-1} . Initial rate data could not

Figure 3.65. pH-velocity profile of NADP⁺-linked activity of FLADH. Assays contained 1.0 mM NADP⁺, 10 mM ethanol and 15.3 μ M enzyme at 25°C.

be obtained at pH above 9.0 due to high rates of spontaneous hydrolysis of the ester.

3.3.7.3 pH dependence of the dismutase activity of FLADH

The low dismutase activity of the enzyme hindered a detailed pH study on this activity. A pH profile was generated from single point data.

Initial rate data were obtained at substrate concentration of 0.2 mM at different pH values. In the pH-velocity profile of dismutase activity (Figure 3.67), two maximum analogous to NADP⁺-linked activity profile were noted. The primary maximum was at acidic pH 5.5 again, however with low enzyme activity of $0.750 \times 10^{-3} \text{ Umg}^{-1}$. The second maximum had higher enzyme activity of $2.55 \times 10^{-3} \text{ Umg}^{-1}$ and occurred at pH 9.0. An interesting feature in this activity-dependence pH profile is the nonzero plateau. This indicates presumably that more than one ionic form of the enzyme was active as observed early in the NAD⁺-linked activity profile. The second deprotonated form catalytically less active; i.e. $\gamma < 1$.

Figure 3.66. pH-velocity profile of esterase activity of FLADH. All assays were at 25°C, contained 0.2 mM of p-nitrophenyl caprylate and 1.44 μ M enzyme.

Figure 3.67. pH-velocity profile of dismutase activity of FLADH. All assays contained 0.2 mM octanal, 1.0 mM NAD⁺ and 1.4 μ M enzyme at 25°C.

3.3.7.4. The pH dependence of NADP⁺-linked activity of TBADH

As with pH studies done on FLADH, a pH dependence of NADP⁺-linked activity of TBADH was obtained. Single point assays at saturated concentrations of propan-2-ol and coenzyme performed over the pH range of 5.0 to 11.0. As the pH increased, activity of enzyme experienced a progressive increase to a maximum value between pH 10.0-10.5. Further increase in pH resulted in a decrease in enzyme activity. The pH-dependence of the NADP⁺-linked activity of this enzyme was a bell shape curve (Figure 3.68). It was not possible to assign pK values with this data.

3.4. Chemical Modifications

3.4.1. General

The principle reason for chemically modifying an enzyme or protein in general is to identify those amino acid residues that function intimately in catalysis. In order to interpret the effect of such modification unambiguously, the reaction should be specific and limited, that is the more unreactive a reagent is, the more specific it is likely to be (145). In practice, chemical modification suffers from a lack of specificity of the reagents towards a specific residue. If modification is carried out under the mildest conditions, it will suffice to provide an

Figure 3.68. pH-velocity profile of NADP⁺-linked activity of TBADH. All assays were at 25°C, containing 10 mM of propan-2-ol, 1.0 mM NADP⁺ and 0.56 μ M enzyme.

information for the functional group of interest. It is useful also to attack a given residue with several different modifying reagents if possible to provide supportive evidence. During a chemical modification of specific residue, susceptibility of the enzyme activity to specific modification can be useful in identifying the essential residues and may provide insight into the mechanism.

In this study, number of chemical modifications were performed on alcohol dehydrogenases isolated from rat liver, fish liver and thermophilic bacteria. In most cases both NAD^+ and NADP^+ -linked activities were monitored throughout the modifications in an effort to identify the catalytically essential amino acids of each activity. In both FLADH and RLADH, dismutase as well as the esterase activities in most cases were monitored during the chemical modifications. Its purpose is to demonstrate the selective chemical inactivation or activation if there is any of these multifunctional activities.

Quantitation of the change in the number of a specific residues affected by a chemical modification is not always essential. Of course, many meaningful protein modifications have been performed without quantitative analysis. Unfortunately, quantitation frequently requires chromatographic procedures to isolate the product. Isolation of a product requires a fair amount of protein (which was not possible in most of the cases) in order to get a

reliable analysis. Nevertheless, effect of modification and correlation of the change in the catalytic activity with affected residues was possible.

In most of the chemical modifications, in particular, concentration of the modifying reagent and pH of the reaction were chosen so as to observe inactivation or activation within a time span of an hour. For each modification, a control curve was included which follows enzyme activity of the free unmodified enzyme but subjected to identical pH and temperature conditions over the same period.

Tables that provide summary of the chemical modifications results that were obtained for each enzyme are presented at the end of the section (p.248, 250 and 255). The specific modification are described in the following subsections.

3.4.2. Carboxymethylation

It is well documented now that several dehydrogenases possess, at each active centre, a thiol group which is essential for enzyme action (4, 18). Horse liver alcohol dehydrogenase (HLADH) is one among them. Li and Vallee (122) demonstrated that iodoacetate selectively carboxymethylates two of the enzyme's 24 thiol groups with consequent complete loss of enzyme activity.

Inactivation of HLADH by the iodoacetate (or haloacids in general) is due to the selective alkylation of Cys-46 and is facilitated by reversible complex formation (18, 67-69). Iodoacetate inactivates HLADH 53 times faster than does iodoacetamide (145). It was suggested that ionic interaction of the iodoacetate's carboxyl group with the guanidine group of Arg-47 facilitates the reaction (73). The Arg-47 is part of the coenzyme binding site, and is where the pyrophosphate group of coenzyme binds. That is, the rate of inactivation by haloacetates or haloacetamides is reduced 100-fold by NAD⁺ or NADH (146, 147). The NAD(H) pyrophosphate groups bind to Arg-47 and Arg-369 and can thus prevent access of the reagent to Cys-46 (4, 21).

Haloacids, such as iodoacetic, and chloroacetic acids are the most widely used protein alkylating group reagents. The nucleophilic substitution reactions of alkyl halides with enzymatic sulfhydryl groups is given by equations 11:

Modification of RLADH with iodoacetic acid resulted in a 40% decrease in NADP⁺ activity within 10 minutes of reaction (Figure 3.69) and 30% of activity remained after 50 minutes. Curiously NAD⁺ activity was drastically affected and only 10% of the activity remained after 10 minutes (Figure 3.69). After 40 minutes of reaction with iodoacetate, the NAD⁺-linked activity was completely

eliminated. The esterase activity of RLADH dropped to about 27% after 60 minutes of reaction (Table 3.23). Although there was differential sensitivities to the alkylating agent, both NAD^+ and NADP^+ -linked activities were deactivated when enzyme was carboxymethylated with the haloacid. The FLADH was modified with the same alkylating agent (iodoacetate). Again the NAD^+ -linked activity was affected to a greater extent than the NADP^+ -linked activity. At 10 minutes of reaction time there were 45% and 20% reduction in the NAD^+ and NADP^+ -linked activities, respectively. While the NAD^+ -linked activity was progressively deactivated as reaction time elapsed, NADP^+ -linked activity remained constant after 35 minutes. Total loss in NADP^+ activity was about 47% after 60 minutes of reaction. Both dismutase and esterase activity were also followed throughout this modification. Retained activities were 22% and 65% for the dismutase and esterase activities, respectively after 60 minutes of reaction time (Table 3.24). It is obvious that esterase activity was the least affected by the alkylating agent (iodoacetate).

Often, but not always, carboxymethylation of proteins can be performed by the corresponding acid amides which are interchangeable for the acids (148). Iodoacetamide ($\text{ICH}_2\text{CONH}_2$) performs a similar carboxymethylation function but has an amide functionality replacing the carboxylic group.

Figure 3.69. Carboxymethylation of RLADH activities. Reaction was at 0-4°C, pH 7.5, 31.9 μ M enzyme, and 8.0 mM iodoacetate. Activities followed were: NAD⁺ control (O), NADP⁺ control (●), NAD⁺ modified (Δ) and NADP⁺ modified (□).

Figure 3.70. Carboxymethylation of NAD⁺ and NADP⁺-linked activities of FLADH. Enzyme (6.6 μ M) was treated with iodoacetate or iodoacetamide (76.4 mM) at 0-4°C, pH 7.4. Activities monitored for the enzyme reacted with iodoacetate were; NAD(P)⁺ control (○), NAD⁺ (Δ) and NADP⁺ (□). Enzyme was reacted with iodoacetamide and NAD⁺ (○) activity was monitored.

The response of NAD⁺-linked activity of FLADH to the iodoacetamide modification is given in Figure 3.70. The extent of modification by the amide is much less when compared to the deactivation that the haloacid acid exerts on NAD⁺-linked activity. This differential response and that the reactivities of iodoacetate and uncharged iodoacetamide could be due partially to the presence of an attractive interaction between the anionic alkylating agent (the haloacid) and an active site residue.

The ineffectiveness of iodoacetamide as modifying agent was illustrated sufficiently even though only the NAD⁺-linked activity of FLADH was modified with this reagent. In Figure 3.70, NAD⁺ activity dropped to 10% when modified by iodoacetate compared to 80% when modified by the iodoacetamide after 60 minutes.

Similarly, the rate of inactivation of the TBADH by iodoacetate was faster than the rate of inactivation by iodoacetamide. This was observed in both NAD⁺ and NADP⁺-linked activities (Figures 3.71a and 3.71b).

The NAD⁺-linked activity was less sensitive than NADP⁺-linked activity to the modification by either iodoacetate or the iodoacetamide. In Figure 3.71a, the NADP⁺-linked activity dropped to < 10% after 60 minutes with iodoacetate, while NAD⁺-linked activity dropped to 40%.

Figure 3.71 a. Carboxymethylation of NAD^+ and NADP^+ -linked activities of TBADH by iodoacetate. Activities are: NAD(P)^+ control (○), NAD^+ (Δ), NADP^+ (□) and NADP^+ -activity of protected enzyme with 0.5 mM NADP^+ (○). Reaction was at 0-4°C, pH 7.5, enzyme (30 μM) and iodoacetate (39 mM).

Figure 3.71 b. Carboxymethylation of TBADH by iodoacetamide. Activities are NAD(P)⁺ control (O), NAD⁺ (Δ), NADP⁺ (□) and NADP⁺-activity of enzyme protected with 0.5 mM NADP⁺ (○). Other conditions see Figure 3.71a.

Like iodoacetate, TBADH alkylated with iodoacetamide retained about 35% of its original NADP⁺-activity, and nearly 70% of the NAD⁺-linked activity (Figure 3.71b).

Modification of NADP⁺-linked activity with iodoacetamide in the presence of 17 fold excess NADP⁺ added prior to reaction is given in Figure 3.71b. The NADP⁺ completely inhibits the inactivation by iodoacetamide. The residual NADP⁺-linked activity of TBADH was 107% in presence of NADP⁺ compared to 35% in its absence after 60 minutes. On the other hand, NADP⁺ slightly stimulates the rate of inactivation by iodoacetate (Figure 3.71a). In the absence of NADP⁺, the enzyme retained about 13% of its NADP⁺-linked activity after 15 minutes and less than 8% after 60 minutes. In the presence of NADP⁺, NADP⁺-linked activity was reduced to 2.5% after 15 minutes. Lack of protection by NADP⁺ in presence of iodoacetate as modifying reagent was in contrast to the complete protection offered by NADP⁺ in presence of iodoacetamide.

3.4.3 Dye-sensitized photo-oxidation

Dye-sensitized photo-oxidation is mostly carried out under mild conditions and allows the modification of a restricted number and types of amino acid residues. A large number of dyes can be used for the photo-sensitized oxidation (125, 149). Methylene blue (cationic dye) and rose bengal (anionic dye) are the two dyes most widely used to

photosensitize the oxidation of proteins. Their specificity for the various amino acids are partially determined by pH. Evidence has shown rose bengal to be more selective for histidine (150). Being anionic is thought to facilitate the rose bengal specificity by favouring the formation of short-lived dye-imidazole complexes (149). In enolase, creatine kinase (150), and aspartate transaminase (151) rose bengal oxidizes them more efficiently. In glyceraldehyde-3-phosphate dehydrogenase, only histidine was oxidized by rose bengal (152). Histidine was also the only amino acid residue affected in the photo-oxidation of ribonuclease (153), *Escherichia coli* tryptophanase (154) and spinach nitrate reductase (155). It is therefore, possible to assess the necessity of histidine for the enzyme function and its importance in catalysis.

Reaction of histidine with sensitizing dye is given by equation 12 below:

Photo-oxidation of histidine results in conversion of histidine to aspartate and urea (156). Photo-oxidation of proteins have been proposed to proceed via two mechanisms (157, 158). In the first type (type I), the substrate (sub) reacts initially with excited triplet sensitizer and

thereafter with oxygen via a radical intermediate. In the type II mechanism as shown in equation 13, energy is transferred from the excited singlet oxygen (1O_2), which in turn, reacts with the acceptor. The Δ_g is the one to be effective due to its longer half-life in solution (159-161).

Whenever possible both NAD^+ and $NADP^+$ -linked activities were followed during this modification, as well as, the other nonphysiological activities.

The $NADP^+$ -linked activity of the RLADH is very low after purification steps with the consequence that a reproducible assessment of this activity during this modification was not possible. Rose bengal was the most effective dye in photo-oxidating NAD^+ -linked activity of this enzyme. The RLADH was modified by methylene blue, rose bengal, and the high energy sensitizer riboflavin. The RLADH retained 3.9% of its NAD^+ activity when modified by rose bengal after 30 minutes. Under the same conditions, NAD^+ -activity remaining after 40 minutes was 9% and 16% of the original catalytic activity when modified by methylene blue and riboflavin respectively (Figure 3.72). The response of esterase activity to the photo-oxidation was different from that of the dehydrogenase. While the dehydrogenase activity was almost completely lost after 30 minutes, esterase

Figure 3.72. Photo-oxidation of NAD⁺-linked activity of RLADH by different sensitizers. Reaction was at 25°C, pH 8.0, enzyme (2.2 μ M), and dye (0.11 mM). Activities are NAD⁺ control (O), NAD⁺ in presence of methylene blue (□), rose bengal (Δ) and riboflavin (○).

activity retained 80% of its original activity after 60 minutes of reaction with rose bengal (Table 3.23). The difference in the responses for these two activities probably indicated the differential dependency of activities on the photooxidizable residues.

The response profiles of NAD^+ and NADP^+ , and dismutase activities of FLADH to the photo-oxidation by rose bengal are given in Figure 3.73. They all showed identical inactivation trends with dismutase activity being the least sensitive. The NAD^+ -linked activity was completely eliminated (1.4% residual activity) by 30 minutes, whereas NADP^+ residual activity was 11.5% by 60 minutes. The dismutase activity was not as sensitive as NAD^+ or NADP^+ activities with 35% residual activity at 60 minutes (Table 3.24).

Finally, NAD^+ and NADP^+ activities of the third enzyme, TBADH were also found to be very sensitive to photo-oxidation by rose bengal. The two activities responded similarly as shown in Figure 3.74. The NAD^+ -linked activity was completely lost by 40 minutes and NADP^+ -linked activity was eliminated by 37 minutes of reaction time (Table 3.25). Identical inactivation profiles suggest a similar dependency of both activities on the photooxidizable residue(s), presumably the histidine.

Figure 3.73. Photo-oxidation of FLADH activities. Activities are: NAD(P)⁺ control (○), NAD⁺ (Δ), NADP⁺ (□), and dismutase (○). Reaction was at 25°C, pH 7.5 rose bengal (0.65 mM) and enzyme (22.5 μM).

Figure 3.74. Photo-oxidation of TBADH activities. Reactions were at 25°C, pH 7.5, rose bengal (0.77 mM) and enzyme (8.3 μM). Activities are NAD(P)⁺ control (O), NAD⁺ (Δ) and NADP⁺ (□).

3.4.4. Glyoxalation

During the past 15 years, information on the functional role of arginyl residues in a wide variety of enzymes as well as other biologically active protein molecules has been obtained from chemical modification studies with α -dicarbonyl reagents (74, 90). It seems that arginyl residues have important roles in the binding of anionic substrates and cofactors by serving as the complementary, positively charged recognition sites in a wide variety of enzymes (74, 88-93). The arginine residues interact with NADP^+ via 2'-phosphate group and/or NAD(P)^+ through their pyrophosphate moiety. Lange et al. (74) investigated effects of two arginyl reagents, 2,3-butandione and phenylglyoxal on alcohol dehydrogenase from human liver, horse liver, and yeast. Reaction of another dicarbonyl; 1,2-cyclohexanedione with arginine (88) requires relatively strong alkali. The dicarbonyl 1,2 cyclohexandione has been useful in antigenicity studies of myoglobin (162) as well as other proteins in less alkaline solutions (163). Phenylglyoxal however, appears to be more useful as a mild arginine reagent (164). It reacts with the guanidino groups of proteins giving derivatives containing two phenylglyoxal moieties per guanidino group as in equation 14:

Initially two α -dicarbonyls reagents; 1, 2-cyclohexandione and phenylglyoxal were employed in an effort to assess their effect on the NAD⁺-linked activity of RLADH and the implication of the arginine residue(s). Modifications were carried in different buffered solution with concentration of reagents ranging between 110-164 fold excess. Buffers used were tris/HCl, sodium borate, and sodium bicarbonate; all at pH 8.2.

Although there was a very minor variation in the extent of inactivation from one buffer to another (results are not shown), but the highest degree of inactivation occurred in the borate buffer. For subsequent studies, 50 mM borate buffer pH 8.2 was adopted for the site-specific modification of RLADH, FLADH and TBADH. Under identical conditions (50 mM borate, pH 8.2 and concentrations of the dicarbonyls), the inactivation of RLADH proceeded more rapidly using phenylglyoxal than when using 1,2-cyclohexandione (Figure 3.75). Rapid inactivation occurred after 10 minutes of reaction time, and NAD⁺-linked activity dropped to 60% and 50% of original catalytic activity when RLADH was modified

Figure 3.75. Glyoxalation of RLADH. Activities are: NAD(P)⁺ control (○), NAD⁺ modified with phenylglyoxal and 1,2-cyclohexandione (Δ,▲), NADP⁺ in presence of phenylglyoxal (□), NAD⁺- activity of enzyme protected with 1.0 mM NAD(H) in presence of phenylglyoxal (○). Reaction was at 0-4°C, in borate pH 8.2, enzyme (66 μM) and modifying reagent (10.9 mM).

with 1,2-cyclohexandione and phenylglyoxal, respectively. Minimal variation in the inactivation degree was observed after extended time of exposure to either of the modifying reagents. Under the same condition, phenylglyoxal inactivated NADP⁺-linked activity of RLADH. The trend and extent of inactivation differed from that of the NAD⁺-linked activity. There was a progressive inactivation with 20% residual NADP⁺ activity after 50 minutes (Figure 3.75). Protection of NAD⁺-linked activity by 1.0 mM NAD⁺ or NADH was almost equal. Residual NAD⁺-activity were 100% and 103% after 60 minutes when enzyme was protected by NADH and NAD⁺ respectively. Esterase activity of RLADH enzyme was not at all affected by phenylglyoxal (100% esterase activity after 60 minutes, Table 3.23).

Progress curves for phenylglyoxalation of FLADH activities in 50 mM borate buffer pH 8.2 are presented in Figure 3.76. The concentration (50.6 mM) of phenylglyoxal was significantly higher than that employed for the RLADH, or HLADH (results are not shown). The inactivation of NAD⁺ and NADP⁺-linked activities was insignificant with residual activities of 81% and 76% respectively. The insensitivities of esterase and dismutase activities as well (Table 3.24) agreed with the results obtained with NAD⁺ and NADP⁺ activities above. This will be discussed further.

The NADP⁺ activity present in dihydrofolate reductase is attributed to the electrostatic interaction between an

Figure 3.76. Phenylglyoxalation of FLADH. Reaction was at 0-4°C, borate pH 8.2, enzyme (2.3 μ M) and phenylglyoxal (50.6 mM). Activities are: NAD(P)⁺ control (O, ●), NAD⁺ (Δ) and NADP⁺ (□).

arginine and the dianionic 2'-phosphate group of the coenzyme as verified by the crystal structure determined by X-ray of the enzyme (44, 100). This same phenomenon is observed in erythrocyte glutathione reductase (GRD) which is an NADP(H) specific enzyme. A favourable binding energy is contributed by the two arginines; Arg-218 and Arg-224 in the coenzyme binding domains that are interacting with 2'-phosphate group of NADP⁺. The TBADH is an NADP⁺ specific, although NAD⁺ can also serve as an electron donor, but to lesser extent. It is of interest to investigate the involvement of arginine residues if any in the binding or catalysis of TBADH. The TBADH was modified by phenylglyoxal in borate buffer, pH 8.2 as was the case for the two previous enzymes. With 300 fold molar excess of the phenylglyoxal, NAD⁺-linked activity was moderately affected. There was a gradual inactivation with 52% of the original activity remaining after 60 minutes of reaction (Figure 3.77). No protection was offered by 0.1 μ M NAD⁺ and NAD⁺-linked activity was reduced to 60% of the original activity within 10 minutes and remained relatively unchanged thereafter. As for NADP⁺-linked activity, TBADH was treated with varying concentrations of phenylglyoxal. Concentration ranged between 160-535 fold molar excess of the reagent. In all cases there was less inactivation than that observed with 300 fold molar excess used in the modification of the NAD⁺-linked activity. Inactivation of NADP⁺ activity decreased with excessive amounts of the

reagent. The NADP⁺-linked activity (in presence of 347 fold molar excess of phenylglyoxal) reduced to 70% of original activity within 10 minutes (Figure 3.77) and remained relatively unchanged throughout the whole modification (Table 3.25). The coenzyme NADP⁺ offered little protection of the NADP⁺-linked activity against inactivation by phenylglyoxal (Figure 3.77). The protected enzyme retained up to 80% of its original catalytic activity after 60 minutes. The slight sensitivity of both NAD⁺ and NADP⁺ activities to phenylglyoxal modification will be discussed further.

3.4.5. Amidation

Conservation of the Asp-223 residue in HLADH, YADH and fourteen other alcohol dehydrogenases (4, 43) demonstrates the importance of this residue. One of the carboxyl oxygens of Asp-223 is involved in hydrogen bond to O-2' hydroxyl of the adenosine ribose (21). Whereas charge repulsion between the anionic 2'-phosphate group of NADP⁺ and the carboxyl oxygen of Asp-223 would weaken the binding of this coenzyme, and explains the high degree of NAD⁺ specificity in these enzymes.

Carboxyl groups of proteins can be converted into amides by a two-step reaction. The procedure involves initial activation of the carboxylic acid by a water-soluble carbodiimide (WSC) to form an O-acylisourea intermediate

Figure 3.77. Phenylglyoxalation of NAD^+ and NADP^+ -linked activities of TBADH. Reaction was at $0-4^\circ\text{C}$, borate pH 8.2, enzyme (28.5 and $11.9 \mu\text{M}$) and phenylglyoxal (8.5 and 4.2 mM) for NAD^+ and NADP^+ activity respectively. In protection experiments, $0.1 \mu\text{M}$ NAD^+ or $3 \mu\text{M}$ NADP^+ was added prior to reaction. Activities are: NAD(P)^+ control (○), NAD^+ modified (△), NADP^+ modified (□), NAD^+ protected (▲) and NADP^+ protected (■).

(96, 165). This highly reactive intermediate in aqueous solution react by two routes. First in the presence of an excess of appropriate nucleophile, O-acylisourea condenses to form the corresponding amide and urea. Second route, O-acylisourea rearranges to an N-acylurea via an intramolecular acyl transfer as shown in scheme 7 below:

Scheme 7. Reaction of a WSC with a protein carboxylic group followed by rearrangement or nucleophilic attack.

Several water soluble carbodiimide have been used for the modification of proteins (163). In this study, all reactions were performed in presence of 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (EDC) for which the structure is shown below:

Just as the water soluble carbodiimide (WSC) can be varied, so the amine component can be varied. The character of the product will vary considerably depending upon the amine employed (163). Its ionic character can be altered as in equations 15, 16, and 17:

Two other amine nucleophiles, ammonium chloride and methylamine were employed.

It was mentioned previously that Asp-223 plays an important role in dictating the NAD⁺ specificity of several dehydrogenases such as HLADH, YADH, and many others. With the various nucleophiles available, the negative charge on targeted carboxyl groups can be kept, removed or replaced by a positive charge. Any change in the NAD⁺/NADP⁺ specificity of enzymes studied can then be monitored. Thus, it becomes possible to determine the importance of carboxyls to the biological activity by monitoring the changes in the enzymic activity.

Knowledge of the three dimensional structure of HLADH and the importance of Asp-223 in HLADH and YADH allowed the use of these two enzyme as models in preliminary experiments. The amidation of HLADH was performed in the presence of excess EDC and ammonium chloride. The NAD^+ -activity decreased to 80% after 60 minutes (Figure 3.78). Under identical conditions, the NADP^+ -linked activity however increased slightly. A gradual increase occurred in the activity reaching a maximum at 30 minutes. At this point, a 1.6 fold increase in the NADP^+ -activity was observed, after which activity gradually decreased and dropped to 116% of original catalytic activity at 60 minutes.

The YADH when subjected to this modification using identical conditions, the NAD^+ -linked activity also experienced an inactivation. Eighty % of its activity remained after 60 minutes of reaction (results are not shown). On the other hand this modification failed to induce any changes in the NADP^+ -linked activity of the YADH. It was noted that YADH has a higher NAD^+ specificity than HLADH, and the very low NADP^+ -linked activity makes it hard to follow it throughout modification.

Treatment of the RLADH with 4×10^3 fold molar excess of ammonium chloride and EDC resulted in inactivation of both the NAD^+ and NADP^+ -linked activities though to different extent (Figure 3.79). The NAD^+ -linked activity was

Figure 3.78. Amidation of HLADH activities. Reaction was at 0-4°C, MES pH 6.0, enzyme (6.17 μ M), (50 mM) of EDC and NH_4Cl . Control contains same reagents except NH_4Cl . Activities are: NAD(P)^+ control (O, ●), NAD^+ (Δ) and NADP^+ (□).

Figure 3.79. Amidation of RLADH activities. Reaction was at 0-4°C, MES pH 6.0, enzyme (12.5 μ M), (50 mM) EDC and NH_4Cl . Activities are: NAD^+ control (○), NADP^+ control (●), NAD^+ modified (Δ), and NADP^+ modified (□).

slightly inactivated with 80% of the original activity remained after 60 minutes of reaction time, similar to HLADH and YADH. Under the same conditions, the NADP⁺-linked activity unexpectedly was inactivated as well. There was a drastic reduction at 10 minutes, and NADP⁺ activity was progressively reduced to 33% of original activity after 60 minutes (Figure 3.79). The esterase activity was reduced to 50% after 60 minutes of exposure to EDC and NH₄Cl (Table 3.23).

The slight responses of NAD⁺-linked activity of the three enzymes (HLADH, YADH, and RLADH) to the modification with ammonium chloride may be due to the fact that ammonium chloride is not fully reacting with the activated carboxyls. The ammonium chloride did not appear to be the preferred reagent for this study essentially because of its low nucleophilic capacity. Therefore a strong nucleophile was required which is capable of reacting rapidly with the O-acylisourea to effectively compete with the rearrangement reaction (95).

In modification of FLADH, the same activating carbodiimide (EDC) was utilized but the nucleophilic agent was varied. In this way, the nucleophile groups varied in charges and size, and exhibited different influences on the activity of the enzyme. The effect of four different nucleophiles under comparable conditions on the NAD⁺-linked activity of FLADH is given in Figure 3.80a. Slight

Figure 3.80 a. Amidation of NAD⁺-linked activity of FLADH in presence of various nucleophiles. Reaction was at 0-4°C, MES pH 6.0, enzyme (26.7 μM), amine (25.6 mM), and EDC (21.2 mM). Activities are: NAD⁺ control (O), NAD⁺-linked activity modified by the nucleophiles; ethylene diamine, (□) n-propylamine (O), taurine (▲) and methylamine (Δ).

inactivation was observed with both methylamine and n-propylamine, with activities retained after 60 minutes were 85% for both nucleophiles. Highest inactivation was observed in the presence of ethylenediamine cationic amine. The activity dropped to 48% of original activity after 60 minutes of reaction in presence of this amine. Anionic amine on the other hand enhanced NAD⁺-linked activity with 1.23 fold activation at the end of the reaction (Table 3.24).

Effect of the same nucleophiles (under the same conditions) on NADP⁺-linked activity of FLADH is presented in Figure 3.80b. All the amines used enhanced NADP⁺-linked activity. Enhancement in the NADP⁺-linked activity by three amines differed in the extent but not in trend of activation. The greatest activation was achieved by methylamine. A 4.5 fold increase in activity observed after 5 minutes of modification, followed by decline in the activity and a final 3.5 fold activation at 60 minutes. Although n-propylamine resembled methylamine in its activation profile, the increase in activity was much lower. A 1.5-fold increase in the NADP⁺-linked activity was observed in presence of n-propylamine within 5 minutes, after which the activity dropped slightly. Unexpectedly taurine induced an increase as well in NADP⁺-linked activity. At 10 minutes a 1.9 fold increase was observed followed by slight decline in enhanced activity. Finally, the ethylenediamine activation trend differed from the

Figure 3.80 b. Amidation of NADP⁺-linked activity of FLADH. Reaction condition same as in Figure 3.80a. Activities are NADP⁺ control (○), enzyme modified in presence of ; ethylenediamine (□), n-propylamine (○), taurine (▲) and methylamine (△).

previous three amines (Figure 3.80b). This amine enhanced NADP⁺-linked activity by 1.8 fold at 5 minutes. The enhancement in activity was continuously increased to reach a 3.4 fold enhancement in 60 minutes (Table 3.24). A reproducible increase in the NADP⁺-linked activity was also observed by EDC alone. In the absence of any amine, a 1.3 fold increase in NADP⁺ activity by EDC was observed after 60 minutes. The effect of all these nucleophiles on the NAD⁺/NADP⁺ specificity of FLADH is given in Figure 3.81. The maximum increase in ratio was, in the presence of methylamine as a nucleophile. That is to say, when FLADH was modified with methylamine, there was an increase in NADP⁺-activity as well as decrease in NAD⁺-activity, resulting in ratio of $\text{NADP}^+/\text{NAD}^+ = 0.5$. This may indicate that the distinction between the two coenzymes made by the enzyme was relatively decreased when the acidic residue(s) are blocked. Ethylenediamine exerted its effect by a continuous increase in the ratio.

The n-propyl amine achieved a 2 fold increase in NADP⁺/NAD⁺ ratio at 2 minutes followed immediately by a decline in the ratio. The taurine on the other hand, was the least effective amine to induce and increase NADP⁺/NAD⁺ ratio (1.5 fold). Ratio was slightly reduced after prolonged exposure of enzyme to n-propylamine and dropped to 1.2 fold at 60 minutes. The EDC effect on the NADP⁺/NAD⁺ ratio in absence of any nucleophile was given by the control curve.

Figure 3.81. Change in $\text{NADP}^+/\text{NAD}^+$ specificity of FLADH during amidation. Reaction conditions same as in Figure 3.80a and b. The $\text{NADP}^+/\text{NAD}^+$ ratio determined in presence of: EDC (○), ethylenediamine (□), n-propylamine (○), taurine (▲), and methylamine (△).

Figure 3.82 a. Amidation of Dismutase activity of FLADH with methylamine. Reaction was at 0-4°C, MES pH 6.0, enzyme (10.6 μ M), EDC (21.2 mM) and methylamine (25.6 mM). Activities are: control (O), modified (Δ).

Figure 3.82 b. Amidation of esterase activity of FLADH by methylamine. Conditions same as for 3.82a. Esterase control (O), modified esterase (Δ).

There was 1.3 fold increase which remained relatively unchanged throughout the whole modification.

Amidation of both dismutase and esterase activities of FLADH were also followed (Figures 3.82a and 3.82b respectively). Under the same conditions, both dismutase and esterase activities were enhanced by 2.6 fold in 10 minutes. There was a rapid inactivation immediately after in the dismutase activity with 40% of original catalytic activity retained. The esterase activity remained constant up to 60 minutes reaction (Table 3.24). The effect of methylamine on these two activities was not unique, as the same effect was observed in its absence as indicated by the control curves for the respective activities. Change in dismutase activity in the presence of EDC alone resembled that of the modified. A 2.4 fold increase was observed in 10 minutes followed by decline in dismutase activity though to a less extent. There was 60% of dismutase activity retained in the control at 60 minutes compared to 40% in the modified sample. Similarly, the esterase control (EDC only) experienced a 2.6 fold increase in activity just as in the modified sample. The increase in the esterase activity (3 fold increase) was maintained for 60 minutes.

The presence of the nucleophile (methylamine) thus was not necessary for the enhancement of esterase and dismutase activities. When methylamine, ethylenediamine or taurine were the nucleophiles used in the NADP⁺-linked activity

modification, they did affect the activity profile. This phenomenon will be discussed later.

Modification of the third enzyme TBADH by EDC in presence of an amine is given in Figure 3.83. Both NAD⁺ and NADP⁺-linked activities were slightly activated (110%) in presence of EDC only. The presence of a nucleophile did affect the response of both NAD⁺ and NADP⁺ activities to the modification. Slight inactivation in the NADP⁺-linked activity in the presence of n-propylamine as a nucleophile was observed. The activity decreased gradually with 70% of activity retained at 60 minutes. Ethylenediamine drastically reduced the NADP⁺-linked activity, with 15% retained activity at 60 minutes. Inactivation of the NAD⁺-linked activity by ethylenediamine was less drastic, with 36% reduction in activity at 10 minutes and 54% of activity retained at 60 minutes. The inactivation extent of NAD⁺ and NADP⁺ activities was similar in the presence of NH₄Cl as nucleophile (Table 3.25). Modification of the NADP⁺-linked activity by ethylenediamine was repeated in the presence of 0.5 mM NADP⁺ as an active site protector. There was no protection offered by NADP⁺ (Figure 3.83). The response of NADP⁺-linked activity of TBADH to the amidation reaction differed from that of FLADH and HLADH which would be expected as TBADH is an NADP⁺ specific enzyme.

Figure 3.83. Amidation of NAD⁺ and NADP⁺-linked activities of TBADH. Reaction was at 0-4°C, MES pH 6.0, enzyme (31.2 μM), EDC (26.5 mM) and amine (44.9 mM). Activities were: NAD(P)⁺ control (○), (●) NAD⁺ (Δ) and NADP⁺ (□) in presence of methylamine, NADP⁺ activity protected with NADP⁺ (0.5 mM) in presence of methylamine (■), and NADP⁺ activity in presence of n-propylamine (◐).

3.4.6. Glycosylation

Lysine residues have been implicated as essential to the activity of various dehydrogenases and are involved in the binding of coenzyme (166, 167). Lysine residues have also been implicated as the recognition site of 2'-phosphate moiety of the coenzyme in many NADP(H)-dependent enzymes (168). Three *in vivo* post-translational modifications that lysine residues are amenable to, has been used *in vitro* to enhance HLADH activity (83). Of the three modifications; acetylation, glycosylation, and methylation, D-Glucoses specifically glycosylate Lys-315 located at the cleft of the subunit interaction region (82).

Although glycosylation is the least reactive, is the most specific of the three modifications.

The glucosylation of HLADH is presented by Scheme 8 and has been demonstrated to occur at ϵ -amino group of lysine (83).

Scheme 8. A plausible mechanism for glucosylation of HLADH

Treatment of RLADH with D-glucose at pH 7.5 resulted in an enhanced NAD⁺-linked activity (Figure 3.84). The activation, reached a maximum within 10 minutes, and was followed by subsequent gradual decrease in the activity. The gradual reduction in the enhanced activity continued with remaining activity (105%) at 100 minutes. The time-dependent stimulation of the NAD⁺-linked activity by D-fructose follow a similar profile. Retained activity after maximum activity was reached in presence of D-fructose is higher (122%) than when D-glucose was the stimulating reagent. The activity in presence of D-fructose also peaked up at 10 minutes to the same degree achieved by D-glucose (140%). The NADP⁺-linked activity of RLADH stimulation profile differed completely from the NAD⁺-activity. Figure 3.84 shows that with D-glucose there was an initial activation in NADP⁺-linked activity up to 150% at 10 minutes followed by a rapid inactivation. The NADP⁺-linked activity dropped to 50% of the original at 60 minutes. Esterase activity was reduced to 60% after 60 minutes of reaction (Table 3.23).

The Glycosylation of FLADH is given in Figure 3.85. Both NAD⁺ and NADP⁺-linked activities were activated by D-glucose. Under the same conditions the NADP⁺-linked was stimulated to a higher degree. With 3.6×10^4 -fold molar excess of D-glucose, a 5.5 and 3.2-fold increase was observed in the NADP⁺ and NAD⁺ activities, respectively at 10 minutes. The enhanced NADP⁺-linked activity remained

Figure 3.84. Glycosylation of RLADH with D-fructose and D-glucose. Reaction was at 0-4°C, pH 7.5, enzyme (6.14 μ M) and D-glucose or D-fructose (1.8 mM). Activities are: NAD(P)⁺ control (O), NAD⁺ in presence of D-glucose or D-fructose respectively (Δ , $\square$), NADP⁺ in presence of D-glucose (O).

Figure 3.85. Glucosylation of FLADH activities. Reaction was at 0-4°C, pH 7.4, D-glucose (0.12M) and enzyme (3.3 μ M). Activities are: NAD⁺ control (○), NADP⁺ control (●), modified NAD⁺ (Δ) and modified NADP⁺ (◻).

Table 3.23

Summary of the effects of chemical modifications on the activities of RLADH

Modifications ^b	Target	Reagent/pH	% Residual activity ^a		
			NAD ⁺	NADP ⁺	Esase
Carboxymethylation	Cysteine	ICH ₂ COOH/7.5	0.38 (40)	30 (50)	27
Photo-oxidation	Histidine	Riboflavin/8.0	16 (40)	--	--
	Histidine	Methylene blue/8.0	9 (40)	--	--
	Histidine	Rose bengal/8.0	4(30)	--	80
Glyoxalation	Arginine	Phenylglyoxal/8.2	55	20(50)	100
	Arginine	1,2-Cyclohexanedione/8.2	65	--	--
Glycosylation	Lysine	D-Glucose/7.5	140 (10)	150 (10)	60
	Lysine	D-Fructose/7.5	140 (10)	--	--
Amidation	Asparatic, Glutamic	NH ₄ Cl, EDC/6.0	80	33	50

a % Residual activities are those observed at 60 minutes unless otherwise indicated by numbers in brackets.

b Modification results are average of at least two experiments.

-- Not determined.

relatively constant with prolonged glycosylation. Enhanced NAD⁺-activity slightly increased with prolonged treatment resulting in a 4.0-fold increase in activity at 60 minutes. The dismutase activity was not affected by this modification with 100% retained activity at 60 minutes. The esterase activity however, was inactivated, with 35% activity remaining at the end of the reaction (Table 3.24).

The time-dependent response of the NAD⁺ and NADP⁺-linked activities of TBADH is presented in Figure 3.86. Under comparable conditions, the increase in NAD⁺-linked activity was greater than the increase in NADP⁺-linked activity. Both NAD⁺ and NADP⁺-linked activities peaked at 10 minutes to 148% and 124%, respectively. While there was a slight continuous enhancement in NADP⁺-linked activity with 141% at 60 minutes, the enhanced NAD⁺-linked activity was slowly reduced to 112% after prolonged exposure to D-glucose (Figure 3.85). The trend of NAD⁺-activation differed from that of the NADP⁺-linked activity (Figure 3.86). Changes in the D-glucose concentration did not achieve better enhancement in the NADP⁺/NAD⁺ linked activities of TBADH. Under comparable conditions the NAD⁺/NADP⁺ linked activities of FLADH were the most affected by D-glucose activation.

Table 3.24

Comparison of the effects of chemical modifications on the activities of HLADH and FLADH

Modification ^c	Reagent/pH	% Residual activity ^a							
		FLADH				HLADH ^b			
		NAD ⁺	NADP ⁺ Dmase	Esase		NAD ⁺	NADP ⁺ Dmase	Esase	
Carboxymethylation	ICH ₂ COOH/7.4	10	53	22	65	37	--	36	39
	ICH ₂ CONH ₂ /7.4	80	--	--	--	--	--	--	--
Photo-oxidation	Rose bengal/7.5	1.4	12	35	--	18	0(18)	2	90
Glyoxalation	Phenylglyoxal/8.2	81	76	102	109	38	38	26	100
Glycosylation	Glucose/7.4	425	640	100	35	132	--	111	60
Acetamidination	Methylacetimidat/7.5	77	--	--	300				
Amidation	NH ₄ Cl,EDC/6.0	85	137(30)	82	--	75	160(30)	55	52
	Methylamine,EDC/6.0	85	343	38	310				
	Ethylenediamine,EDC/6.0	48	340						
	n-propylamine,EDC/6.0	86	134						
	Taurine,EDC/6.0	123	145						

a % Residual activities are those observed at 60 minutes unless otherwise indicated by numbers in brackets.

b Data are obtained from ref (61), as well as from this study.

c Modification results are at least an average of two experiments.

Figure 3.86. Glucosylation of TBADH activities. Reaction conditions were 0-4°C, pH 7.5, D-glucose (11.1 mM) and enzyme (4.2 μM). Activities followed are: NAD(P)⁺ control (O), NAD⁺ modified (Δ), NADP⁺ modified (◻).

3.4.7. Acetamidination

The imidoesters have been shown to react specifically with amino groups in proteins (120, 169) at moderately alkaline pH's to form imido-amide or what is called an amidine derivatives. The reaction may occur at either the α -amino group of a polypeptide chain N-terminal, or on lysine ϵ -amino group. The α -amino derivative is unstable to acid protein hydrolysis, but the ϵ -amidino groups are partially stable (120). Quantitation of the extent of amidination cannot therefore be done simply by amino acid analysis of the acid-hydrolyzed protein, since the ϵ -amidino group partially hydrolyze in 6N HCl at 110°C (169, 170).

The reaction with methylacetimidate proceeds as shown below by equation 18:

Mechanism of amidine formation with amino groups.

Studies with both model compounds and proteins performed in aqueous solution between pH 7 and 10 at room temperature (169) have provided no evidence for reaction of imidoesters with protein groups other than the amino groups.

In order to investigate further the role of lysine residue in the dehydrogenase catalysis or in any other activity, both FLADH and TBADH were reacted with methylacetimidate hydrochloride.

The time dependence of the dehydrogenase activity change is given in Figure 3.87 for TBADH. It showed that the NADP⁺-linked activity was slightly deactivated while the NAD⁺-activity was not affected at all after one hour of reaction (Table 3.25). On the other hand, in FLADH, the NAD⁺-linked activity experienced 25% deactivation when FLADH was modified by methylacetimidate. Although this modification resulted in minor changes in the NAD⁺ activity of FLADH (Table 3.24), it caused an enhancement in the esterase activity of up to 300% after one hour of reaction time (Figure 3.88). The dehydrogenase activity of HLADH (170) as well as other enzymes were enhanced when modified by methylacetimidate. The difference in the effect of this modification observed on different alcohol dehydrogenases could be due to the lack of selectivity of this particular reagent (methylacetimidate) in its reaction with lysine residue(s).

Figure 3.87. Acetamidination of TBADH with methylacetimidate (38.4 mM) at 0-4°C, pH 7.5 and enzyme (1.8 μM). Activities are: NAD(P)⁺ control (O), modified NAD⁺ (Δ), modified NADP⁺ (□).

Table 3.25

Summary of the effects of chemical modifications on NAD⁺ and NADP⁺-linked activities of TBADH

Modifications ^b	Target	Reagent/pH	% Residual activity ^a	
			NAD ⁺	NADP ⁺
Carboxymethylation	Cysteine	ICH ₂ COOH/7.5	45	8.0
	Cysteine	ICH ₂ CONH ₂ /7.5	72	35
Photo-oxidation	Histidine	Rose bengal/7.5	1.5 (30)	0.37 (30)
Glyoxalation	Arginine	Phenylglyoxal/8.2	52	70
Glycosylation	Lysine	Glucose/7.5	148 (10)	124 (10)
Acetamidination	Lysine	Methylacetimidat/7.5	95	66
Amidation	Asparatic, Glutamic	NH ₄ Cl, EDC/6.0	69	74
	Asparatic, Glutamic	n-propylamine, EDC/6.0	--	70
	Asparatic, Glutamic	Ethylene-diamine, EDC/6.0	54	15

a % Residual activities are those observed at 60 minutes unless otherwise indicated by numbers in brackets.

b Modification results are average of at least two experiments.

-- Not determined.

Figure 3.88. Acetamidination of FLADH activities with methylacetimidate (35.9 mM) at 0-4°C, pH 7.5 and enzyme (69.5 μM). Activities followed are: NAD⁺ and esterase control (O), modified NAD⁺ (Δ), modified esterase (□).

Although two other chemical modifications (acetylation and reductive methylation) are available for modifying lysine residues, both are less selective than glycosylation (83).

3.4.8. Incorporation of radioactive methylamine

The enhancement of NADP⁺-linked activity, esterase and dismutase activities of FLADH were all observed in the presence of EDC alone (Figures 3.80b; 3.82a,b). The presence of a nucleophile regardless of its change or size, did affect the rate and extent of activation in NADP⁺-linked activity. Methylamine as a nucleophile, however, did not affect the rate, extent or the profile of dismutase or esterase activation. These results raised a question concerning EDC role in the modification and the possibility of cross-linking of acidic residues. To determine the degree of incorporation of methylamine the FLADH was modified with $^{14}\text{CH}_3\text{NH}_2$. The reaction of FLADH with $^{14}\text{CH}_3\text{NH}_2$ in the presence or absence of EDC was followed up to 1 hour. Figure 3.89 shows no incorporation of radioactivity after 60 minutes of reaction occurred.

There was less than 1 mole of methylamine incorporated per mole of FLADH after 1 hour of reaction. A 1.2×10^4 fold molar excess of radioactive methylamine was used knowing that molar excess less than this (0.43×10^3) elicit a 4.5-fold enhancement in the NADP⁺-linked activity of FLADH

Figure 3.89. Incorporation of $^{14}\text{CH}_3\text{NH}_2$ into FLADH and YADH. Reaction mixtures contained: $^{14}\text{CH}_3\text{NH}_2$ (35.2 mM), EDC (26.5 mM) and (2.9 or 3.8 μM) of FLADH or YADH respectively. Control mixtures were the same as above but with no EDC, where (O, ●) are control and modified FLADH, (Δ , $\blacktriangle$) control and modified YADH.

within 5 minutes (Figure 3.80b). The same result was obtained when less molar excess of methylamine was used in the amidation of YADH. There was 9.2×10^3 -fold molar excess of methylamine which induced a 2.6 fold increase in the NADP⁺-linked activity of YADH, but showed no incorporation as well. There was 0.5-0.8 mole of amine incorporated per mole of enzyme over the 1 hour (Figure 3.89). This confirmed that methylamine failed to incorporate although its presence induced the change in NADP⁺-linked activity of either YADH or FLADH.

3.5. ³¹P NMR studies of the NAD(P)⁺ binding to enzymes

Recently, ³¹P NMR spectroscopy has been used to study interaction of several enzymes with phosphate-containing ligands (171) allowing selective observation of the ³¹P-resonances of phosphate moieties of free and bound ligands. Three enzymes; YADH, FLADH, and TBADH were purified to homogeneity and used to study the influence of the enzyme on ³¹P NMR spectra of NAD⁺ and NADP⁺. These three enzymes were selected due to the difference in their NAD⁺/NADP⁺ specificity. The increase in NADP⁺ specificity (lower NAD⁺/NADP⁺ ratio) is in the following order YADH, FLADH and the TBADH; i.e. YADH is the least in its NADP⁺ specificity (highest NAD⁺/NADP⁺ ratio).

A series of spectra were obtained for variable concentration of NAD⁺ and NADP⁺ in the presence of fixed concentration of each enzyme and will be discussed below.

3.5.1. Yeast alcohol dehydrogenase (YADH)

The ³¹P spectra of free NAD⁺ in solution and bound to YADH are shown in Figure 3.90a-f. The ³¹P-NMR spectrum of free NAD⁺ consists of high field signal at -9.09 ppm attributed to the 5'-pyrophosphate moiety. This signal arises from the two phosphorus nuclei in the pyrophosphate group, which displays the same chemical shift. Figure 3.90a-f show spectra of the binary complex of the YADH with NAD⁺ at pD 7.5 at various coenzyme/enzyme ratios (not all spectra are shown). In the presence of fixed concentration of enzyme (11.0 μM), the 5'-pyrophosphate doublet experienced a very slight upfield shift, which shifted back towards that of the free coenzyme as the coenzyme concentration was increased.

A significant increase in the resolution of the pyrophosphate doublet resonance was observed as the ratio of coenzyme/enzyme increased, as seen from the expansion plots (Figure 3.91 a-f). Increase of the ligand NAD⁺ concentration to 10 mM leads to the appearance of two additional resonances (numbered as 3 and 4) of free nucleotide (probably) in addition to signals (numbered as 1 and 2) of the bound ligand (Figure 3.91d). The additional small peaks

Figure 3.90 a-f. ^{31}P -NMR spectra of free NAD^+ and varied NAD^+ concentration in presence of native YADH ($11.0\ \mu\text{M}$) at pD 7.5, 25°C . where (a) free $15\ \text{mM}\ \text{NAD}^+$, b-f in presence of YADH with NAD^+ varied as follows: (b) $1.0\ \text{mM}\ \text{NAD}^+$, (c) $2.0\ \text{mM}\ \text{NAD}^+$, (d) $10\ \text{mM}\ \text{NAD}^+$, (e) $15\ \text{mM}\ \text{NAD}^+$, and (f) $20\ \text{mM}\ \text{NAD}^+$.

Figure 3.91 a-f. Expansion spectrum of the 5'-pyrophosphate resonance of NAD^+ in presence of YADH. NAD^+ concentrations were varied as follows (a) 1.0 mM, (b) 2.0 mM, (c) 8.0 mM, (d) 10 mM, (e) 15 mM, and (f) 20 mM. For other conditions see Figure 3.90.

increased slightly in their intensity as NAD⁺ concentration increased further (Figure 3.91 e and f).

The ³¹P-NMR spectrum of free NADP⁺ consists of two absorption bands (172). The low field singlet at 82.21 ppm arises from the 2'-phosphate group and the doublet at 6-8.81 ppm derive from the two phosphorus nuclei in the 5'-pyrophosphate group. A series of spectra with varying NADP⁺ concentration were obtained under same conditions used to obtain NAD⁺ spectra. A very slight up field shift in the 5'-pyrophosphate doublet of NADP⁺, as well as increased resolution of the doublet was observed similar to that observed in NAD⁺ spectra. The 2'-phosphate signal is similar to that of the free coenzyme (results not shown) even at the lowest coenzyme concentration. This is a consequence of the sample containing predominantly free coenzyme. In order to obtain value for chemical shifts of the bound coenzyme, it was necessary to obtain spectra under conditions where essentially all the coenzyme is bound to enzyme (i.e. low coenzyme/enzyme ratio). As a compromise between this and signal-to-noise requirements, enzyme concentration used was higher (21.3 μM) for obtaining NADP⁺ spectra. Typical spectra are shown in Figure 3.92 a-e. In the binary complex of 2.0 mM of NADP⁺ with YADH (Figure 3.92b), the 2'-phosphate resonance was shifted downfield to 84.5 ppm, although the resonance of two pyrophosphate phosphorus

Figure 3.92. ^{31}P -NMR spectra of NADP^+ in presence of YADH ($21.3 \mu\text{M}$) at 25°C , pH 7.5. Where (a) Free 5.0 mM NADP^+ . Other spectra are in presence of enzymes and varied NADP^+ concentrations as follows: (b) 2.0 mM , (c) 3.0 mM , (d) 4.0 mM , and (e) 10 mM .

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nuclei are hardly affected (Figure 3.92b). The same chemical shift (84.49 ppm) was observed when NADP⁺ concentration was increased to 3.0 mM (Figure 3.92c). Further increase in the NADP⁺ concentration to 4.0 mM caused downfield shift in the 2-phosphate to 84.35 ppm, thus reverting the resonance peak back to that of the free coenzyme (Figure 3.92d). There was a gradual shift back towards that of the free coenzyme when NADP⁺ concentration was increased up to 10 mM. A chemical shift of 3.76 ppm was observed for the 2'-phosphate resonance at this concentration (Figure 3.92e). An enzyme concentration dependence shift was observed for the 2'-phosphate resonance of NADP⁺, with maximum downfield shift observed at the lowest coenzyme/enzyme ratio. A decrease in the downfield shift occurred progressively with an increase in coenzyme/enzyme ratio. This indicates that exchange of NADP⁺ between the free and bound states is fast on the NMR time scale (99).

The association constant for the ligand NADP⁺ can be calculated using the ³¹P chemical shifts of the 2'-phosphate group at the various concentration (2.0 mM to 10.0 mM) in the presence of YADH. The observed chemical shift δ_{obs} is described by equation 19:

$$\frac{1}{\delta_{obs} - \delta_a} = \frac{1}{K_a E_t (\delta_{aa} - \delta_a)} + \frac{1}{E_t (\delta_{aa} - \delta_a)} \quad \text{At } \dots\dots\dots 19$$

Where δ_{obs} is the observed chemical shift, δ_{c} is the chemical shift of complexed ligand, and δ_a is the chemical shift of free ligand. The parameters A_t , E_t and K_a refer to total ligand concentration, total enzyme concentration and association constant respectively.

A plot of $1/\delta_{obs} - \delta_a$ versus A_t allows the evaluation of K_a (173). Such plot was prepared for the chemical shift observed for 2'-phosphate resonance of NADP⁺ (Figure 3.93). The association constant was determined to be $K_a = 7.51 \times 10^{-2} \text{ mM}^{-1}$. This value is much lower than the value ($4.5 \times 10^4 \text{ M}^{-1}$), observed for the binding of NADP⁺ to dihydrofolate reductase by Feeney et al. (100). This indicates NADP⁺ binds very weakly to YADH.

3.5.2. Fish Liver alcohol dehydrogenase (FLADH)

A study of NAD⁺ as well as NADP⁺ binding to the FLADH was performed as for YADH. The ³¹P-NMR spectra of varied concentration of NAD(P)⁺ at fixed concentration of FLADH (98.2 μM) are given in Figure 3.94 a-e and Figure 3.95 a-f, respectively. A wide range of concentration was used, however not all spectra are shown. The NADP⁺ spectra were chosen to represent those that either exhibited maximum downfield shift or indicated the saturation of enzyme. No discernible shift (0.2 ppm) in the pyrophosphate resonance was observed for both NAD⁺ and NADP⁺ in presence of enzyme (Figure 3.94, b-e and Figure 3.95, b-f). However, the 2'-

Figure 3.93. Concentration dependent shifts in the ^{31}P -resonance of 2'-phosphate for NADP^+ in presence of YADH ($21.3 \mu\text{M}$) at pD 7.5 and 25°C .

Figure 3.94 a-e. ^{31}P -NMR spectra of NAD^+ in the presence of FLADH ($98.2 \mu\text{M}$) at 25°C , pD 7.5. ^{31}P -NMR spectra of NAD^+ were obtained as (a) Free 5.0 mM NAD^+ and in presence of FLADH, NAD^+ concentrations were as follows (b) 1.0 mM , (c) 3.0 mM , (d) 10 mM and (e) 25 mM .

Figure 3.95 a-f. ^{31}P -NMR spectra of NADP^+ in presence of FLADH ($98.2 \mu\text{M}$). Spectra of free NADP^+ and in presence of FLADH were obtained at pD 7.5 and 25°C . (a) Free 5.0 mM NADP^+ . In presence of enzyme, NADP^+ concentrations were as follows: (b) 1.0 mM , (c) 2.0 mM , (d) 5.0 mM , (e) 10 mM and (f) 15 mM .

phosphate of NADP^+ was substantially shifted downfield to 5.93 ppm at the lowest ratio (coenzyme/enzyme = 10). When coenzyme concentration was increased by two fold, the 2'-phosphate resonance shifted further downfield to 6.08 ppm. The 2'-phosphate resonance reverted back toward that of free ligand with further increase in NADP^+ concentration (Figure 3.95 b, c, e, and f). The plots of chemical shifts as a function of ligand concentration at constant enzyme concentration were prepared for the 5'-pyrophosphate of NAD(P)^+ (Fig. 3.96a) and 2'-phosphate of NADP^+ (Figure 3.96b). There was no (significant) systematic dependence of the shift of 5'-pyrophosphate resonance of either NAD^+ or NADP^+ on the concentration of the coenzyme. The dependence of the chemical shift of 2'-phosphate as a function of ligand concentration was a rectangular hyperbola (Figure 3.96b). The association constant K_a can be determined as previously using equation 19. The position of the ^{31}P resonance of the 2-phosphate in the spectra was relatively invariant ($\delta=6.0$ ppm) in the concentration range (1.0-5.0 mM). It indicated that NADP^+ was not in excess in this concentration range yet (Figure 3.95 b, c, and d). In order to employ the equation, data was extrapolated from the titration curve (Figure 3.96b) that indicated a maximum variation in the 2' phosphate chemical shift. A plot (Figure 3.97) was constructed using these data and K_a value was found to be equal to ($K_a=1.56 \text{ mM}^{-1}$, i.e. $1.56 \times 10^3 \text{ M}^{-1}$). This value is 20 fold higher

Figure 3.96 a,b. Effect of FLADH on the ^{31}P -NMR resonance of (a) 5'-pyrophosphate of NAD⁺ (O) and NADP⁺ (Δ) and (b) ^{31}P -NMR resonance of 2'-phosphate of NADP⁺. Spectra were obtained in presence of FLADH (98.2 μM) at pD 7.5, 25°C.

Figure 3.97. Concentration dependent shifts in the ^{31}P -resonance of 2'-phosphate and determination of K_a for NADP^+ in presence of FLADH. Other conditions are as in Figure 3.96a,b.

than K_a obtained for YADH indicating the higher NADP⁺ specificity for the fish enzyme.

3.5.3 *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH)

The same set of experiments were performed with the NADP⁺ specific enzyme, TBADH. The ³¹P spectra for the 5'-pyrophosphate resonance of NAD(P)⁺, as well as the 2'-phosphate resonance of NADP⁺ as a function of coenzyme concentrations in the presence of TBADH (fixed at 104 μM) are given in Figure 3.98 a-e and Figure 3.99 a-e respectively. As previously, there was no significant chemical shift (< 0.2 ppm) of the 5'-pyrophosphate resonance in either NAD⁺ or NADP⁺ in the presence of the enzyme. The chemical shift dependence of 5'-pyrophosphate resonance in NAD(P)⁺ on ligand concentration is given in Figures 3.100a and b. The trend in the chemical shifts of 5'-pyrophosphate resonance was relatively similar where both exhibited a maximum shift, but at different concentration of the ligand. The maximum shifts were at 8.0 and 10.0 mM concentration of NADP⁺ and NAD⁺, respectively.

Figure 3.99b shows the 2'-phosphate signal of 1.0 mM NADP⁺ in the presence of TBADH. A singlet 2'-phosphate resonance is (substantially) shifted downfield to $\delta=5.52$ ppm at this lowest coenzyme concentration. The resonance signal shifted gradually from that of a bound state to that of the

Figure 3.98 a-e. ^{31}P -NMR spectra of NAD^+ in presence of TBADH ($104\ \mu\text{M}$) at 25°C , pH 7.5. Where (a) Free $5.0\ \text{mM}$ NAD^+ . In presence of TBADH, NAD^+ concentrations were as follows: (b) $1.0\ \text{mM}$, (c) $3.0\ \text{mM}$, (d) $5.0\ \text{mM}$ and (e) $10\ \text{mM}$.

Figure 3.99 a-f. ^{31}P -NMR spectra of NADP^+ in presence of TBADH ($104\ \mu\text{M}$) at 25°C , pD 7.5. Free $5.0\ \text{mM}$ NADP^+ is presented by (a). Other spectra are in presence of TBADH and NADP^+ concentrations were as follows: (b) $1.0\ \text{mM}$, (c) $2.0\ \text{mM}$, (d) $3.0\ \text{mM}$, (e) $4.0\ \text{mM}$ and (f) $10\ \text{mM}$.

Figure 3.100 a-c. Effect of TBADH on the ^{31}P -NMR resonance of 5'-pyrophosphate of (a) NAD $^{+}$, (b) NADP $^{+}$ and ^{31}P -NMR resonance of 2'-phosphate of NADP $^{+}$ (c). Other conditions are as in Figures 3.98 and 3.99..

free state as the NADP^+ concentration was increased (Figures 3.99c, d, e and f). Similar to FLADH, the plot of chemical shift of $2'$ -phosphate as a function of NADP^+ concentration at constant enzyme concentration was a rectangular hyperbola (Figure 3.100c). From this plot, the association constant K_a can be obtained using data at the lowest coenzyme/enzyme ratio. Such plot is represented in Figure 3.101 with the value of $K_a = 19.0 \text{ mM}^{-1}$ or $19.0 \times 10^3 \text{ M}^{-1}$. This value is 12 fold larger than that of the FLADH ($1.5 \times 10^3 \text{ M}^{-1}$) indicating the higher NADP^+ specificity of TBADH.

In general, for the three enzymes studied (YADH, FLADH, and TBADH), it was found that changes in the chemical shift of the pyrophosphate resonance on binding to the enzyme are much less than those of the $2'$ -phosphate signal. The pyrophosphate signals in presence of enzymes are close to that of the free coenzyme. As a consequence, K_a could not be obtained for the NAD^+ coenzyme. A probable reason was that enzymes were saturated by the coenzyme which is present mostly in the free state. A concentration of NAD^+ lower than 1.0 mM gave irreproducible scans with very low signal-to-noise ratio. The other alternative is to increase the enzyme concentration to give lower coenzyme/enzyme ratio. This would introduce a new complication due to the viscosity of concentrated enzyme solution. A common feature was observed in the 2 -phosphate chemical shift of NADP^+ in presence of all enzymes. The downfield shifted single signal of the $2'$ -

Figure 3.101. Concentration dependent shifts in the ^{31}P -NMR resonance of 2'-phosphate and determination of K_a for NADP^+ in presence of TBADH. Other conditions are as in Figure 3.99a-f.

phosphate suggests a fast exchange of ligand molecules between the "free" and "bound" states on NMR time.

3.6. Effect of modified enzyme on ^{31}P -NMR spectra of coenzymes

In an attempt to further delineate the contribution of residue(s)-cofactor interactions to the overall binding, enzymes were subjected to chemical modifications prior to ^{31}P -NMR analyses. The effect of these modified enzymes on the chemical shifts of the 2'-phosphate of NADP^+ was compared with that of the native enzyme. Essentially, in most modifications only the NADP^+ spectra in presence of modified enzymes were obtained.

3.6.1. Modified yeast alcohol dehydrogenase

The YADH enzyme was amidated with methylamine in the presence of EDC for 1 hour. There were 3.5 and 4.1 fold increase in the NADP^+ -linked activity of control (subjected to EDC alone) and modified YADH respectively. The spectra of 2.0 mM NADP^+ were obtained in the presence of native, modified and control enzymes (Figure 3.102 b, c and d).

The ^{31}P -NMR spectra suggest that the 2'-phosphate signal was not affected when enzyme was reacted with methylamine in the presence and absence of EDC. The 2'-phosphate resonance in the presence of the native enzyme was shifted downfield ($\delta=4.5$ ppm). In comparison the chemical shifts observed in

Figure 3.102. ^{31}P -NMR spectra of 2.0 mM NADP^+ in presence of native, modified and amidated YADH at pH 7.5 and 25°C. (a) Spectra of free 5.0 mM NADP^+ . Spectra of 2.0 mM NADP^+ in presence of (b) native YADH (21.3 μM), (c) amidated YADH (69.3 μM) and (d) control YADH (52.0 μM).

presence of control ($\delta=4.87$ ppm) and modified ($\delta= 5.06$ ppm) are only slightly higher. The 5-pyrophosphate resonance in the presence of either the modified or control YADH was hardly affected, as was observed for native enzyme (Figure 3.102 c and d).

3.6.2 Modified fish liver alcohol dehydrogenase

The FLADH was subjected to two modifications; amidation and glyoxalation (with phenylglyoxal). The spectra of free NADP^+ , 1.0 mM NADP^+ in presence of native, amidated and phenylglyoxalated enzyme are given in Figure 3.103 a-d. The 2'-phosphate resonance of 1.0 mM NADP^+ shifted downfield ($\delta=5.82$ ppm) in presence of the native enzyme (5.0 μM) as observed previously. The phenylglyoxalated enzyme (with 60% of its original activity) caused the same trend. The 2'-phosphate resonance shifted downfield as well ($\delta= 6.07$ ppm) in the presence of phenylglyoxalated FLADH. This higher value in chemical shift could be rationalized on basis of higher phenylglyoxalated enzyme concentration (6.6 μM).

The amidated enzyme (7.9 μM) affected the 2-phosphate resonance of NADP^+ (1.0 mM) by shifting it downfield to $\delta=6.12$ ppm. Although there was a 3.4 fold increase in the NADP^+ activity, the amidated enzyme did not cause any detectable differential effect on the 2'-phosphate signal as compared to native enzyme.

Figure 3.103 a-d. ^{31}P -NMR spectra of NADP $^{+}$ at 25°C and pH 7.5 in presence of native and modified FLADH. Where (a) free 5.0 mM NADP $^{+}$. Spectra of 1.0 mM NADP $^{+}$ in presence of (b) native FLADH (5.0 μM), (c) amidated FLADH (7.9 μM) and (d) phenylglyoxalated FLADH (6.6 μM).

A higher enzyme concentration (7.9 μM) of the amidated enzyme would account for the further downfield shift observed in the 2'-phosphate signal in comparison to native enzyme. The 5'-pyrophosphate resonance of NADP^+ was not affected by amidation or phenylglyoxalation although there was slight decrease in its intensity (Figure 3.103 d, as well as expansion plot that is not shown). The decrease in intensity of 5'-pyrophosphate resonance of NADP^+ in presence of phenylglyoxalated enzyme is more pronounced than in presence of amidated FLADH. Similarly, the 5'-pyrophosphate of NAD^+ in presence of amidated FLADH was not affected (results not shown as well).

3.6.3. Modified *Thermoanaerobium brockii* alcohol dehydrogenase

The TBADH was subjected to phenylglyoxalation and amidation modifications. The spectra of 1.0 mM NADP^+ as free, in the presence of native, amidated and phenylglyoxalated TBADH are presented in Figure 3.104 a-d. The 2'-phosphate resonance was almost equally affected in presence of either native or modified enzyme. The 5'-pyrophosphate as usual was not affected by the presence of native or modified enzyme.

The 2'-phosphate resonance was shifted downfield ($\delta = 6.07$ ppm) in the presence of the native enzyme (Figure

Figure 3.104. ^{31}P -NMR spectra of NADP $^{+}$ in presence of native and modified TBADH, where (a) free 5.0 mM NADP $^{+}$. Spectra of 1.0 mM NADP $^{+}$ in presence of (b) native, (c) amidated TBADH (5.7 μM) and (d) phenylglyoxalated TBADH (7.0 μM). Spectra obtained at pD 7.5, 25°C.

3.104b) indicating its involvement in strong electrostatic interaction with residues on the enzyme active site.

The phenylglyoxalated enzyme also shifted the 2'-phosphate signal ($\delta = 6.22$ ppm). Therefore, modification of arginine residue(s) does not somehow interfere with the electrostatic interaction of the 2'-phosphate signal. The modification of TBADH with methylamine in presence of EDC did not induce any differential effect on 2'-phosphate of NADP⁺. There was a downfield shift ($\delta = 6.01$ ppm) of the 2'-phosphate signal in presence of amidated enzyme compared to native enzyme ($\delta = 6.07$ ppm).

These results indicate that 5'-pyrophosphate resonance was not affected by the presence of either native enzyme or modified enzymes. On the other hand, the 2'-phosphate signal was shifted in the presence of enzymes. But no differential effect was observed whether the enzyme was native, phenylglyoxalated or amidated.

CHAPTER FOUR

DISCUSSION

There has been a great deal of advance in the knowledge of alcohol dehydrogenases (ADHs), especially of the horse liver enzyme. Alcohol dehydrogenase has a widespread occurrence in nature (3), even when limiting the reports to those for which ethanol is a good substrate, and pyridine nucleotides act as coenzymes. As mentioned earlier in Chapter 1, ADH occurs in a variety of organisms. In higher organisms, isozymes frequently have different organal distributions (174). Although mammalian ADH is found mainly in liver, it also occurs in other organs outside the liver (175, 176). The ADH has been partially purified and characterized to various degrees of purity from sources other than horse liver and yeast.

The recent progress on evolutionary connections between different alcohol dehydrogenases and dehydrogenases in general has been considerable and allowed extensive inferences to be drawn. These inferences in general appear to be valid regarding relationships and properties of these enzymes. Often there is a significant difference in the physical properties of the enzymes from different sources and one should be careful about making generalization. Consequently, additional data are required to substantiate the generalization derived from these comparisons.

Based on the present experimental work, this discussion will include a comparison of the properties of our homogeneous preparations to those of HLADH and occasionally to YADH.

The purification procedure in this study was developed initially for RLADH independently. The purification procedure consists of ammonium sulfate fractionation, anion exchange column chromatography, gel filtration and affinity chromatography step. Lad and Lefferet (56) isolated alcohol dehydrogenase from male Fischer-344 rat livers. Their isolation procedure consisted of differential centrifugation, $(\text{NH}_4)_2\text{SO}_4$ precipitation and sequential chromatographies over a Sephadex G-100, DEAE-Affi-Gel-Blue, affinity Gel Blue and AMP-agarose. The RLADH in this study was isolated from Sprague-Dawley rat livers. It is obtained as homogenous preparation (Plate 2) only when all steps were performed. The first chromatographic step was the DEAE-A50 anion exchange. The pH of the purification buffer (8.4) was slightly lower than pI of the enzyme (pH 8.6-8.8), thus no binding of enzyme to exchanger would be expected. Despite the nonbinding of the enzyme protein to the column, reasonable purification fold was obtained, because contaminating acidic proteins were retained on column. Generally, purification folds obtained by gel filtration chromatography are low. Collection of very small fractions improved resolution and separated enzyme peak from the major

nonactive protein band. Gel filtration was a necessary step, as trials in which this step was cancelled resulted in nonhomogeneous enzyme preparation (Plate 1). The affinity chromatography using AG-NAD⁺ type 1 resulted in homogeneous concentrated enzyme fraction. This medium has not been used previously in the preparation of RLADH. Other affinity media such as 4-[3-(N-6-amino caproyl) aminopropyl]-pyroazole, N6-(aminohexyl)-AMP, and Cibacron blue dye coupled to Sepharose were used in other studies to obtain different degrees of purified enzyme (177-179). Enzyme yield was only 2.5% compared to 4% yield obtained by Lad and Leffert (56) in their isolation.

Unfortunately the lability of the NADP⁺-linked activity of RLADH hindered its assessment throughout the purification procedure. However, it was possible to determine the elution profile for this activity from the first chromatographic step only (Figure 3.2).

The purification procedure developed for RLADH was adopted to the purification of FLADH. The use of single buffer system throughout purification sequence further simplifies the procedure developed for RLADH, FLADH and TBADH as well. The FLADH also proved to be multifunctional as the RLADH and HLADH (Table 3.7). Ability of this enzyme to use both NAD⁺ and NADP⁺ efficiently enabled the assessment of both activities throughout different steps. In general, the behaviour of NAD⁺-linked activity of FLADH on

all columns were similar to the RLADH. Absence of ethanol from purification buffers used for FLADH was an advantage which allowed the dismutase activity to be followed as well. The purification and yield of the NAD⁺-linked activity of FLADH were both higher by 2 fold than for NADP⁺-linked activity. The NAD⁺-linked activity yield (4.6%) of FLADH was slightly higher than the yield obtained for NAD⁺-linked activity of RLADH. Elution of all activities from two absorbants (DEAE-A50 and Sephadex G-100) whose selectivity is based on different molecular parameters provide evidence that activities are associated with same protein. Both Fractogel TSK DEAE-650 S and Fractogel TSK HW 55 F that replaced the DEAE-A50 and Sephadex G-100 respectively, proved to have increased sample capacity, better resolution and sharper chromatographic peaks. The use of AG-NAD⁺ type 3 affinity medium resulted in emergence of an unretarded contaminating peak corresponding to inactive protein. The NAD⁺-linked activity of FLADH was released in one peak on addition of 10 mM NAD⁺. Dialysis or gel filtration on Bio-gel- P-6 column removed NAD⁺. Esterase activity eluted in two peaks from AG-NAD⁺ type 3 affinity chromatography. A low activity peak was coeluted with the NAD⁺-linked activity. An appreciably high esterase activity was eluted in subsequent second peak emerged immediately after the first one. In light of this behaviour, and considering the lower purification of esterase activity, the possible existence of

other esterases or isoenzymes is suggested. About 100% more esterase activity was measured in 30-60% ammonium sulfate fraction than in crude extract. This is likely due to low-molecular weight inhibitors contained in liver crude extract that prevented maximal enzyme activity or inhibit enzyme activity. Similarly, greater esterase activities were measured in subsequent steps (ultracentrifugation and anion exchange) than in freshly prepared crude extracts. Ultracentrifugation accomplishes the removal of high-molecular weight inhibitors, while DEAE-A50 removes acidic contaminants. There was no significant purification or yield in dismutase activity as a result of its instability.

The ratio of $\text{NAD}^+/\text{NADP}^+$ specific activities of FLADH was variable throughout purification procedures (Table 3.3). This is partly due to existence of isoenzymes. Chromatofocusing of ammonium sulfate fraction indicated the presence of two dehydrogenase activity bands and five other protein bands (Figure 3.15b). Variation in $\text{NAD}^+/\text{NADP}^+$ ratio was a consequence of the presence of these two NAD^+ -linked activity isoenzymes. However, it seems that DEAE-A50 anion exchange step was capable of separating these two isoenzymes. Isoenzyme with pI 8.05 was unbound because its pI was higher than the pH of purification buffer (pH 7.5). At the same time, pH 7.5 of purification buffer is higher than isoelectric point of second isoenzyme (pI 6.5). This results in binding of the second isoenzyme to the anion

exchanger and would be eluted only by a salt gradient or change in buffer's pH. Taroni et al (104) provided evidence for two distinct mitochondrial malic enzymes in human skeletal muscle. One is strictly NADP⁺-dependent (with pI 6.6) and the other (pI 5.25) reacting with either NAD⁺ or NADP⁺. These two mitochondrial enzymes were separated on a DEAE-Sephadex chromatography at pH 7.0. Strictly NADP⁺-dependent malic enzyme was not bound and was recovered in the fraction following isocratic elution. The NAD(P)⁺-dependent enzyme however, was eluted by a linear KCl gradient, with NADP⁺-linked activity paralleled NAD⁺-linked activity. Similarly, two isoenzymes were separated from fish liver by DEAE-A50 Sephadex chromatography. Their possible separation is supported by the NAD⁺/NADP⁺ ratio which seemed to be relatively constant. The NAD⁺/NADP⁺ ratio was within the range of 6.04 ± 2.3 after the DEAE-A50 step (Table 3.3). The ratio 6.04 is within 37% error of NAD⁺/NADP⁺ ratio of 4.4 observed for isoenzyme 1. Total recovery of NAD⁺ activity from chromatofocusing was only 27%, probably caused by exposure of the enzyme to extremes of pH in this step. This low enzyme activity recovery was the basis for rejecting chromatofocusing as purification step. Higher flow rates as well as higher NAD⁺-activity recovery (61.3%) from DEAE-A50 was advantageously used by including anion exchange in the purification procedure. Further support of the association of both NAD⁺ and NADP⁺ activities with isoenzyme

1 is the activity staining of the electrophoretogram for FLADH as illustrated in Figure 3.20. Quantitative recovery of enzyme activity cannot be rigorously accounted for because of the electrophoretic conditions (long running time, necessarily small sample volumes, dilution and long assay times). As a result, activity staining could not be performed on ultrapure samples. It is important also to realize that under most circumstances, electrophoresis under nondenaturing conditions gives a qualitative rather than a quantitative assessment of the component activities. However, it is possible to obtain comparative information on these two activities at any one substrate concentration. From the stained electrophoretogram of DEAE-A50 Sephadex fraction, no high absorbance distinctive peak was observed, for NAD⁺ or NADP⁺ activity but absorption peaks of both activities were superimposed.

Alcohol dehydrogenase from thermophilic bacteria; *Thermoanaerobium brockii*, unlike last two enzymes (FLADH and RLADH) is NADP⁺ preferring (Table 3.8). It has a higher activity toward secondary alcohols as well (10). Enzyme was purified to homogeneity (Plate 5) from heat-treated cell crude extract (Plate 4) available commercially. The purification procedure consists of the same steps that purify both FLADH and RLADH with few changes. This enzyme is more acidic than animal liver enzyme just as plant alcohol dehydrogenases (61). TBADH elution from anionic exchanger

(DEAE-A52 cellulose at pH 7.5) required a salt gradient. It was eluted at very low salt concentration, indicating that TBADH was weakly held by anion exchanger at this pH. The NAD^+ and NADP^+ activities with secondary alcohol as well as the NADP^+ activity with primary alcohol coeluted from both anion exchange and gel filtration media. It suggests these activities are associated with the same protein. Although enzyme had lower activity with primary alcohol, it indicated TBADH is capable of oxidizing both primary and secondary alcohol and utilizes both NAD^+ and NADP^+ but to different degrees.

Differences in affinity for a given enzyme to different affinity columns are reflected by permitted binding of the particular enzyme. Often, problems arise and require different matrix. Dramatic enzyme purifications have been achieved using affinity technique, yet method of achieving the very specificity somewhat hampers the wider application of the affinity technique. In most cases, for each individual enzyme a different specific ligand has to be chosen and bound to matrix, at a certain distance to permit binding of the enzyme (180, 181). The ligands NAD^+ or NADP^+ are covalently attached to an insoluble support either through adenine base (182, 183), hydroxyl group of ribose moiety (184, 185) or the phosphate group (186) with proper carbon chain spacers. These immobilized ligands contain various modifications of adenine nucleotides or NAD(P)^+ .

Ability of affinity columns to retain certain enzymes may very well depend on whether the geometrical orientations of the modified ligands properly fit the binding sites of these enzymes (187). The enzyme TBADH showed very little affinity to Sigma AG-NADP⁺ in which NADP⁺ is bound to the spacer (hexanoic acid) through an ester linkage with a ribose moiety of pyridine nucleotide. In contrast, the AG-NADP⁺ type 3 purchased from P.L. Biochemicals has a clearly defined matrix. It contains no unreacted spacer groups (1, 6-hexandiamine) with coenzyme bound to spacer at the C-8 position of the adenine nucleotide. The TBADH showed greater affinity for the latter matrix (AG-NADP⁺ type 3). Bound enzyme eluted with 10 mM NADP⁺ is homogeneous (Plate 5). This indicates location of spacer at C-8 position of the pyridine nucleotide causes enzyme to bind to NADP⁺ although not tightly enough resulting in washing off some of the enzyme (Figure 3.11). Purification is low although it could not be compared to the untreated extracts. Yields of both NAD⁺ and NADP⁺-linked activities of TBADH are higher than yields obtained for these two activities from FLADH or RLADH.

Ratio of NADP⁺/NAD⁺ activities of TBADH was not constant throughout purification. Activity stain (Figure 3.21) of DEAE-A52 cellulose fraction subjected to polyacrylamide gel electrophoresis under non-denaturing conditions indicated two activities shared an identical

protein band. This is supported by coelution of the two activities from all columns. Inconsistency in $\text{NADP}^+/\text{NAD}^+$ ratio is due to differential stabilities of the two activities.

Purification of crude YADH available commercially was achieved by single step. Unfortunately, neither AG- NAD^+ type 3 nor AG- NAD^+ type 1 affinity media that are available exhibit good binding for YADH from crude extracts. While YADH did not bind at all to AG- NAD^+ type 3, it exhibited some binding to AG- NAD^+ type 1. The geometrical orientations of the modified NAD^+ in both affinity media did not properly fit binding site of the YADH. As a result, YADH showed no affinity at all for AG- NAD^+ type 3 and some affinity for AG- NAD^+ type 1. Recovery of YADH in eluent during the loading of extracts deserves further enumeration. Saturation of the affinity column often results in the exclusion of weakly bound enzyme. Nevertheless, YADH obtained by NAD^+ pulse was electrophoretically pure (Plate 6).

Purification and recovery of YADH were low. The aim of purifying YADH was to obtain a pure homogeneous preparation to use in some experiments, such as $^{14}\text{CH}_3\text{NH}_2$ incorporation, ^{31}P -NMR and others. Use of commercial crude YADH would have interfered with above experiments.

The YADH is NAD^+ specific (Table 3.8), and no NADP^+ activity was detected with enzyme concentration in the range used in NAD^+ -linked activity assay, although slight NADP^+ -

linked activity was detected by the use of high enzyme concentration. In fact, in staining for NADP⁺ activity, a high concentration of crude enzyme was necessary to obtain significant staining. Instability of NADP⁺-linked activity in purified YADH hindered attempts to stain for the NADP⁺ activity in this fraction.

Molecular weights of native animal liver enzymes (RLADH and FLADH) are within the range of $82-83 \times 10^3$ dal. Subunit weight is within the range of $40-42 \times 10^3$ dal, implicating a dimeric structure for these two enzymes similar to HLADH. The TBADH appears to resemble plant ADH and YADH in their physical properties as well as their lack of multifunctionality. The native and subunit molecular weights of TBADH are relatively similar to YADH (Table 3.6). Both YADH and TBADH have tetrameric structure and Stokes radius in the range of 42.0-45.0 Å.

Liver alcohol dehydrogenase from man (188), monkey (189), horse (190, 191) and rat (53, 124) have been found to exist in multiple forms. Number of isoenzyme forms found by various investigators for the rat liver alcohol dehydrogenase varied. While Arslanian et al. (54) reported only one form of RLADH, others reported multiple forms of the enzyme (56, 57-60). Markovic et al. (53) in their study demonstrated that the multiple forms of RLADH were influenced by the presence of DTE. In the present study, two activity peaks were obtained on chromatofocusing (Figure

3.15a). Markovic et al. (53) noted that purification of RLADH with DTE present resulted in detection of only two isoenzymes. Distribution of the total enzyme activity of the isoenzymes was 94.8% for isoenzyme 1 with pI 8.8 and 5.2% for the isoenzyme 2 with pI 8.65-8.62. Isoenzyme 1 with pI 8.8 is the well studied cathodic form of RLADH in the recent work by Julia et al. (59,60).

Crude extract of FLADH subjected to chromatofocusing yielded several protein peaks, however only two activity peaks were detected. One isoenzyme with pI 8.05 had both NAD⁺ and NADP⁺-linked activities in the ratio of 4.4. The second isoenzyme with pI 6.5 displayed only NAD⁺-linked activity. This is the first report on the existence of two isoenzymic forms of FLADH from pickerel fish.

The *Thermoanaerobium brockii* ADH exhibited the absorption spectrum of a typical protein with an absorption maximum at 272 nm in tris/HCl, pH 9.0 at 25°C with no evidence of any prosthetic group. Extinction coefficient at this wavelength is $2.3 \times 10^7 \text{ cm}^2 \text{ mol}^{-1}$; this value is higher by 10^2 - 10^3 fold than extinction coefficients of HLADH and YADH (Table 3.6). This suggests a higher content of aromatic amino acids; tyrosine and tryptophan in TBADH than in HLADH or YADH.

Overall shape of molecule is determined by the frictional ratio or also known as dissymmetry ratio f/f_0 . This ratio is an index of deviation of the molecule shape

from a perfect sphere (192), and for a perfect sphere, $f/f_0=1$. The frictional ratios for the alcohol dehydrogenases were all greater than 1 but less than 2.5 ($1 < f/f_0 < 2.5$) implicating oblate shape for all proteins.

Alcohol dehydrogenases in general are relatively temperature-labile enzymes. Yeast alcohol dehydrogenase is somewhat unstable even at 25°C (193). Commercial use of HLADH is not recommended above 30°C because 60% of activity is lost on heating at 43°C for 50 minutes (194). Dependence of NAD⁺-linked activity of RLADH on temperature was biphasic. Activity lost was 30% when enzyme was incubated for 1-2 minutes at temperatures up to 50°C. Incubation at temperatures above 55°C resulted in rapid deactivation indicating a conformational change of the enzyme. The RLADH is more labile than its counterparts from other mammals. Addition of different reagents to increase enzyme stability resulted in either temporary stabilization or interfered with analysis. The use of FLADH (which is more stable) in detailed analysis is thus convincing.

Differences between the liver and yeast enzymes, in the residues which are in the active site pocket region is consistent with strict substrate specificity of YADH. As mentioned previously in section 3.3.4., these changes are the residues Phe-93 and Ser-48 in HLADH to tryptophan and threonine, respectively in YADH. Change of Val-58 to tryptophan and insertion of two prolines on both sides of

residue 58 in the YADH as well, results in a smaller narrow active site pocket. These changes are reflected by the inactivity of YADH toward secondary alcohols and cyclohexanol (4). The TBADH is similar to YADH in that alcohols with decreasing chain length are better substrates for these two enzymes.

In general, animal ADHs (HLADH RLADH nad FLADH) exhibit broad substrate specificity toward primary and secondary aliphatic alcohols as well as aromatic alcohols. They all exhibited maximum activity with primary alcohols with exception of FLADH. Maximum activity was observed with the unsaturated alcohol 2-propen-1-ol. Activity of the above enzymes toward secondary alcohols were less than primary alcohol in contrast to TBADH.

In view of the similarities in substrate specificities of the ADHs from animal sources, shape of the active "pocket" may be conserved since all enzymes accomodate same substrates or part of the substrate within it.

Glucose-6-phosphate dehydrogenases (G6PD) can be distinguished on the basis of their nucleotide specificity (105). One group, exemplified by enzymes from brewers' yeast, *Candida utilis* and *Escherichia coli* reacts exclusively with NADP⁺ (105 and references therein). A second group, appears to include animal G6PDs in general, react with NADP⁺ but also displays weak activity with NAD⁺ and some of its analogues. The differential response of NAD⁺

and NADP⁺ activities of rat mammary G6PD to various reagents results from alteration in equilibrium between two enzyme forms (105, 195). The two enzyme forms are presumably conformational isomers each reacts preferentially with one of the coenzymes (105). The third group of G6PD can react approximately equally well with NAD⁺ and NADP⁺. This class includes enzymes from *Leuconostoc mesenteroides*, *Pseudomonas aeruginosa* and *Hydrogenomans H 16*. Steady state kinetic analysis of NAD⁺ and NADP⁺-linked reactions of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase revealed differences in their kinetic mechanisms (105, 196). The reactivity of this enzyme with NAD⁺ and NADP⁺ appears to involve two distinct conformational forms (103, 196, 197). The two enzyme forms do not preexist but are generated by the coenzymes and glucose 6-phosphate (198). The reaction with NAD⁺ and NADP⁺ serve catabolic and anabolic roles respectively in *Leuconostoc mesenteroides* (103). Coenzyme utilization by G6PD in this microorganism must be therefore carefully regulated *in vivo*.

Other dehydrogenases that display dual nucleotide specificity are animal mitochondrial glutamate dehydrogenase, homoserine dehydrogenase in plants and many others (196, and references therein).

Alcohol dehydrogenases from various sources are either NAD⁺ (43, 61) or NADP⁺ specific (10). However enzymes in Table 3.8 did not show strict specificity for one coenzyme,

but displayed weak activity towards the other (61). In fact, HLADH, RLADH and FLADH utilized NADP⁺ to different degrees except for YADH (Table 3.8), which was absolutely specific for NAD⁺ in the concentration range used. Enzymes (HLADH, RLADH, FLADH and TBADH) in this study on basis of their nucleotide specificity are similar to second group of G6PD according to Olive et al. (105). This second group reacts with one coenzyme (NAD⁺ or NADP⁺), but also display weak activity with the other coenzyme as well. FLADH is the least specific for NAD⁺ and had the lowest NAD⁺/NADP⁺ ratio (Table 3.8) compared to HLADH, YADH and RLADH. Little is known about differential coenzyme specificity especially in alcohol dehydrogenases. In this connection, it was of interest to examine mechanisms of both NAD⁺- and NADP⁺-linked reactions catalyzed by FLADH to ascertain whether they differed. The FLADH did not display dual nucleotide specificity, as it did not react equally with NAD⁺ and NADP⁺. The TBADH is NADP⁺-preferring, but it utilized NAD⁺ to less extent. Investigation of mechanisms with the two coenzymes in FLADH and TBADH would help in understanding how enzymes that can utilize both coenzymes differ from those which are NAD⁺ or NADP⁺-specific.

Steady-state kinetic analysis of NAD⁺- and NADP⁺-linked reactions catalyzed by FLADH revealed differences in their kinetic mechanisms. Initial velocity studies with FLADH demonstrated this enzyme to catalyze a sequential mechanism

with either NAD^+ or NADP^+ as the coenzyme. Product inhibition patterns observed for the NADP^+ -linked reaction at nonsaturating concentration of nonvaried substrates were all linear. Acetaldehyde as a product inhibited noncompetitively with respect to NADP^+ but competitively with respect to ethanol. Inhibition by NADPH was competitive with respect to NADP^+ and noncompetitive with respect to ethanol. From the product inhibition patterns obtained (Table 3.19) and according to Table 3.17 two possible mechanisms may be considered. Mechanism a, Theorell-Chance mechanism with no abortive complexes; mechanism b, Rapid Equilibrium Random Bi Bi with abortive ternary complexes. A tentative choice among the two mechanisms can be still made on the basis of the data obtained. Mechanism a does not account for the inhibition observed by NADP^+ which is not relieved by increased ethanol concentration. Nonlinear reciprocal plots of $1/v$ versus $1/[\text{NADP}^+]$ indicate the complexity of mechanism (Figure 3.26a). One plausible explanation of nonlinear kinetics of the NADP^+ -linked activity is the interaction of NADP^+ with more than one enzyme species. According to Bardsley and Childs diagnostic plots, the nonlinearity in NADP^+ reaction (Figure 3.27b) is derived from $n:m$ ($m-n=1$) function. A mechanism consistent with kinetic results and compatible with product inhibition patterns obtained is described by $m>n$ ($m-n=1$) function. Such function describes the random mechanism with the formation

of dead-end substrate inhibition (Scheme 4). The contribution of the dead-end ternary complex is substantiated by the following observations:

(a) nonlinear reciprocal plots with respect to NADP^+ . Inhibition is asymptotic to the reciprocal velocity axis indicating formation of dead end complex.

(b) intercepts and slopes (obtained from initial rates plotted reciprocally versus $1/[\text{Ethanol}]$) replots against $1/[\text{NADP}^+]$ were nonlinear (Figure 3.28b) and reached infinity as $1/[\text{NADP}^+]$ approached zero; and

(c) inhibition at relatively low NADP^+ concentration (1.7 mM) with inhibition increasing as NADP^+ concentration increased. Saturation with ethanol did not eliminate inhibition as mentioned in section 3.3.1.2. Product inhibition patterns obtained predicted existence of dead-end complexes of the type enzyme-substrate-product. Inhibition by NADP^+ is due to enzyme- NADP^+ -acetaldehyde complex (EAP). At high ethanol concentration a second ternary complex enzyme-ethanol- NADPH (EBQ) contribute to inhibition as well. Participation of EBQ explains the ineffectiveness of high ethanol concentration in reducing the inhibition by NADP^+ .

Initial velocity studies of NAD^+ -linked activity of FLADH demonstrated this enzyme to catalyze a sequential mechanism with NAD^+ as well. Product inhibition patterns at non-saturating concentration of nonvaried substrate were all linear. Both of acetaldehyde and NADH were noncompetitive

inhibitors with respect to ethanol and NAD^+ at nonsaturating concentrations of the nonvaried substrates. The "slope-hyperbolic, intercept-hyperbolic" noncompetitive inhibition patterns obtained are termed so, as all slopes and intercepts replots were hyperbolic. Inhibition patterns obtained (Table 3.20) unequivocally distinguish the steady-state random mechanism according to Table 3.17 from any other bireactant mechanisms (140). This steady-state random mechanism is supported by the curvature observed near the ordinate in the initial velocity plots (Figure 3.25a). Bardsley and Childs diagnostic plots (136) for NAD^+ -linked activity identified λ as $\lambda = 0$ ($m=n>1$). This function agrees with a random addition of NAD^+ . Results of kinetic studies and product inhibition are consistent therefore, with a steady-state random mechanism for NAD^+ -linked activity and a rapid equilibrium random mechanism with formation of dead-end complexes for NADP^+ -linked activity. Ability of FLADH to promote NAD^+ -linked at the expense of NADP^+ -linked activity seems to result from interplay of these two kinetic mechanisms. The two kinetic mechanisms can be readily accommodated by the mechanism postulated in Scheme 9:

Scheme 9: Proposed kinetic mechanism of NAD⁺ and NADP⁺-linked activities of FLADH. Scheme shows the dead-end complexes for NADP⁺-linked activity.

Increase in alcohol (ethanol) or NADP⁺ would lead to formation of dead-end complexes and inhibition of NADP⁺-linked reaction. In NAD⁺-linked activity, reaction is not inhibited by low alcohol or NAD⁺ concentration and no dead-end complex of the type enzyme-substrate-product forms. Inhibition of NADP⁺-linked activity was observed at 1.7 mM of NADP⁺, a value that is 14 times greater than Michealis constant. In NAD⁺-linked activity, 5% inhibition was observed at 13.3 mM concentration of NAD⁺, a value which is 81 times greater than Michaelis constant (Figure 3.24). It is reasonable to believe then such high concentration of NAD⁺ are not attained in fish liver. The important, regulatory role for both NADP⁺ and alcohol in inhibiting NADP⁺-linked reaction thus postulate the preference of FLADH for NAD⁺ as a coenzyme. Whether same biochemical properties determined *in vitro* here are manifested *in vivo* remains to be determined. Metabolic role of alcohol dehydrogenase in pickerel fish is not established yet. This question can not be answered definitely or seriously considered unless subcelullar localization of the enzyme is established.

The general idea is that alcohol dehydrogenases from eukaryotic organisms are mainly NAD⁺ enzymes, while those from prokaryotic organisms are either NAD⁺ or NADP⁺-dependent. Alcohol dehydrogenase isolated from the extreme thermophilic archaebacterium *Sulfolobus solfataricus* required NAD⁺ as a coenzyme (199). Alcohol dehydrogenase

isolated from two thermophilic bacteria were shown to utilize NADP⁺ as a coenzyme (10, 200). Lamed and Zekuis indicated (200) NAD⁺-linked ethanol dehydrogenase of the obligate anaerobe *Thermoanaerobium brockii* was less active and was not stable towards O₂ in comparison to NADP⁺-linked ethanol dehydrogenase. The substrate range and reversibility of NADP⁺-linked activity of TBADH appears well related to the suggested metabolic functions of enzyme during growth (10, 200). Most notably *Ta.brockii* produced ethanol during glucose fermentation and catabolizes ethanol as sole energy source in co-culture with an H₂-consuming methanogen. Addition of ketones to glucose-fermenting cells results in formation of stoichiometric amounts of respective alcohols.

In this study, NADP⁺ and NAD⁺-linked activities of TBADH with secondary alcohols were purified and characterized. Steady-state kinetic analysis of TBADH with secondary alcohol in the presence of NAD⁺ or NADP⁺ indicated distinct behaviour.

The NADP⁺-linked reaction with secondary alcohol of TBADH displayed sequential mechanism represented by linear converging lines (Figure 3.29a). Diagnostic test from $n:m$ function of Bardsley and Childs (136) did not give a finite positive horizontal asymptote as $[NADP^+] \rightarrow \infty$. This reflects NADP⁺-linked activity mechanism of TBADH has no complexity, randomness or dead-end inhibition as was seen in both NAD⁺ and NADP⁺ mechanisms of FLADH. Product inhibition studies

(Table 3.18) using NADPH and 2-propanone, are consistent with an ordered mechanism with oxidized coenzyme being bound first and reduced coenzyme released last (140) under conditions where no substrate activation or inhibition is observed. Significant differences between NAD^+ and NADP^+ -linked activities of TBADH with secondary alcohols were observed in their reciprocal plots. The curvilinear reciprocal plot of $1/v$ versus $1/[\text{NAD}^+]$ in Figure 3.30a indicated that steady-state behaviour exhibited a higher degree of dependence on coenzyme concentration. Clearly NAD^+ -linked mechanism is not consistent with a simple ordered sequential mechanism. Bardsley and Childs diagnostic plots (Figure 3.32a) failed as well to identify a finite positive horizontal asymptote for NAD^+ -linked reaction of TBADH in presence of secondary alcohol. Obviously this variety in NAD^+ and NADP^+ kinetic mechanisms may reflect differences in prevailing physiological conditions, necessitating different control mechanisms. Increase in kinetic parameters (K_m , K_{12}) of NAD^+ -linked reaction by 613 and 1.8×10^3 fold respectively with decrease in turnover number (Tables 3.9 and 3.10) significantly contributed to the pronounced activity of TBADH for NADP^+ than for NAD^+ at low concentrations of coenzymes. Dependence of reaction velocity on the concentration of NAD^+ in the presence of lowest constant concentration of propan-2-ol (0.8 mM) is sigmoidal. Sigmoidicity is indicated by the divergence from

linearity in Lineweaver-Burk plot for the NAD^+ -linked activity of TBADH (Figure 3.30a). Curvature at high $1/[\text{NAD}^+]$ is characteristic of nonrectangular hyperbolic kinetics typical of enzymes with sigmoidal substrate saturation curves (201). Hill coefficient of 1.18 was obtained at 0.8 mM concentration of propan-2-ol (Figure 3.30c) suggests positive cooperativity. With increasing propan-2-ol concentrations, Hill coefficients attained similar values with insignificant increase in value as alcohol concentration increased.

It is likely then that NAD^+ is the activator demonstrated by the positive cooperativity (inset in Figure 3.30c) observed in kinetics. In addition it was found that NAD^+ at a concentration 20-fold in excess of that of NADP^+ caused no inhibition of the enzyme activity. This finding may indicate that locus of NAD^+ binding is not operationally adjacent to the NADP^+ active site(s). Even in the absence of any information on NAD^+ and NADP^+ concentration in *Ta.brockii* and possible coenzyme compartmentation, it is still reasonable to assume that high NAD^+ concentration (>2.0 mM) is not encountered by enzyme *in vivo*. At low concentration (0.5-2.0 mM), TBADH can not therefore react with NAD^+ without some mechanism for increasing its affinity for this coenzyme (or decreasing its affinity for NADP^+). These results suggest an important role for NAD^+ in regulating coenzyme specificity of TBADH.

Steady-state kinetic approach is adequate for studying regulatory enzymes with sigmoidal saturation curves but can not substitute for thermodynamic binding data. Thermodynamic studies are essential for an understanding of ligand-enzyme interactions, binding stoichiometry and interacting binding sites (202). Until sufficient information become available from thermodynamic studies, the question of NAD⁺-linked mechanism of TBADH with secondary alcohol can not be answered definitely. Two or more alternative pathways for NAD⁺-mechanism which also lead to sigmoid kinetics might be operative (203).

Preference of TBADH for secondary alcohols can be deduced from the relatively higher turnover number, lower K_b as well as lower K_{1a} as compared to corresponding values for primary alcohols (Table 3.10). Initial rate data displayed curvilinear rate plots (Figure 3.31a) at high NADP⁺ concentrations. Inhibition by NADP⁺ is asymptotic to reciprocal velocity axis indicating formation of dead-end complex. Bardsley and Childs (136) test indicated $m-n=1$ (Figure 3.32c) , implicating abortive inhibition by high concentrations of NADP⁺ of the ethanol oxidation by TBADH. The inhibition by high concentration of NADP⁺ with ethanol as substrate is observed for FLADH as well.

The TBADH showed both low affinity for ethanol and inhibition by high concentrations of NADP⁺. Whether this inhibition by NADP⁺ is physiologically significant remains

to be seen, but it may be a characteristic which serves to reduce the potential activity of ethanol oxidation by TBADH.

Horse liver alcohol dehydrogenase (HLADH) operates by an ordered ternary-complex mechanism (Scheme 10), coenzyme binding preceding binding of the alcohol substrate (4, 204).

Scheme 10: The ordered ternary-complex mechanism usually considered for evaluation of the kinetics of HLADH analysis. E, R, and O denote enzyme, NADH and NAD⁺, respectively. Alc and Ald stand for an alcohol and a corresponding aldehyde product.

Investigation of the effect of pH on the catalytic steps of alcohol binding (205, 206) and ternary-complex interconversion (207, 208) have shown that the enzyme NAD⁺-alcohol complex participates in a proton dissociation equilibrium which regulates the reactivity of reaction intermediate. Alcohol desorption occurs exclusively from the protonated form of the ternary complex, while hydride transfer from the alcohol substrate to NAD⁺ requires the unprotonated form of the complex.

Affinity of HLADH for its coenzyme NAD⁺ increases drastically when pH is raised from 7 to 10. Affinity of enzyme for NADH remains essentially constant up to pH 9 and then decreases (209). Theorell suggested this differential effect of pH on binding of oxidized and reduced coenzyme to

derive from electrostatic interactions of the NAD^+ nicotinamide ring charge with an ionizing water molecule at the active-site zinc ion of the enzyme subunit (210). Kvassman and Pettersson (206) proposed that all main effects of pH on the catalytic reaction in pH range 6-10 reflect analogous ionizations of either zinc-bound water or of the zinc-bound alcohol substrate. The pH dependence of NAD^+ binding was envisaged to derive from combined effects. The combined effects of coenzyme pyrophosphate group and nicotinamide ring charges cause a net perturbation of the pK_a of zinc-bound water to 7.6 in the enzyme NAD^+ complex. It was established recently beyond reasonable doubt by Pettersson and Eklund (211) that the major effects of pH on coenzyme binding to HLADH derive from ionization of zinc-bound water and consequent electrostatic interactions with the charged groups on the coenzyme.

Often effects of pH on enzyme kinetic parameters are interpreted in terms of highly simplified kinetic mechanism (142). With more complicated but perhaps more realistic systems, number of assumptions that are not easy to justify will still remain. In this situation, uncertainties are introduced into the interpretations obtained.

None of the FLADH activities (NAD^+ and NADP^+ -linked, dismutase, and esterase) gave a "bell-shaped" pH-velocity curves (Figures 3.64-3.67). A "bell-shaped" curve is interpreted in terms of a model in which the enzyme

contained only two ionizable groups that were essential for activity.

A biphasic V_{max} versus pH plot was obtained for NAD⁺-linked activity of FLADH which exhibited continuous increase in activity as pH increased (Figure 3.64). Compared to the activity at pH 9.0, relative activity was 19% at pH 7.0, 57% at pH 8.0, 160% at pH 10.0, and 170% at pH 11.0. A limiting nonzero plateau indicates that more than one ionic form of the enzyme is catalytically active (143). The NAD⁺-linked unusual pH dependence curve may be due to the deprotonation of two ionizable groups in a proton relay system. A Gln-68 mutant of alcohol dehydrogenase I from *Saccharomyces cerevisiae* exhibited unusual log V/K vs pH dependence curve (212), similar to log V/K_a versus pH curve (inset of Figure 3.64) of our NAD⁺-linked activity of FLADH. The unusual pH dependence curve of Gln-68 mutant was explained on basis of the deprotonation of two ionizable groups in the proton relay system such as: His-51 and the zinc alcohol (23). Evidence for two active deprotonated states in the liver enzyme (40, 213) and in the yeast enzyme (214) has been obtained previously.

The pH profile obtained for NAD⁺-linked activity of FLADH is suggestive of a two-functional group model (Scheme 6), and two ionizable species were identified. One group possessing $pK=9.45$, the other group with $pK=7.2$. These pK values are very similar to those obtained by Takahashi et

al. (144) for the mitochondrial (pI=5) isoenzyme of horse liver aldehyde dehydrogenase (HLALDH). The pK_a 's identified from their data were suggested to be involved in the rate-determining step. One group that was identified had $pK_{a1}=7.2$, while the other group with $pK_{a2}=9.5$. The groups with these pK_a 's are those of general base (B) in structure I and the electron rich component (A:) shown in structure II.

Where B: is general base, and N is a nucleophilic side chain of an amino acid such as the sulfur of cysteine for papain (215) or GAPDH (216). Histidine has been identified as the general base (B) in chymotrypsin, papain and GAPDH (217) as in structure I. With all three enzymes, the histidine is in close proximity to an electron sink such that a "charge relay" system can function as in structure II.

Here A: is the electron sink such as aspartate-carboxylate in chymotrypsin, asparagine-carboxyl oxygen with papain (217) and tyrosine-phenol with GAPDH (216). In mitochondrial

($pI=5$) isoenzyme of HLALDH, at physiological pHs, only the group with $pK_{a1}=7.2$, presumably a histidine would be involved (structure I) analogous to histidine-176 in GAPDH. The charge relay system function in the nonphysiological alkaline region (structure II). Tyrosine, analogous to tyrosine-311 in GAPDH could be the group involved in this possible relay system (144). A second possible group which could function in "charge relay" system is an acidic species. The hydrolysis of acyl intermediate at physiological pHs does not require the action of charge relay system. With the second ionization which would permit the charge relay system to function, only a 3-fold increase in turnover number per active site of HLALDH was observed (144). The overall role of this charge relay system in hydrolysis of acyl intermediates is hence of dubious importance in HLALDH.

The biphasic pH profile obtained for NAD^+ -linked activity of FLADH lend support to the involvement of a general base with $pK_a=7.2$ suggesting a histidine residue. Tyrosine, acidic species or any other group with $pK_a=9.45$ would be involved in the charge relay system at alkaline pH. On the basis of these identification, chemical modifications were performed with intention to demonstrate the identity of active site groups involved. In FLADH, there was about 22-fold ($\gamma=22.4$) increase in the rate of NAD^+ reaction activity as enzyme was deprotonated at alkaline pH. In comparison

with pH-velocity plot of NAD⁺-linked activity obtained, the pH dependence of velocity in NADP⁺-linked, dismutase and esterase activities were complex. The pH dependence of the rate of these three activities at fixed substrate concentration is of restricted usefulness. One common feature was observed in the pH-velocity profiles of NADP⁺-linked, dismutase and esterase activities is the double maxima. The pH values at which these maxima occurred differed from one activity to another. Such double maxima plots were obtained with partially purified enzyme preparation, and thus may be due to the presence of FLADH two isoenzymes and the possibility of their incomplete separation by ion exchange chromatography. The biphasic pH-velocity plot of NAD⁺-linked activity (obtained with a pure sample) may arise from the presence of these two isoenzymes as well. This is highly speculative but admittedly a possibility. Further investigation is needed to clarify the pH behaviour of FLADH activities.

The pH-profile obtained for NADP⁺-linked activity of TBADH revealed a slightly asymmetric bell-shaped profile, indicating only two ionizable groups are essential for activity.

An efficient method of fast screening for those amino acids that function intimately in catalysis is chemical modifications. On this basis, series of chemical modifications were performed on the alcohol dehydrogenases

from rat, fish and thermophilic bacteria. The intention of these modifications is to distinguish the responses of these enzymes to each modification in comparison to HLADH responses to the same modifications. An additional purpose was to observe any differential action of these modifications on dehydrogenase (with NAD⁺ and NADP⁺ as coenzyme), dismutase as well as esterase activities. Differential responses of these activities for each enzyme to each modification would be used then to support either separate or common active sites. Our approach in understanding the information gained from chemical modifications is based on comparing the responses of enzymes to each modification. A general discussion of modifications effects on all tested enzymes will make it possible and easier to reveal any significant differences in the interaction between each enzyme and the modifying reagents. This allows the screening for any homology of alcohol dehydrogenase from fish liver to that from horse liver. The TBADH response to chemical modification will allow the extension of this comparison and observe if this enzyme (TBADH) shares any degree of homology with other NAD-dependent ADH irrespective of their coenzyme specificity or evolutionary origin.

The modifications of the RLADH, FLADH and TBADH with alkylating agents such as, iodoacetic acid clearly showed the necessity of intact thiol species for both NAD⁺ and

NADP⁺-linked activities in all three enzymes (Figures 3.69-3.71a). The modifications were also repeated on FLADH and TBADH, using iodoacetamide. Iodoacetamide was much less effective in deactivating NAD⁺ and NADP⁺-linked activities of both FLADH and TBADH (Figures 3.70 and 3.71b). The sensitivities of NAD⁺ and NADP⁺-linked activities of RLADH, FLADH and TBADH to inactivation by iodoacetate is linked to a charge effect. Such charge effect possibly an attracting interaction between the anionic alkylating agent and an active site residue stimulate the reaction to take place to a greater extent. Iodoacetamide, on the other hand, lacks the acidic moiety and subsequently does not experience such attractive interactions. This explains the insensitivities of both FLADH and TBADH to inactivation by iodoacetamide. There were 72% and 35% residual activity of NAD⁺ and NADP⁺-linked activity respectively in TBADH when reacted with iodo-acetamide. In FLADH, NAD⁺-linked activity dropped to 80% of original catalytic activity at the end of reaction when modified with iodoacetamide. Such differential responses to alkylating agents has been observed previously in HLADH. Iodoacetate inactivates HLADH 53 times faster than does iodoacetamide (145). Iodoacetate selective alkylation of Cys-46 was facilitated by formation of a reversible complex (67-69). Modification is preceded by reversible binding of the iodoacetate to an anion binding site for chemical attack on Cys-46 (72). Arginine-47 is involved in

this general anion binding site (73). Attachment of iodoacetate to this anion binding site is a typical example of an-active-site-directed irreversible inhibition. The crevice that binds the coenzyme in HLADH is unchanged by iodoacetate modification (122, 146). Inactivated carboxymethylated HLADH can still binds coenzyme (146) and also has a small residual enzyme activity (71). Decreased activity of carboxymethylated HLADH results from changed electronic configuration of the zinc atom, the presence of iodide ion coordinated to the zinc atom (73), interference with the necessary conformational change, or a combination of all these factors (4). The small residual NAD⁺ activity was also observed in our carboxymethylated enzymes with RLADH having the least NAD⁺ activity and TBADH retaining the highest NAD⁺ activity.

In HLADH, the rate of inactivation by haloacids or haloacetamides is reduced 100-fold by NAD⁺ or NADH (146, 147). Pyrophosphate group of NAD⁺ or NADH binds to Arg-47 and Arg-369, thus prevent access of the reagent (haloacids or haloacetamides) to Cys-46 (4, 21). In contrast, Chen and Plapp (218) observed that carboxyethylation of Cys-174 in HLADH by long ω -bromoacetamide-substituted fatty acids is facilitated in the presence of NAD⁺. In this case, these reagents apparently fit nicely into the active site in the presence of NAD⁺, and Met-306 in the substrate binding pocket could be alkylated (218, 219). Similarly,

inactivation of TBADH by iodoacetate was enhanced in presence of NADP⁺ (Figure 3.71a). This may be due to the residues in active site are more exposed to iodoacetate to enable the binding of iodoacetate in a productive manner.

The present data indicate that the cysteinyl residues modified in RLADH, FLADH and TBADH are essential to enzyme activity. It is not possible to distinguish at this point whether cysteine residues participate in catalysis directly in these enzymes. Modifications of these enzymes by iodoacetate may interfere indirectly with the catalytic mechanism through steric factors.

Fourteen histidine residues or seven per subunit are present in HLADH, two of them; His-51 and His-67 are strategically situated within the catalytic domain (19). Histidine-67 serves as a ligand to catalytic zinc, while His-51 serves as a proton sink during enzymatic catalysis (23, 40). Dye-sensitized photo-oxidation (with methylene blue, and rose bengal) carried out under mild conditions, allowed the modification of seven histidine and one tyrosine residues per subunit of HLADH (87). A histidine residue was identified from the pH profile obtained for NAD⁺-linked activity of FLADH. The NAD⁺-linked activity of FLADH was seen to be highly dependent on the presence of intact histidine residues (Figure 3.73). After 30 minutes of reaction with rose bengal, only 1.4% of NAD⁺ activity remained, and 11.5% of NADP⁺-linked activity remained after

60 minutes (Table 3.24). Both TBADH and RLADH were as sensitive as FLADH to dye-sensitized photo-oxidation (Figures 3.72 and 3.74). In RLADH, after 30 minutes of reaction with rose bengal, only 4% of NAD⁺ activity remained.

The NAD⁺-linked activity of RLADH was also sensitive to photo-oxidation in presence of methylene blue and riboflavin which is a high energy sensitizer (Table 3.23). Both of NAD⁺ and NADP⁺ activities of TBADH were completely eliminated by 40 and 37 minutes respectively of reaction with rose bengal (Table 3.25), indicating the vital importance of intact histidine residue for these activities. This similar responses of all dehydrogenases (HLADH, RLADH, FLADH and TBADH) to photo-oxidation (Table 3.23-3.25) in presence of rose bengal established the identity of this modification and functional importance of residues (histidine) modified. Photo-oxidation of histidine is usually the fastest reaction at neutral pH, tyrosine is most reactive at higher pH's, where it is anionic (125). Tryptophan and methionine are the only amino acids readily oxidized below pH 4. This implies that inactivations of FLADH, RLADH and TBADH resulted presumably from destruction of histidine(s). The dismutase and esterase activities in FLADH and RLADH respectively were not affected as much as the dehydrogenase activity (Tables 3.23 and 3.24). Esterase activity in HLADH as well was not

affected by photo-oxidation with 90% remaining activity at the end of reaction.

The pH-velocity profile and chemical modification data of FLADH lend support to the involvement of histidine in "charge relay" system, as described previously in structure I and II. This explains the slight difference in NAD⁺ and NADP⁺ responses to photo-oxidation by rose bengal. Detailed structural information of FLADH, which is at present not available is required to confirm the presence of histidine(s) in the active site. Until sequence analysis become available for FLADH and TBADH, the role of histidine as well as cysteine residues in catalysis can not be assigned.

Bränden and Eklund (4, 21 and 220) had discussed arginine-pyrophosphate interactions in lactate and alcohol dehydrogenases. Matthews et al. (44) from X-ray crystallography attributed the NADP⁺ specificity of dihydrofolate reductase to an attractive interaction between an active site arginine and the 2'-phosphate of NADP⁺.

The specificity of α -dicarbonyl reagents for arginine had been confirmed by Lange et al. (74) in their study on HLADH inactivation by above reagents. The modification of arginyl residues by α -dicarbonyl and essentiality of these residues to HLADH-coenzyme interaction was confirmed by amino acid analysis, radioactivity incorporation, and differential labeling using NADH. Two arginyl residues per

subunit were modified by either butandione or phenylglyoxal and neither lysyl nor histidyl residues were modified (74). The two arginyl residues modified are responsible for loss of NADH binding and hence enzyme activity. The presence of NADH prevent both loss of these two arginyl residues and the concomitant loss of activity.

The RLADH was modified by both phenylglyoxal and 1,2-cyclohexandione in different buffers to establish the procedure for modification and analysis (Figure 3.75).

Treatment of proteins with cyclohexandione in alkaline solutions (pH 11 to 12 or less) results in modification of both guanidino and amino groups (86, 127). Among arginine-directed chemicals, phenylglyoxal appears to be the handiest because of the stability of the arginine adduct under non-alkaline conditions (93, 164). Use of phenylglyoxal has been critized because of uncertainty in the stoichiometry of its addition in a non-borate buffer (164).

Inactivation of NAD⁺-linked activity of RLADH in borate buffer by phenylglyoxal differs from that obtained using 1,2-cyclohexandione. Phenylglyoxal inactivated the NAD⁺-linked activity of RLADH more rapidly than cyclohexandione. This indicates that different structures of the two carbonyl reagents play a major role in directing the chemical towards arginine residues localized in different portions of the protein. Such differential behaviour exerted by different

carbonyls have been also observed in the inactivation of D-amino-acid oxidase (93 and references therein).

There was a significant difference in the rate and extent of loss in the NAD^+ and NADP^+ activities of RLADH when both were subjected to phenylglyoxal (Figure 3.75). The NADP^+ activity was substantially reduced with 20% activity remaining after 50 minutes (compared to 55% of NAD^+ activity remaining). The observation that NADP^+ activity is deactivated more rapidly on modification by phenylglyoxal implies that arginine residue(s) are located within the active site of RLADH. Arginine would then provide an attractive center toward NADP^+ .

Complete protection against inactivation of NAD^+ activity in RLADH was afforded by the coenzyme NAD^+ and its reduced form (NADH). The protection by NADH against inactivation by phenylglyoxal or butanedione was also observed in HLADH (74) as mentioned previously. The protection offered by NAD(H) in RLADH implies that arginine residues are located within the active site of enzyme.

Higher concentration of phenylglyoxal were employed in both FLADH and TBADH modifications (Figures 3.76 and 3.77). However, inactivation of NAD^+ and NADP^+ activities in both enzymes proceeded in a much slower rate than in RLADH or HLADH. The lack of complete inactivation of FLADH and TBADH by phenylglyoxal indicate that arginine(s) modified are not essential for catalysis. This is supported partially by the

protection experiments (Figure 3.77) performed on TBADH in presence of NAD(P)^+ . Both coenzymes offered no protection for TBADH against inactivation by phenylglyoxal. One reason could be that concentrations of NAD(P)^+ were too low to offer any protection. This may imply that arginine residues modified may not be located within active site. Clearly it is not possible to assign functional roles to amino acids in enzymes based solely on protection experiments. However, further support for the non-essential arginine residues in TBADH as well as FLADH is given by ^{31}P NMR experiments.

In order to probe any changes in the nature of NAD(P)^+ binding to FLADH and TBADH upon phenylglyoxation, ^{31}P NMR analysis was employed. The effect of modified enzymes (FLADH, TBADH) compared to natives on the resonance of NADP^+ will be discussed below.

The absence of any NADP^+ -linked activity from lactate and glyceraldehyde-3-phosphate dehydrogenases (LDH and GPDH) was attributed to the repulsion of the 2'-phosphate of NADP^+ with an active site acidic residue (4, 11, 21, 42). Charge repulsion between the anionic 2'-phosphate group of NADP^+ and carboxyl oxygen of Asp-223 weaken the binding of NADP^+ to HLADH (21). The residue Asp-223 in HLADH is conserved in YADH, as well as fourteen other alcohol dehydrogenases (4, 43), and explains the high NAD^+ specificity in these enzymes.

Presence of an appreciable NADP^+ -linked activity in FLADH as well as the NADP^+ preference of TBADH evokes speculation regarding presence of acidic group within the active sites of these two enzymes. The enzymes (HLADH, RLADH, FLADH and TBADH) were amidated and the effect of acidic species loss on NAD^+ and NADP^+ activities of each enzyme were determined. In preliminary experiments, HLADH was used as a model due to the knowledge of its three dimensional structure.

The HLADH and in particular YADH are strictly NAD^+ specific due to the involvement of Asp-223 residue as described earlier. Amidation of HLADH (Figure 3.78), YADH and RLADH (Figure 3.79) with molar excess of ammonium chloride in the presence of EDC inactivated NAD^+ -linked activity slightly (80% of activity remained in all three enzymes after 60 minutes). It is conceivable to assume that loss of acidic species in an enzyme by amidation would remove the ability of enzyme to distinguish between NAD^+ and NADP^+ as coenzymes thereby increasing the NADP^+ -linked activity. Responses of NADP^+ -linked activity in HLADH, YADH and RLADH to ammonium chloride in the presence of EDC varied. While there was slight activation in NADP^+ activity of HLADH, no effect was observed on this activity of YADH, and NADP^+ -linked activity of RLADH was inactivated (Figures 3.78, 3.79 and Tables 3.23 and 3.24). This indicates that ammonium chloride is not a strong nucleophile and lacks the

capability to effectively compete with the rearrangement reaction (Scheme 7) leading to incomplete reaction with activated carboxyls (95).

Four different amines which varied in size and charge were used to amidate FLADH in the presence of EDC. These different nucleophiles induced differential responses in the NAD⁺-linked activity (Figure 3.80a), but similar influences on NADP⁺-linked activity of FLADH (Figure 3.80b).

Methylamine which causes a reduction in the negative charges on acidic moieties inactivated the NAD⁺-linked activity of FLADH slightly. Uncharged n-propylamine influenced NAD⁺-linked activity in a similar way to methylamine. If charge attraction by an acidic group is responsible for binding NAD⁺ to FLADH, then one would expect n-propylamine to have no effect on the NAD⁺ activity. This was not observed.

Ethylene diamine (positively charged) on the other hand, would theoretically maximize charge repulsion between NAD⁺ and modified acidic moieties, leading to inactivation. In presence of ethylenediamine, NAD⁺-linked activity was in fact deactivated more rapidly than in the presence of methylamine or n-propylamine. Taurine (negatively charged) would enhance the NAD⁺ activity of FLADH due to negative charges now introduced on acidic moieties. Modified FLADH did indicate activation in its NAD⁺-linked activity in presence of taurine. Theoretical expectations from these different nucleophiles and their effect on NAD⁺ activity of

FLADH seems to be valid partially. The behaviour of NADP⁺-linked activity of FLADH in the presence of EDC and the different nucleophiles contradicted results obtained with the NAD⁺-linked activity of FLADH. In presence of EDC, all the nucleophiles (methylamine, ethylenediamine, n-propylamine and Taurine) regardless of their charge or size, activated NADP⁺-linked activity of FLADH, though to different extent. Negative charge(s) on acidic group(s) will still exist when modified with taurine and theoretically would lead to inactivation of NADP⁺-linked activity. Such results were not obtained. This indicates that charge repulsion or attraction has little influence on the binding of NADP⁺ to FLADH. One other important piece of data is the control experiments. In the presence of EDC only, NADP⁺ activity of FLADH was enhanced as well, indicating that EDC alone is sufficient to cause an increase in NADP⁺-linked activity. Both esterase and dismutase activities of FLADH were activated as well (Figure 3.82a and b) by EDC in the absence and presence of methylamine. Again this raises the question of EDC role in the amidation reaction and the possibility of polymerization or a combination of groups are reacting. Unfortunately, amidation reaction can not be followed up with amino acid analysis (95). In order to monitor the role of nucleophile in the amidation reaction, incorporation of radiolabelled amidating reagent into FLADH and YADH was followed (Figure 3.89). Of the four nucleophile

studied (methylamine, ethylenediamine, n-propylamine and taurine) it was found that methylamine was the nucleophile that induced the greatest increase in $\text{NADP}^+/\text{NAD}^+$ ratio (Figure 3.81). Methylamine is available in ^{14}C -labelled form, and was used as the amidating nucleophile in incorporation studies. No incorporation of methylamine into FLADH or YADH was observed in the presence or absence of EDC. In the presence of a high molar ratio of methylamine, no change was observed in incorporation over one hour, even though there was 4.5 and 2.6 fold increase in NADP^+ -linked activity of FLADH and YADH respectively. Results thus obtained confirm the involvement of EDC in the activation of NADP^+ -linked activity in FLADH. It suggests that EDC is affecting the structure of enzyme in such a manner that steric hindrances to extra phosphate group of NADP^+ diminish. Further support for this is provided by ^{31}P -NMR analysis of 2'-phosphate resonance of NADP^+ in the presence of amidated FLADH (will be discussed below).

In TBADH, NADP^+ is the favored coenzyme with appreciably higher activity than with NAD^+ . Therefore, presence of acidic residue(s) in active site would be unrealistic since it will cause repulsive effect, thereby weakening NADP^+ binding. If active site acidic group(s) are present, then there must be an active site species that counteract the repulsive effect in order for NADP^+ to bind. Ethylenediamine (positively charged) inactivated both NAD^+

and NADP⁺-linked activities of TBADH (Figure 3.83). There was no difference in the inactivation of NADP⁺-linked activity when TBADH was amidated with ethylenediamine in presence of NADP⁺. This indicates that acidic species are not involved in the active site of TBADH or play a role in binding. Similarly, n-propylamine inactivated NADP⁺-linked activity of TBADH though to a less extent than ethylenediamine did. EDC alone activated both NAD⁺ and NADP⁺-linked activity slightly in TBADH. The carbodiimide, EDC is primarily used to activate carboxyl groups in the protein to form O-acylisourea intermediate (96, 165). The EDC is known to have three effects: it may form covalent linkages with the protein; in the absence of added amine, formation of linkages between protein carboxyl and amino groups are induced by EDC; and finally it may induce conformational change (165). Thus, EDC is implicated to take part in the effect(s) imparted on the NAD⁺ and NADP⁺-linked activities of TBADH in resemblance to FLADH. The role of EDC was investigated further by ³¹F NMR analysis of amidated TBADH and will be discussed below.

Arginine residues have been shown to serve as the recognition site for anionic cofactors for a number of enzymes (44, 220). The positive charge can be likewise provided by lysine residues (221). A number of NADP(H)-dependent enzymes has been shown to contain lysyl residues at the active site (168, and references therein). One such

example is NADPH-cytochrome P-450 reductase where one residue of lysine is located at the binding site of the 2'-phosphate group on the adenosine ribose of NADP(H) (168).

Lysine-315 located in the cleft of the subunit-interaction region of HLADH was glycosylated by either D-glucose or D-fructose. Glycosylation of HLADH stimulated dehydrogenase activity.

Incubation of RLADH with D-Glucose or D-fructose results in an enhanced NAD⁺-linked activity, with activation reaching a maximum within 10 minutes and then gradually decreasing (Figure 3.84). Similarly, NADP⁺-linked activity of RLADH was activated in presence of D-glucose within 10 minutes, but activity decreased rapidly over the next 50 minutes (Figure 3.84). The activation of NAD⁺ activity of HLADH exhibited a transient activation just as in RLADH. However, activation of NAD⁺ activity of HLADH in the presence of D-glucose occurred within 40-50 minutes and then decreased slowly over the next 60 minutes (170).

From the pH-profile of NAD⁺-linked activity of FLADH, a group with pK=9.45 was identified. Such pK value would implicate cysteine, lysine or tyrosine. It has been discussed earlier that cysteine was essential for both NAD⁺ and NADP⁺ activities. Glucosylation of FLADH with D-glucose enhanced both NAD⁺ and NADP⁺-linked activities with maximum stimulation at 10 minutes just as in the RLADH activation (Figure 3.85). The NAD⁺ and NADP⁺-linked activities of FLADH

were enhanced to 4.0 and 6.0 fold respectively in presence of D-glucose. Compared to RLADH, HLADH and even TBADH (Figure 3.86), NAD⁺ and NADP⁺ activities of FLADH were enhanced the most by D-glucose. Stimulation of NAD⁺ and NADP⁺ activities of TBADH by D-glucose were similar to stimulation of NAD⁺ activity of RLADH.

The stimulatory effect of glycoslation on NAD⁺ and NADP⁺-linked activities is remarkably similar with respect to all enzymes (RLADH, TBADH, and FLADH and compared to HLADH).

Monitoring the changes in dehydrogenase activity of HLADH enabled the correlation of stimulatory effect with the structure and process of glycoslation (82). It was concluded (82, 83) that initial reaction between ϵ -amino group of Lys-315 and C-1 of glucose yields the N-glycosidic ADH(III). This N-glycosidic ADH accounts for the initial phase of activation (Scheme 8, results section 3.4.6). It is followed by the slow Amadori rearrangement(IV) which is associated with the inactivation. Relationship of k_2 to k_3 presumably dictates whether stimulation or suppression of dehydrogenase activity of HLADH would occur. It can be speculated that activation of NAD⁺ and NADP⁺ activities in RLADH, FLADH and TBADH is operating in an analogous manner.

The multifunctional activities of FLADH and RLADH responses to glucosylation (Tables 3.23 and 3.24) were different from the dehydrogenase activities in presence of

NAD(P)⁺. Esterase was inactivated in both RLADH and FLADH similar to HLADH, while dismutase activity was not affected in FLADH similar to HLADH. The differential sensitivities of dehydrogenase (in presence of NAD(P)⁺), dismutase and esterase activities to glucosylation implicates nonequivalent binding/catalytic domains for RLADH and FLADH multifunctionality similar to HLADH.

Reaction of imido esters with either a amino group of a polypeptide chain N-terminal or ϵ -amino group of lysine is specific between pH 7-10 (120, 169). Modification of TBADH resulted in moderate inactivation of NADP⁺-linked activity, and no effect on its NAD⁺ activity (Figure 3.87). The slight inactivation may result from inaccessibility of lysine residues to the reagent. Similarly, NAD⁺-linked activity of FLADH was moderately inactivated while esterase activity was enhanced by 3 fold (Figure 3.88). The results of this modification warrant further investigation.

It was mentioned earlier that ³¹P-NMR analysis was used to define more precisely the effect of modified enzymes on NAD⁺ and NADP⁺ binding. It is desirable to point out initially the effects of native enzymes on 5'-pyrophosphate of NAD⁺ and NADP⁺ and the 2'-phosphate of NADP⁺ resonances. This allows the characterization of the differences in the mode of binding of these coenzymes to modified enzymes. Coenzymes, upon binding to the active site of the enzyme, will be influenced by residues that are responsible for

interactions with the specific coenzyme. Presumably, if the interactions involve the phosphate moieties, then any change in the phosphate resonances will reflect a change in the above interactions.

The 5'-pyrophosphate resonance of free NAD^+ and NADP^+ under the conditions employed gave rise to a broad peak. In the presence of enzyme (YADH, FLADH or TBADH) there was no change in chemical shifts or resolution of these resonances (Figures 3.90-3.99). However, ^{31}P signals of 5'-pyrophosphate of NAD(P)^+ -enzyme complexes were somewhat broader than of the free NAD(P)^+ . Mas and Colman (98) observed a substantial variation in the chemical shifts of the pyrophosphate resonances in the presence of pig-heart NADP^+ -dependent isocitrate dehydrogenase. In contrast to single resonance of ^{31}P nuclei of pyrophosphate moiety in the spectrum of free nucleotide (NADPH), a quartet in the spectrum of bound NADPH was observed. This indicates that the two phosphoryl groups experience different environments and/or undergo some structural changes (98) upon binding of NADPH to the enzyme. In our study, the presence of enzyme (YADH, FLADH or TBADH) did not induce any significant shift in the resonance of ^{31}P nuclei of pyrophosphate moiety in either NAD^+ or NADP^+ . No apparent distinction is made by any of those enzymes on the pyrophosphate of NAD(P)^+ under the conditions employed.

All spectra of the nucleotide NADP^+ complexes with enzyme (YADH, FLADH or TBADH), indicated a single 2'-

phosphate resonance, which was sensitive to the molar concentration of coenzyme (Figures 3.92, 3.95 and 3.99). The large downfield shift of 2'-phosphate resonance of NADP⁺ upon binding to the enzyme indicates the contribution of this moiety to the binary complex formation. The downfield direction of the chemical shift of the ³¹P resonance in the binary complexes of these enzymes (YADH, FLADH and TBADH) with NADP⁺ suggest that the 2'-phosphate group exist in its dianionic form. In the binary complex of NADP⁺ with dihydrofolate reductase, the significant downfield shift of 2'-phosphate resonance was attributable to strong electrostatic interaction of dianionic 2'-phosphate with a cationic site on enzyme (100). This cationic species was identified as an arginine based on X-ray data of the enzyme dihydrofolate reductase (44).

Association constant K_a obtained from a plot of ³¹P chemical shifts of 2'-phosphate versus NADP⁺ concentration for YADH, FLADH and TBADH were 75.1 M⁻¹, 1.56X10³ M⁻¹ and 19.0X10³ M⁻¹, respectively (Figures 3.93, 3.97 and 3.101). These association constants are consistent with NADP⁺ binding less tightly to YADH than to FLADH or TBADH, and agree well with NADP⁺ specificity of these enzymes.

The strategy used in this study for identifying the particular interactions between residues in active sites of the enzymes and phosphate moieties of coenzymes, was examining the ³¹P NMR spectrum of 2'-phosphate moieties in

the presence of modified enzymes. Arginine and acidic group have been implicated in the binding of NAD(P)⁺ to many enzymes (21, 44, 220).

The ³¹P NMR resonances of 2'-phosphate were obtained in presence of the enzyme in which arginine or acidic group were modified (Figures 3.102-3.104). The 5'-pyrophosphate signals experienced no chemical shift or broadening effects in presence of amidated (YADH, FLADH or TBADH) or phenylglyoxalated enzymes (FLADH or TBADH).

The resonance of 2'-phosphate were previously seen to experience downfield shift (with respect to free NADP⁺) in presence of native enzymes. The large downfield shift for the 2'-phosphate resonance was still observed in the presence of phenylglyoxalated FLADH or TBADH. The ³¹P signal of 2'-phosphate of NADP⁺ has no dependence then on presence of intact arginine residues. This supports the modification results obtained for phenylglyoxalated FLADH as well as protection experiment by NAD(P)⁺ of phenylglyoxalated-TBADH. ³¹P-NMR analysis of phenylglyoxalated TBADH and FLADH effects on 2'-phosphate resonances established and supported the conclusion that arginine residue(s) modified are not likely to be active site residues, or are involved in coenzyme binding.

In the presence of amidated and control YADH, the downfield shift of 2'-phosphate resonances were still observed with slightly further downfield shift of 0.46 and

0.37 ppm respectively compared to native YADH. This may imply that increased attractive interactive has resulted through the removal of repulsive effects caused by the amidation of acidic residue(s). The slight change in chemical shift of 2'-phosphate was observed in the absence of amine as well (i.e. control sample) and again support the proposition given earlier of EDC involvement in the NADP⁺-linked activity activation.

Similarly both amidated FLADH and TBADH did not cause any detectable differential effect on the 2'-phosphate downfield shift as compared to the native enzyme just as in amidated and control YADH. The relatively small discrepancy between the downfield shifts of 2'-phosphate resonance in presence of native and amidated enzymes (YADH, FLADH or TBADH) suggests acidic acid residue(s) amidated are not involved in the binding of NADP⁺ to above enzymes. The 2'-phosphate chemical shift is virtually constant in the various binary complexes of NADP⁺ with the various forms of enzymes (YADH, FLADH and TBADH). This supports the involvement of EDC in the activation observed in NADP⁺-activity of YADH and FLADH as was suggested earlier from incorporation data.

Neither the three-dimensional structure nor the amino acid sequence is known for both FLADH and TBADH. The structural insights regarding specific coenzymes binding interactions obtained in present studies depend on the

results obtained from indirect methods (modification and radioactive incorporation) and analogies with other dehydrogenases. The ^{31}P NMR analysis has been widely applied to characterize enzymes which bind phosphate-containing ligands (98, and references therein). No reliable theory exists yet to correlate observed chemical shifts and coupling constants with molecular structure of enzyme.

Whether specific amino acid residues within the 2'-phosphate binding site of FLADH and TBADH are the same as those in the NADP^+ specific *Lactobacillus casei* dihydrofolate reductase is a possibility. Considering the ^{31}P -NMR spectra for dihydrofolate reductase (99, 100) and the wealth of structural data available (44), it is tempting to speculate about analogies between the nucleotide binding domains of dihydrofolate reductase, FLADH, and TBADH.

The oxygens of the 2'-phosphate in dihydrofolate reductase are involved in hydrogen bonds and salt bridges to protein side chains; Arg-43, Thr-63, His-64 and Gln-65 (222). This unique array of amino acid residues surrounding the 2'-phosphate of the adenosine ribose must be in some way responsible for downfield shift of the ^{31}P resonance of 1.7 ppm beyond that of the dianionic form of free NADPH in solution (100).

The magnitude of the chemical shift of 2'-phosphate observed for FLADH- NADP^+ and TBADH- NADP^+ complex is slightly larger than shifts observed for dihydrofolate reductase.

From our chemical modifications the presence of histidyl and cysteinyl residues in TBADH and FLADH have been implicated as to be essential for catalytic activity, but not arginine or acidic amino acids. The question of these amino acids role in binding and catalysis can be answered clearly when detailed X-ray structure of FLADH and TBADH become available and compared to the detailed X-ray structure of HLADH or dihydrofolate reductase.

CONCLUSIONS AND ORIGINAL CLAIMS

A common procedure for the purification of alcohol dehydrogenase from various sources is reported in the present study. It achieves about 125 fold purification for animal enzymes. Alcohol dehydrogenases isolated from rat liver (RLADH) and pickerel fish (FLADH) are both dimer, while *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH) is tetramer. A partial characterization of the physical and structural properties of the enzymes is given and comparison was made with respect to horse liver alcohol dehydrogenase (HLADH). The enzymes isolated differ in their coenzymes, substrate and multifunctional specificities. This is the first report to show that FLADH has two isoenzymes. One isoenzyme (pI 8.05) is active with either NAD^+ or NADP^+ , and the other (pI 6.51) is active only with NAD^+ . The NAD(P)^+ specific isoenzyme (isoenzyme 1) of FLADH is potentially interesting in several aspects. No information is available yet about the activity, distribution and the role of FLADH in pickerel fish liver. Kinetic studies were done with respect to NAD^+ and NADP^+ -linked activities of isoenzyme 1 of FLADH. It provided an understanding of the mechanisms of these two activities. The NAD^+ and NADP^+ -linked activities of isoenzyme 1 of FLADH are catalyzed by two different mechanisms, and account for the difference in activities between the two coenzymes. The difference in the mechanisms

is suggested to play a role in the regulation of both NAD⁺ and NADP⁺-linked activities of FLADH. Kinetic studies done on NAD⁺ and NADP⁺-linked activities of TBADH similarly indicated difference in the mechanisms of these two activities. Chemical modifications, ³¹P-NMR and incorporation studies were used to investigate essential amino acids. Unlike horse liver alcohol dehydrogenase, arginine and acidic residues (aspartic or glutamic) are unlikely to be involved in NAD(P)⁺ binding to FLADH and TBADH. Cysteine and histidine are catalytically essential for NAD⁺ and NADP⁺-linked activities of FLADH and TBADH as well as RLADH. No assessment was done to quantitate the cysteine and histidine residues that are essential for catalytic activities of TBADH or FLADH. It is hoped this thesis has demonstrated by comparison with HLADH that essential amino acids can be identified by the perturbations in activity induced by chemical modifications. A consensus structural details of TBADH and FLADH will enable us to understand the structural requisities for these two enzymes which enable them to use NAD⁺ as well as NADP⁺.

Detailed insight into the role of lysine and/or tyrosine residues in a proton relay system implicated by pH studies for the NAD⁺-linked activity of FLADH is suggested for future work.

Conclusions from our present *in vitro* studies for regulation of NAD⁺ and NADP⁺-linked activities in TBADH and

FLADH in particular is based on conventional assays which employ a single coenzyme. It represents the first study of coenzyme specificity of alcohol dehydrogenases, in particular FLADH and TBADH. With the *in vivo* situation, both coenzymes are present, and interpretation made from *in vitro* studies should be extrapolated cautiously.

Dual wavelength assay is applicable to all pyridine nucleotide-linked dehydrogenases which are not strictly specific for either NAD⁺ or NADP⁺ and can utilize the thionicotinamide analogues with comparable molar activities. The applicability of dual wavelength assays to FLADH and TBADH will permit investigations of the effects of changes in concentrations of substrates, coenzymes, and potential regulatory ligands under conditions more closely approximating physiological than is possible with conventional assays.

APPENDIX I

Method of a two-substrate kinetic analysis

The general kinetic equation for two-substrate systems written in the form used by Cleland (135) is given by equation 1:

$$v = \frac{V_{max} [A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad \dots\dots\dots 1$$

Where V_{max} is the limiting rate at high substrate concentrations when both [A] and [B] are very large. The parameters K_a , K_b and K_{ia} are Michaelis constants for A and B and inhibition constant respectively. For an Ordered BiBi mechanism such as the one described below:

the inhibition constant K_{ia} is given by Cleland's nomenclature (135) as

$$K_{ia} = \frac{k_{-1}}{k_1}$$

Equation 1 above is used to analyze experimental results, with the use of Lineweaver-Burk plots of $1/v$ against $1/[A]$ and $1/v$ against $1/[B]$. The equation may be written as:

$$1/v = \left(1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{12}K_b}{[A][B]} \right) / V_{max} \dots\dots\dots 2$$

A plot of $1/v$ against $1/[A]$ at constant $[B]$, will therefore be linear, with the intercept on the $1/v$ axis as in equation 3:

$$\text{Intercept} = \frac{1 + K_b / [B]}{V_{max}} \dots\dots\dots 3$$

and

$$\text{Slope} = \frac{K_a + \{(K_{12}K_b)/[B]\}}{V_{max}} \dots\dots\dots 4$$

Experimentally, initial rates (v) are measured at varied concentration of one substrate (e.g. A) at different fixed concentrations of the second substrate (B). The kinetic data from primary plots are then averaged from duplicate or triplicate experiments. For an enzyme following bisubstrate kinetics relations in which the above relations are true will yield a family of converging lines, each being obtained for a different fixed concentration of B. Both the slope and the intercept will in general decrease with increasing $[B]$.

From secondary plots of intercepts and slopes described by equation 3 and 4 against $1/[B]$ it is possible to determine all kinetic parameters. A secondary plot of intercepts versus $1/[B]$ yields a plot with

$$\text{Intercept} = \frac{1}{V_{max}}$$

$$\text{Slope} = \frac{K_a}{V_{\max}}$$

The secondary plot of slope against $1/[B]$ yields a plot with

$$\text{Intercept} = \frac{K_a}{V_{\max}}$$

$$\text{Slope} = \frac{K_a K_b}{V_{\max}}$$

These kinetic constants are obtained from secondary plots by regression analysis (223) with standard error calculated from correlation coefficients determined by regression.

APPENDIX II

Inhibition Studies

Three simple types of enzyme inhibitors are known. Assuming that only a single substrate is involved in the reaction, and that only one type of inhibition is present at any time, the effects of these inhibitors are given below. The inhibitor I seems to have two possible sites of interaction with the enzyme.

The velocity equation accomodating these two sites in reciprocal form (203) is

$$\frac{1}{v} = \frac{K_s (1 + [I]/K_{i_s}) + [S] (1 + [I]/K_{i_i})}{V_{\max} [S]}$$

Where I, S, K_{i_s} and K_{i_i} are the inhibitor, varying substrate, slope effect of the formation of dead-end enzyme-inhibitor complex, and K_{i_i} intercept effect. Double reciprocal plots yield:

$$\text{intercept} = \frac{1}{V_{\max}} + \frac{[I]}{K_{i_i} V_{\max}}$$

A secondary plot of intercepts versus the concentration of inhibitor I can be used to obtain K_{i_i} . Similarly, slopes obtained from the double reciprocal plots are:

$$\text{Slopes} = \frac{K_s}{V_{\max}} + \frac{K_s [I]}{K_{i_s} V_{\max}}$$

Then K_{is} can be obtained from the slope of secondary plot of slopes versus concentration of I.

If binding of substrate and inhibitor to an enzyme form are mutually exclusive, the effect is termed competitive, therefore binding of I prevents binding of S. In this case only slope is affected and a value of K_{is} is obtained.

Uncompetitive inhibition results when the inhibitor interacts with the binary complex only, therefore only the intercept term is affected, and K_{ii} only is obtained. Non-competitive or mixed inhibition results when inhibitor binds to both free enzyme and enzyme-substrate binary complex.

Saturation by S does not completely remove the effect of inhibitor. This results in both slope and intercepts being affected and convergence to the $1/v$ axis is not expected. If $K_{is} = K_{ii}$ the inhibition is pure non-competitive, if $K_{is} \neq K_{ii}$, inhibition is considered mixed.

APPENDIX III

The following appendix contains secondary plots of kinetic and product inhibition studies obtained for NAD⁺ and NADP⁺-linked activities of FLADH and TBADH (refer to text).

Fig.(3.25 b)

Fig. (3.26 b)

Fig.(3.33b)

Secondary plots for steady-state kinetics of the NAD^+ , NADP^+ and dismutase activities of FLADH (Figures 3.25b, 3.26b and 3.33b respectively). In every plot, intercepts (O) and slopes (Δ) are obtained from the primary plots. For more information see corresponding figures.

350

Secondary plots for steady-state kinetics of TBADH. Kinetics of NAD(P)⁺ with propan-2-ol (Figure 3.29b and 3.30b) and NADP⁺ kinetics with ethanol as substrate (Figure 3.31b). In every plot, intercepts (○) and slopes (Δ) are obtained from primary plots. For more details see the corresponding figures.

Secondary plots for the product inhibition studies of the NADP⁺-linked activity of TBADH (Figures 3.42a-3.45a). Intercepts (○) and slopes (Δ) obtained from primary plots are replotted versus [product]. For more details see corresponding figures.

Secondary plots for the product inhibition studies of the NADP⁺-linked activity of FLADH (Figures 3.46a-3.49a). Intercepts (O) and slopes (Δ) obtained from primary plots are replotted versus [product]. For more details see corresponding figures.

Secondary plots for the product and cross product inhibition studies of NAD^+ -linked activity of FLADH (Figures 3.50a-3.55a). In each plot, the right axis represents slopes (Δ) and left axis represents intercepts (O) obtained from primary plots and replotted versus [product]. For more details see corresponding figures.

Fig. 3.50c

Fig. 3.51c

Fig. 3.52c

Fig. 3.53c

Fig. 3.55c

APPENDIX IV

Effect of pH

The most convenient and widely used method of treating the effects of pH on enzyme activity is that of Malcolm Dixon (142). It involves plotting the logarithm of the kinetic constant against the pH as shown below;

For the maximum velocity (curve a), taking the logarithm of equation 1

$$V = \frac{\tilde{V}}{1 + [H^+]/K_A^{ES} + K_B^{ES}/[H^+]} \quad \dots\dots\dots 1$$

(where K_A^{ES} and K_B^{ES} represent the molecular dissociation constants for the enzyme-substrate complex) will give equation 2:

$$\log V = \log \tilde{V} - \log (1 + [H^+]/K_A^{ES} + K_B^{ES}/[H^+]) \quad \dots\dots\dots 2$$

The pH dependence will be determined by the second term in equation 2, this is because $\log \tilde{V}$ is independent of pH.

At low pH values where $[H^+] \gg K_A^{ES}$ and $[H^+] \gg K_B^{ES}$, equation 2 will be simplified to equation 3;

$$\log V = \log \tilde{V} - \log [H^+] / K_A^{ES} = \log \tilde{V} + (pH - pK_A^{ES}) \dots 3$$

and thus the slope of the graph will be +1. As the pH is raised there will be a range over which two terms contribute to the slope, and provided that $K_A^{ES} \gg K_B^{ES}$ there will then be a range where $K_A^{ES} \gg [H^+] \gg K_B^{ES}$. Over this range equation 2 simplifies to equation 4:

$$\log V = \log \tilde{V} \dots \dots \dots 4$$

So the graph will have a slope of zero. Further increase in pH will give a region in which the dependence is governed by $\log(1 + K_B^{ES}/[H^+])$, followed by one where $K_B^{ES}/[H^+]$ is dominant and equation 2 simplifies to equation 5:

$$\log V = \log \tilde{V} - \log K_B^{ES} / [H^+] = \log \tilde{V} + (pK_B^{ES} - pH) \dots \dots \dots 5$$

which will give a slope of -1.

Thus, provided that $pK_B \gg pK_A$, a graph of $\log V$ against pH will be composed of linear regions of slopes +1, 0, and -1. Linear regions will be joined by curved sections where two of the terms in the second part of equation 2 contribute appreciably to the slope. If the linear portions are extended they will intersect at the pK values.

Similar behaviour should be seen if $\log (V/K_m)$ is plotted against pH. In this case, the intersection points will give the pK values for ionization of the free enzyme (pK_A^E , pK_B^E) rather than of the enzyme-substrate complex (curve b).

The NADP⁺-linked activity of TBADH gave a bell-shaped pH profile.

The pH-velocity profiles of all FLADH activities were more complicated. Detailed explanation of pH effects on NAD⁺-linked activity of FLADH is given in the results section.

The followings are double reciprocal plots for NAD⁺-linked activity of FLADH at pH 8.0-11.0 and secondary plots for these pH plots.

Figure 3.58 a and Figure 3.59 a. Double reciprocal plots of NAD^+ -linked activity of FLADH in sodium phosphate, pH 8.0 and sodium pyrophosphate, pH 8.5 respectively. Number on each line represents ethanol concentration in mM. Enzyme concentration was $0.28 \mu\text{M}$.

Figure 3.60 a and 3.61 a. Double reciprocal plots of NAD^+ -linked activity of FLADH in sodium pyrophosphate, pH 9.0 and 10.0 respectively. Other conditions are the same as for Figures 3.58a and 3.59a.

Figure 3.62 a and 3.63 a. Double reciprocal plots of NAD^+ -linked activity of FLADH in sodium pyrophosphate, pH 10.5 and sodium bicarbonate, pH 11.0 respectively. Ethanol concentrations in mM are indicated by the number on each line. Enzyme concentrations were $0.27 \mu\text{M}$ for pH 10.5 and $1.22 \mu\text{M}$ for pH 11.0.

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Secondary plots for the pH study of NAD^+ -linked activity of FLADH. Intercepts (○) and slopes (Δ) are obtained from primary plots (Figures 3.56a-3.59a) for pH 7.0, 7.5, 8.0, and 8.5 respectively. For other details see corresponding figures.

Secondary plots for the pH study of NAD^+ -linked activity of FLADH. Intercepts (O) and slopes (Δ) are obtained from primary plots (Figures 3.60a-3.63a) for pH 9.0, 10.0, 10.5 and 11.0 respectively. For other details see corresponding figures.

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