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**PURIFICATION, CHARACTERIZATION AND KINETIC STUDIES
OF ALCOHOL DEHYDROGENASE FROM FISH LIVER (RAINBOW
TROUT) AND WHEAT**

by

Ballari Mukherjee, M.Sc.
Indian Institute of Technology, Kharagpur.

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

Master of Science

Department of Chemistry
Carleton University
Ottawa, Ontario
December, 1999

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**“PURIFICATION, CHARACTERIZATION AND KINETIC STUDIES OF
ALCOHOL DEHYDROGENASE FROM FISH LIVER (RAINBOW TROUT) AND
WHEAT”**

submitted by Ballari Mukherjee, M.Sc.
Indian Institute of Technology, Kharagpur

in partial fulfilment of the requirements for the degree of Master of Science

Prof. G.W. Buchanan

Chairman, Department of Chemistry

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Abstract

Alcohol dehydrogenase from fish liver (rainbow trout) has been purified to electrophoretic and chromatographic homogeneity with 90.4 fold purification. The alcohol dehydrogenase from wheat was partially purified to 60.25 fold. Specific activity for the fish enzyme was 2.4 Units/mg and that for wheat was 5.4 Units/mg (after sephacryl S 300). The purification procedure consists of the combination of ammonium sulphate precipitation, anion/cation exchange (DE-52/50, CM-52) chromatography, gel filtration or size exclusion chromatography using sephacryl S-300, Sephadex G-200 and affinity chromatography (NAD^+ coenzyme resins); and is similar to that employed to purify all dehydrogenases from this laboratory. Partial characterization and comparison of physical, catalytic and mechanistic properties is made with respect to horse liver alcohol dehydrogenase and yeast alcohol dehydrogenase.

Alcohol dehydrogenase from rainbow trout liver (FADH) is a dimer with molecular weights similar to horse liver alcohol dehydrogenase. This enzyme also display multifunctional activity. FADH has aldehyde oxidising and esterolytic activities in addition to alcohol oxidising activity. Plant alcohol dehydrogenase, wheat however exists as a tetramer and shows only the oxidative activity towards alcohol. The crude wheat enzyme did show an esterolytic activity, but upon further purification it yielded more or less purified enzyme which had no esterolytic activity. It was however not possible to purify wheat by NAD^+ affinity chromatography because the enzyme did not bind with the resin. Several other forms of affinity chromatography were tried but failed to give a homogenous enzyme.

Both the enzymes from fish and wheat however, showed a high specificity for NAD^+ . The enzyme from wheat showed a very weak specificity for NADP^+ and this was not investigated here since the wheat enzyme was very unstable and the specificity for NADP^+ was very low. The resemblance in physical properties, and coenzyme specificity of fish liver alcohol dehydrogenase and horse liver alcohol dehydrogenase indicate evolu-

tionary connections.

The steady state kinetic analysis of the two enzymes showed that the NAD^+ linked activity for the fish liver alcohol dehydrogenase proceeds via Steady-State Random BiBi mechanism, whereas the mechanism of NAD^+ linked activity of the wheat enzyme is an Ordered BiBi mechanism.

The NADP^+ activation in fish liver alcohol dehydrogenase is induced by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and an amine. Effects on activity in presence of different amines were observed.

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Abbreviations

°A	Angstrom Unit
ADH	Alcohol dehydrogenase
A ₂₈₀	Absorbance at 280 nm
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CH ₃ CHO	Acetaldehyde
CM-52	Carboxymethyl cellulose
°C	Degree centigrade
DEAE	Diethylamino ethyl ion exchange
DHase	Dehydrogenase activity
Dmase	Dismutase activity
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3'-dimethylamino propyl) carbodiimide
EDTA	Ethylene diamine tetraacetic acid
Ease	Esterase activity
E _t	Total enzyme concentration

EtOH	Ethanol
FADH	Fish (liver) alcohol dehydrogenase (Rainbow trout)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gms	grams
gms/ml	grams per millilitre
μg	micrograms
HLADH	Horse liver alcohol dehydrogenase
HLALDH	Horse liver aldehyde dehydrogenase
HCl	Hydrochloric acid
LDH	Lactate dehydrogenase
μL	microlitre
mA	milliAmpere
mg	milligram
M	Molar
mM	milliMolar
ml	millilitre
NAD(P) ⁺	Nicotinamide adenine dinucleotide (phosphate), oxidized form
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced form

NaCl	Sodium chloride
nm	nanometer
pI	Isoelectric point
R _f	Relative distance
SDH	Sorbitol dehydrogenase
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
tris	Tris (hydroxymethyl) amino methane
U	Enzyme activity unit
UV	Ultraviolet radiation
v	initial velocity rate
V _{max}	Maximum velocity
V _{max} /E _t	Turnover number
WSC	Water soluble carbodiimide
WADH	Wheat alcohol dehydrogenase
w/v	weight by volume
YADH	Yeast alcohol dehydrogenase

Introduction

Alcohol dehydrogenase (ADH) was the first NAD^+ dependent dehydrogenase to be purified in crystalline form, first from brewer's yeast (by Negelin and Wulff in 1943)¹ and later from horse liver (by Bonnichsen and Wassen in 1948)². Besides these two enzymes which were obtained in pure state, the alcohol dehydrogenase (NAD^+ : alcohol oxidoreductases, EC 1.1.1.1, ADH) has a wide occurrence in nature. The enzyme occurs in a variety of organisms³ including different types of bacteria, yeasts, fungi, insects, fishes, amphibians, birds and mammals⁴. Partial purifications and characterizations were made from rainbow trout⁵, chicken⁶, wheat germ⁷, cotton seeds⁸, peanuts⁹, thermophilic bacteria¹⁰ and various other organisms⁴. ADH activity was also found in a great number of animals (hog³, human³, rabbit³, lamb³ and rat liver³), plants (pea³, corn³ and rice³) and microorganisms (*Acetobacter peroxydans*³, *Acetobacter xylinum*³, *Arthobacter*³, *Aspergillus niger*³, *Escherichia coli*³ and *Neurospora crassa*³). Estimation of functional and structural similarities and evolutionary correlations between different alcohol dehydrogenases in general, has 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 alcohol dehydrogenase (HLADH), rat liver enzyme, and partial sequences of alcohol dehydrogenases from other sources had been determined. Knowledge of the three-dimensional structure of the horse liver alcohol dehydrogenase determined by X-ray diffraction had provided the firm ground for the elucidation of functional and evolutionary relationships¹¹. The liver alcohol dehydrogenase belongs to major pathway of alcohol metabolism and catalyses the oxidation of various aliphatic, alicyclic, aromatic, primary and secondary alcohols to the corresponding aldehydes (Scheme 1)¹².

SCHEME 1 Oxidation of alcohols by HLADH.

In addition to its normal dehydrogenase activity HLADH and FADH exhibit multi-functional activities^{14,15}. It catalyses irreversible dismutation of aldehydes to the corresponding carboxylic acids in the presence of NAD^+ . This dismutase activity was found to increase with increasing chain length of aliphatic aldehydes reaching a maximum at octanal¹⁶. The reaction is given in Scheme 2.

SCHEME 2 Dismutation of aldehydes to the corresponding carboxylic acid by HLADH.

The enzyme also catalyses an NAD^+ independent hydrolysis of octanoate esters (namely *p*-nitrophenyl esters¹⁷) into octanoic acid and the corresponding alcohol. *p*-Nitro-

phenyl octanoate is used as the substrate and the progress of the esterase reaction is followed by monitoring the production of p-nitrophenol at 400 nm (Scheme 3).

SCHEME 3 NAD^+ independent hydrolysis of esters by 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 negligible¹⁸. Due to its wide ranging sociological as well as physiological ramifications, ethanol metabolism has been intensely studied. It is an important enzyme in biological systems and deserves a closer investigation.

Three main isozymes of HLADH are formed by the dimeric combinations of two different protein chains known as E for *ethanol active* and S for *steroid active*³, based on a difference in substrate specificity and six amino acid residues. The main isozymes are EE, ES and SS. Commercially active enzyme form has a molecular weight 80,000 daltons and is a dimer of two identical subunits (the *ethanol active* form EE). Each subunit is divided into two domains joined by a narrow neck region and separated by a deep active site cleft. One of these domains, is the coenzyme binding domain and binds the coenzyme. The two zinc atoms are bound within the second domain, the catalytic domain. The coenzyme binding domain comprises of 143 residues and the larger catalytic domain comprises 231 residues. A homologous interaction within regions of the coenzyme binding domain joins the two subunits. The two domains are joined into the dimeric molecule through interactions within the coenzyme binding domains (Figure 1) and is separated by a deep active site cleft surrounded almost entirely by hydrophobic residues. This cleft accommodates the substrates and the nicotinamide moiety of the coenzyme¹⁹. The interactions between the two subunits A and B of HLADH dimer are shown schematically in Figure 1²⁰. Table 1²⁰ lists

all of these subunit interactions.

FIGURE 1 Stereo diagram of the interaction between two subunits A & B of the HLADH dimeric molecule.
The subunit contacts are listed in Table 1.

Table 1: Interactions between the two subunits A and B of HLADH dimer

Interaction type	Residue in subunit A	Residue in Subunit B
Main chain hydrogen bonds	O of Arg-312	N of Gly-316
	O of Trp-314	N of Trp-314
	N of Trp-314	O of Trp-314
	N of Gly-316	O of Arg-312
	N of Leu-301	O of Met-303
	O of Leu-301	N of Met-303
	N of Met-303	O of Leu-301
	O of Met-303	N of Leu-301
Non polar contact between side chains	Met-275	Pro-305
	Ile-291	Leu-308
	Val-294	Pro-305, Met-306, Leu-309
	Pro-296	Met-306
	Leu-301	Met-303
	Met-303	Leu-301, trp-314
	Pro-296	Met-275, Val-294
	Met-306	Val-294, Pro-296
	Leu-308	Ile-291, Trp-314
	Leu-309	Val-294
	Trp-314	Met-303, Leu-308, Trp-314

The catalytic domain consists of residues 1-175 and 319-374 and firmly binds two zinc atoms, one of these ions plays a structural role²¹. The structural zinc is liganded in a

distorted tetrahedral arrangement by four sulphur atoms from cysteine residues 97, 100, 103 and 111 (Figure 2)²¹. The other, known as *active site zinc*, plays an important role in the oxidation-reduction reactions (as a Lewis acid). The catalytic zinc atom is bound at the bottom of this pocket in the middle of the subunit by three protein ligands; two sulphur atoms from Cys-46 and Cys-174 and one nitrogen from His-67 (Figure 2)²¹.

FIGURE 2 Schematic diagram of the binding of the ligands to the two different zinc atoms (catalytic: **a** and structural: **b**).

A water molecule or hydroxyl ion, depending on the pH, completes a distorted tetrahedral coordination¹⁴.

The active site pocket (substrate binding pocket) is about 25 °Å deep from the surface to the zinc atom (Figure 3)¹⁹. The zinc ligands, the nicotinamide moiety of the coenzyme, and the side chain of Ser-48 are the only polar atoms in the pocket near zinc. The hydrophobic side chains from residues Phe-93, Phe-140, Leu-141, Leu-57, Leu-116, Ile-318, Val-294, Phe 110, Pro-295, and Pro-296 which are from the same subunit as the ligands to the zinc and Met-306 and Leu-309 from the other subunit form the remaining part

of the inside wall of the pocket. The substrate binding pocket is shown in Figure 3. Kinetic and spectroscopic measurements shows that substrate binds directly to the catalytic zinc atom. The zinc bound water molecule is involved in a hydrogen bonding system which includes Ser-48 and His-51 (Figure 4)¹⁹. It is believed that these hydrogen bonds are possibly responsible for proton release in the catalytic mechanism (this is not discussed in this thesis).

FIGURE 3 Schematic representation of substrate binding pocket in HLADH viewed from the direction of the zinc atom. Bromobenzyl alcohol molecule is bound in the pocket. The zinc ligands, the nicotinamide moiety of the coenzyme and the side chain of Ser-48 are the only polar atoms in the pocket near zinc. the hydrophobic side chains form the remaining part of the inside wall of the pocket.

Residues 95-113 form a lobe which binds the second zinc atom of the subunit²¹. This lobe projects out from the catalytic domain having a few side chain interactions with the remaining parts of the subunit, which agrees with the variability of its sequence in different species. Stereo diagrams of this lobe with its bound zinc are given in Figure 5²¹.

FIGURE 4 Schematic diagram illustrating the hydrogen bond system of the zinc bound water molecule, Ser-48 and His-51 and a possible mechanism for the proton release induced by NAD^+ binding.

FIGURE 5 Stereodigram of the residues 95-113 and the bound zinc atom (the second structural zinc atom).

The coordination of this zinc atom in HLADH is similar to the tetrahedral arrangement of four cysteine ligands around iron in rubredoxin². It is also more similar to the four cysteine ligands around the iron clusters in the bacterial ferredoxins²¹. The two catalytic zinc atoms of the molecule are separated by 47 Å. Hence, there is no direct interaction between the two catalytic centres.

The structures of catalytic domains of LDH (lactate dehydrogenase) and GAPDH, are different from that of HLADH. In HLADH the catalytic domain is built up by a complicated network of β structures. There are three main regions of pleated sheet which are known as β I, β II and β III. Figure 6 gives a schematic representation of these three regions. In the catalytic domain 18% of the residues comprise of four helical segments, whereas 35% of the residues are in pleated sheet regions and 14% are in reverse bends. From the above data, it can be seen that a large number of residues, 33%, have no regular secondary structure.

FIGURE 6 Schematic diagram of the three pleated sheet regions in the catalytic domain.

Alcohol dehydrogenase is a oxidoreductase and is also known as aldehyde reductase. The broad substrate specificity of HLADH has been confirmed by several investigations. Crystallographic studies of substrate binding have been concentrated on alcohol binding obtained from an equilibrium mixture of enzyme, coenzyme and bromobenzyl alcohol/aldehyde¹⁹. The solution studies have shown that the ternary complex with alcohol is preferred. Jornvall²² determined the sequence of the 374 amino acids in the single polypeptide chain of one subunit of the E isozyme. The X-ray structure of the native enzyme (EE isozyme), has been determined by Eklund et al. to 0.24 nm resolution²³. The two

coenzyme molecules are bound per enzyme molecule independent of each other. Coenzyme binding induces a conformational change of the enzyme from open to closed form²⁴. The coenzyme binding domain consists of residues 176-318. The basic part of the element in this domain is built up from six parallel strands of pleated sheet (β A- β F) flanked by helices (α A, α B, α C, α E and 3¹⁰S) in a regular pattern. The physical sequence of strands from top to bottom of the sheet is C-B-A-D-E-F²⁵. The folding of the polypeptide chain in helices and β structure is given in Figure 7²¹.

FIGURE 7 Schematic diagram illustrating the fold of the polypeptide chain within the coenzyme binding domain and the nomenclature of the elements of secondary structure.

This domain consists of three hydrophobic regions. One of them is involved in the subunit interaction, and the other two play an important role in the folding of the coenzyme binding domain since they form hydrophobic cores between the helices and the parallel pleated sheet²¹. The amount of secondary structure in this domain is considerable: 44% of the residues are helical, mostly α -helices; 32% are in the pleated sheet structure; and 13% in reverse bends²¹. So, only about 10% of the residues have no secondary structure; and no continuous stretch of irregular structure is longer than four residues.

The coenzyme nicotinamide adenine dinucleotide (NAD) has a number of specific interactions that form 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 main chain atoms of the enzyme. Figure 8 shows these interactions schematically. This coenzyme binding site locks the coenzyme in a fixed conformation which is identical in all those ternary complexes of alcohol dehydrogenase which have the closed protein structure conformation¹⁹. Various crystallographic studies on inhibitor binding to LADH have shown that there are two main classes of inhibitors. One kind of inhibitor binds in the coenzyme binding cleft, mainly in the region where the AMP part of the coenzyme binds. Another kind of inhibitor binds in the substrate binding pocket and competes with the substrate for ligation to the active-site zinc atom. The binding site for these inhibitors is limited in space by the side chains of Ser-48, His-67, and Phe-93 as well as the nicotinamide ring and allows only one molecule to bind at the same time. Figure 9¹⁹ shows imidazole binding and illustrates this point.

FIGURE 8 Schematic representation of interactions from coenzyme and substrate to the HLADH enzyme molecule.

FIGURE 9 Stereo diagram of bound imidazole to HLADH. The figure shows the slot between the side chains of Ser-48 and Phe-96 where inhibitors and substrates bind.

The structures of few dehydrogenases like dogfish lactate dehydrogenase LDH, pig soluble malate dehydrogenase s-MDH, HLADH and lobster glyceraldehyde-3-phosphate dehydrogenase GAPDH at high resolution were compared. It showed a remarkable similarity in the structures of those parts of the polypeptide chain whose function is to bind the NAD^+ coenzyme, but the complete subunit conformations are not alike. The catalytic domains of these enzymes however bind different substrate molecules and hence they have different three dimensional structures.

From the X-ray structures of lactate, malate, alcohol and glyceraldehyde-3-phos-

phate dehydrogenases, the general nature of NAD^+ enzyme interactions were confirmed. Table 2²¹ shows other specific interactions and many similar features can be identified

. One similarity is the structurally equivalent aspartic acid residue (Asp-223 in HLADH) that is also chemically identical in the co enzyme binding domains of the above dehydrogenases. This residue forms a hydrogen bond to O-2' hydroxyl of the adenosine ribose. This particular residue is present in HLADH, YADH and fourteen other dehydrogenases and proves its importance. 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. There is an electrostatic attraction exhibited by the adenosine ribose moiety of NAD^+ and the carboxyl oxygen of ASP-223 and an repulsive interaction of such acidic groups with the 2'-phosphate of NADP^+ hence the NAD^+ specificity of the above enzymes. Matthews et al. have identified an arginine residue in the active site of dihydrofolate reductase. Arginine provides an attractive interaction with the 2'-phosphate of NADPH and provides an explanation for the specificity of this enzyme for NADPH.

Before structure determination were accomplished, sequences of different dehydrogenases were aligned and structural similarities were observed. Table 3²¹ shows the available sequence information in relation to the known tertiary and quaternary structures.

The essential cysteine residue was used as a point of alignment between LDH, GAPDH, LADH and yeast ADH by Harris and Fondy et. al. After the structure determination, it was seen that such comparisons were valid only between LDH and GAPDH, where the cysteine is at the end of βF and forms the junction between two different domains. In LADH the essential cysteine-46 is in the middle of the catalytic domain. Also it was seen that the amino acid sequence of baker's yeast enzyme (YADH) was distantly related to HLADH and also their three dimensional structures were distantly related.

Table 2: Enzyme coenzyme interactions in LDH, HLADH and GAPDH

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

Table 2: Enzyme coenzyme interactions in LDH, HLADH and GAPDH (Continued)

Coenzyme moiety	Residues			Type of contact
	LDH	HLADH	GAPDH	
Carboxamide	C=O 139	C=O 292		H bond
	Ser 163	N 319	Asn 313	H bond

Underlined residues in Table 2 on the same line are structurally equivalent according to the alignment by Rossmann et al. ¹¹.

Table 3: Available structural information for dehydrogenases

Dehydrogenases	Species for which complete sequence is known	Species for which three dimensional structure is known	Additional species for which essential thiol sequence is known	Oligometric structure
LDH	Dogfish	Dogfish	Bovine, chicken, pig and bullfrog	Tetramer
HLADH	Horse and rat	Horse	Horse, human liver and B. stearothermophilus	Dimer
GAPDH	Pig, lobster and yeast	Lobster	Human, rabbit, chicken, ostrich, E.coli, honey bee	Tetramer
GluDH	Chicken, bovine			Hexamer

Alcohol dehydrogenase was also found to be related to polyol dehydrogenase. Jornvall compared the sequence of LADH with that of GAPDH¹². The catalytic domain of LADH matched with the nucleotide binding domain of GAPDH which ruled out the idea of an early common genetic origin. Jornvall et al.¹³ aligned sixteen characterized alcohol dehydrogenases and one sorbitol dehydrogenase (SDH) which consists of three different types of molecules: dimeric ADH, tetrameric ADH and tetrameric SDH, metalloproteins with one or two atoms of zinc per subunit and polypeptide chains from different kingdoms and orders (mammals, fungus, yeasts and higher plants). The comparisons revealed extensive variations in alcohol dehydrogenase. They emphasize general importance of particular residues, suggesting related overall functional constraints in the molecules. From all these comparisons it was finally concluded that the structural features of HLADH is common amongst ADHs from different sources, and evolutionary relationship has been inferred.

Numerous kinetic studies have been performed on ADH from various sources. The analysis of the kinetics of a reaction catalysed by the enzyme is an important tool for the study of enzyme mechanism. By this, the order in which substrates bind and product leaves the enzyme, and the velocity at which these processes takes place can be established. Explanation of kinetic studies, inhibition patterns and rate equations are given in results and discussion. The catalytic mechanism was an ordered Theorell-Chance mechanism where the coenzyme binds first and is released last without the formation of a kinetically significant ternary complex (Scheme 4).

SCHEME 4 Ordered Theorell-Chance mechanism.

According to this mechanism, the catalysis is instantaneous upon the approach of

the substrate. A kinetic treatment of this mechanism gives the following rate equation:

$$\frac{v}{E_t} = \frac{n_{ab}AB - n_{pq}PQ}{d_0 + d_aA + d_bB + d_{ab}AB + d_pP + d_qQ + d_{pq}PQ + d_{ap}AP + d_{bq}BQ} \quad (\text{Eq. 1})$$

where A, B and P, Q are concentrations of reactants and products respectively, and n_{ij} and d_{ij} are combinations of simple rate constants. Another mechanism can be shown which would include a ternary complex (Scheme 5).

SCHEME 5 Ordered bi-bi ternary complex mechanism.

A full kinetic treatment of this mechanism showed that it had a rate equation similar to Equation 1 but the denominator includes terms in ABP and BPQ. The appropriate kinetic experiments using A and B with P as an inhibitor can be used to distinguish these two mechanisms as P is a competitive inhibitor of B in a Theorell-Chance mechanism but a non-competitive inhibitor of B in an ordered Bi-Bi mechanism. These experiments showed that HLADH operates by a ternary complex mechanism.

Another kind of variation of the bisubstrate reaction mechanism would involve the random binding and release of the substrates and coenzyme (Scheme 6). Isotope exchange studies have shown that the enzyme can catalyse the reaction when the substrate is bound first. Further investigations and inhibition studies showed that the random pathway only contributes significantly to the reaction rate at high substrate concentrations. At low substrate concentrations the reaction mechanism is ordered bi-bi, with addition of NAD^+ to the free enzyme first.

SCHEME 6 Random bi-bi mechanism.

The accepted mechanism of HLADH catalysis is a preferred random mechanism where the coenzyme is preferentially bound first and released last as shown in Scheme 7.

SCHEME 7 Preferred random mechanism of HLADH.

Kinetic analysis alone does not elucidate the detailed molecular mechanism by which these transformation occur and how the amino acid residues contribute to them. It is essential to study the structural elements of the active site (amino acid side chains) and

three dimensional structure of the active site to learn about the catalytic mechanism. At first two plausible mechanisms were proposed: direct hydride transfer and radical reaction. After detailed experimental observations and studies it was concluded that the oxidation of alcohols in presence of NAD^+ and alcohol dehydrogenase takes place via direct hydride transfer reactions.

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 proteins²⁶. Modifications of amino acid residues also takes place in vivo subsequent to the translational process in protein synthesis. Such modifications cause desirable changes in the properties and activities of proteins without structural effects. It has been seen however that current methodologies of chemical modification of active sites residues mostly lead to an inactivation of enzymes.

A large number of active-site directed chemical modifying reagents have been used to investigate the structure and mechanism of horse liver alcohol dehydrogenase. The halo acids iodoacetate and 3-bromopropionate have been shown to selectively alkylate Cys - 46 and Cys - 174, which are active site zinc ligands in HLADH. It was seen that enzyme alkylated with iodoacetate retains less than 5% of the original catalytic activity. On the basis of X-ray crystallographic studies of the Cys - 46 carboxymethylated enzyme, and chemical modification studies of arginine residues, it has been suggested that iodoacetate binds to Arg - 47 prior to alkylation of Cys - 46 to form the reversible Michaelis-Menten complex. Quite a few number of different modifications that affect the ϵ -amino groups of lysine at the active site of HLADH have been studied. A very important reaction of HLADH is with methyl picolinimidate by Plapp^{27,28}, which modifies 50 of 60 lysine residue, causing the overall enhancement in enzymic activity. Histidine residues in HLADH have been modified by carbethoxylation with diethylpyrocarbonate which resulted in a rapid activation followed by a slower inactivation.

Other than the above mentioned amino acid residues, very few detailed studies on the modification of acidic amino acids have been reported. The presence of carboxyl group, Asp - 223 and its involvement in hydrogen bonding to the adenosine ribose moiety is responsible for the NAD^+ specificity exhibited by HLADH and other dehydrogenases as mentioned earlier²⁹. A selective chemical modification procedure using water soluble carbodiimides and an amine is used for modifying Asp - 223 to determine the role of carboxylic group in proteins³⁰. The effect of such modification of carboxylic residues on proteins that have ability to use both NAD^+ and NADP^+ could provide an explanation of the nature of the active site³¹.

The objectives of this thesis are summarised as follows:

(i) to modify the purification procedure from Tsai et. al.³³ for FADH (Rainbow trout) and WADH (Wheat) and to purify the enzymes in due process.

(ii) to compare properties, multifunctionality, nucleotide specificity, mechanism and amino-acid residues responsible for nucleotide specificity of HLADH and YADH with alcohol dehydrogenase isolated from fish liver (Rainbow trout) and wheat.

(iii) kinetic studies of the reaction mechanism and product inhibition studies which finally leads to the understanding of the reaction mechanism.

(iv) to employ chemical modification to demonstrate the nucleotide specificity.

Experimental Procedures

Materials:

Trout livers were supplied by Thistle Springs Trout Farm & Camp Grounds (Ash-ton, Ontario). Roblin Wheat (treated with insecticide: Carbathiin and fungicide: Thiram) was obtained from Ritchie Feed and Seed. Dithiothreitol (DTT), glycerol, glycine, ammonium sulphate and ethylene diamine tetraacetic acid (EDTA), were from Aldrich Chemical Company, Inc. Milwaukee, WI, USA. Oxidised form of β -nicotinamide adenine dinucleotide phosphate (NADP^+), β -nicotinamide adenine dinucleotide (NAD^+), reduced form of β -nicotinamide adenine dinucleotide (NADH), molecular weight markers for SDS-Page and native Page, phenylmethyl sulphonyl fluoride (PMSF), Tris(hydroxymethyl)aminomethane (Tris), taurine, propylamine, sodium phosphate-dibasic, heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), sodium phosphate-monobasic, anhydrous (NaH_2PO_4), sodium dodecyl sulphate (SDS), coomassie brilliant blue R-250, β -nicotinamide adenine dinucleotide /coenzyme resins (for affinity chromatography) and 5' AMP (for affinity chromatography) were purchased from Sigma Chemical Company, St. Louis. Matrex Red Gel A was from Amicon Corp. Tris-HCl ready gels were purchased from Bio-Rad laboratories. Bromophenol blue, dioxane and octanal was from Fisher Scientific, Fairlawn, NJ, USA. Crystalline bovine serum albumin for protein concentration was supplied by Bio-Rad laboratories. DEAE-52 was from Whatman. DEAE, Sephadex G-100, G-200 and Sephacryl S-300 (high resolution) were from Pharmacia Biotech. Octanal, dioxane were purchased from Fisher Scientific. Other reagent grade chemicals and biochemicals used in this experimental study were purchased from Sigma Chemical Company, Bio-Rad Laboratories, BDH Chemicals and Aldrich Chemical company.

Stock buffers:

The stock buffers Tris-HCl and phosphate buffers were prepared as follows:

a. Tris-HCl buffer, pH 8.0, 8.5 and 9.0 (0.2 M): 24.2 gm Tris was taken in a 1 litre volumetric flask and made upto the mark with deionised water. The required pH 7.5, 8.0, 8.5, 9.0 were obtained by adjusting with HCl/NaOH.

b. Phosphate buffer, pH 7.4 (0.2 M): 5.28 gm NaH_2PO_4 and 43.46 gm of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ was taken in a 1 litre volumetric flask and made upto the mark with deionised water. The pH was measured and adjusted with the acidic or basic part of the buffer as required.

Assays:

All enzymic activities were assayed spectrophotometrically in a Varian-Cary spectrophotometer in quartz cells.

Assay for Dehydrogenase activity. Alcohol dehydrogenase (ADH) activity was monitored at 340 nm in a reaction mixture (1.0 mL) containing 0.1 mL of NAD^+ (10.0 mM), 0.1 mL of ethanol (100.00 mM), 0.5 mL of Tris-HCl buffer (0.2 M, pH 9.0), 0.2 mL of deionised water and 0.1 mL of enzyme solution. One unit of dehydrogenase activity is defined as that amount of enzyme that produces 1 μmol of NAD(P)H ($\epsilon = 6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) per minute.

Assay for Dismutase activity. ADH (dismutase) activity was measured at 340 nm in an assay mixture (1.0 mL) containing 0.1 mL NAD^+ (10.0 mM), 0.2 mL of octanal (1.0 mM), 0.5 mL of phosphate buffer (0.2 M, pH 7.4), 0.1 mL of deionised water and 0.1 mL of enzyme solution. One unit of dismutase activity is defined as that amount of enzyme needed to produce 1.0 μmol of NADH per minute.

Assay for Esterase activity. Esterase activity was followed at 400 nm of the release of p-nitrophenol ($\epsilon = 16.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) from a reaction mixture (1.0 mL) containing 0.02 mL of p-nitrophenyl octanoate (5.0 mM) in dioxane, 0.5 mL of Tris-HCl buffer (pH 8.5),

0.38 mL of deionised water and 0.1 mL of enzyme solution. One unit of esterase activity is defined as that amount of enzyme required to release 1.0 μ mol of p-nitrophenol per minute.

Extraction of enzyme:

Wheat (100-200 gms.) was cleaned with lots of deionised water and 30% commercial bleach solution till all the fungicide were removed. It was then soaked overnight at room temperature (22°C) in deionised water. It was then homogenized in a blender with 3 volumes of ice cold 10mM phosphate buffer (pH 7.0) containing 1.0 mM DTT and 1.0 mM EDTA at high speed in three 40 seconds bursts spaced 1 minute apart. The semisolid mixture was then filtered through a cheese cloth and the turbid solution was collected and centrifuged at 5200 rpm at 4°C for 30 minutes. The supernatant i.e. the clear solution was again filtered through a cheese cloth and collected in a clean beaker (washed with deionised water) and placed on ice for further purification.

Fish livers (30-60 gms.) were chopped and homogenized with 3 volumes of ice cold 10 mM Tris-HCl buffer, pH 7.5 containing 1.00 mM DTT and EDTA. Homogenization was carried out on ice using Sorvall-Omni mixer at speed 5 in three 30 seconds bursts, spaced 1 minute apart. The homogenate was centrifuged at 5200 rpm for 30 minutes. The supernatant was filtered through a cheese cloth for further purification.

Purification of alcohol dehydrogenase from wheat and fish:

Ammonium sulphate fractionation. The supernatant i.e. the enzyme extract, from the previous step was placed on an ice bath and ammonium sulphate was added slowly to the enzyme extract (wheat and fish) whilst stirring to 30% saturation (176 gms./litre) for 1 hour. The extract was then centrifuged at 5200 rpm for 30 minutes and the precipitate was removed by collecting the supernatant.

The supernatant was then placed on an ice bath and its ammonium sulphate concentration was increased to 70% saturation for wheat (gms./litre) and 80% saturation for fish

liver (gms./litre) by gently stirring for 1-2 hours. After centrifugation at 5200rpm for 30 minutes the supernatant was discarded and the enzyme pellet was dissolved in minimum volume of buffer (phosphate buffer for wheat and Tris-HCl buffer for fish liver). The above concentrated enzyme extract was then dialysed for 16-17 hours against 1 litre of corresponding buffer with 1-2 buffer changes.

Ion exchange chromatography. About 10-15 mls. of the above dialysed fraction was applied to an anion exchange column (30.0 X 2.5 cm), DEAE-52 for fish liver enzyme and CM-52 for wheat enzyme. The columns were equilibrated with Tris-HCL buffer (pH 7.50) and phosphate buffer (pH 7.00) respectively. The column was first eluted with Tris-HCl buffer (at a flow rate of 32 ml/hr and 2 ml fractions were collected) till the A_{280} of the eluate was same as the A_{280} of the buffer. Then the column was eluted with a linear NaCl gradient (0.00-0.5 M) to elute other isozymes (if present). The wheat enzyme was eluted with a linear NaCl (0.00 - 0.50 M) gradient at a flow rate of 32 ml/hr and 2 ml fractions were collected. The peak fractions were located by measurement of absorbency at 280 nm and activity assays. The dehydrogenase active fractions were pooled and concentrated from sucrose by leaving the enzyme extract contained in a dialysis bag immersed in dry sucrose for 5-6 hours. The concentrated enzyme was then dialysed in 1 litre of corresponding buffer for 3 hours with at least one buffer change.

Size exclusion chromatography. The dialysed protein solution was loaded onto a Sephadex G-200 column (35 X 2.5 cm.) equilibrated with corresponding buffers for each enzymes. The enzymes were eluted with the same buffers at a flow rate of 40 ml/hr and 2 ml fractions were collected. Sephadex G-200 was later replaced by Sephacryl S-300. Sephacryl S-300 demonstrated better separation from the most important impurity esterase. The peak fractions were located by absorbency at 280 nm and dehydrogenase active fractions were again concentrated from sucrose and dialysed in corresponding buffers for 3 hours with at least one buffer change.

Affinity chromatography. The active enzyme solution from last step after dialysing

was applied onto an β -nicotinamide adenine dinucleotide/coenzyme resin (for affinity chromatography) column (3.0 X 0.9 cm). The flow rate was 10 ml/hr and 2 ml fractions were collected. The column was washed extensively with respective buffers until no more UVabsorbing material was detected, and A_{280} of the eluate dropped to that of the buffer. The enzyme was eluted off the column immediately on addition of buffer that contained 10 mM NAD^+ , and dehydrogenase active fractions were collected. The enzyme was concentrated from sucrose and the coenzyme was then removed by 24 hours dialysis against three changes of 1 litre buffer.

The above method did not work for wheat enzyme, therefore Matrex Red gel was tried instead, without satisfactory result. As a consequence, the wheat alcohol dehydrogenase was not homogeneous.

Protein assay:

Protein concentrations were determined by monitoring the absorbance of a standard solution at 280 nm at different concentrations. Crystalline bovine serum albumin was used as a standard and the standard curve was constructed by using bovine albumin in the concentration range of 2×10^{-3} gms/ml to 20×10^{-3} gms/ml. The absorbance of alcohol dehydrogenase was measured and its concentration was determined from the standard curve.

SDS-discontinuous gel electrophoresis:

For SDS-discontinuous gel electrophoresis, gels with a 10% separating gel and 4% stacking gel was used. The ready gels (Tris-HCl gels, SDS-PAGE) were purchased from Bio-Rad laboratories. This follows Laemmli's³² method with a small modification. For running the ready gels Mini-PROTEAN II cell (from Bio-Rad) was used. The standard proteins were obtained from Sigma. Two different kind of standards were used. Low molecular weight standard (range of approximate molecular weight 14,000-66,000), containing the following proteins:

Lysozyme, Egg White (14,300)

β -Lactoglobulin, Bovine Milk (subunit 18,400)

Trypsinogen, Bovine Pancreas, PMSF treated (24,000)

Pepsin, Porcine Stomach Mucosa (34,700)

Albumin, Egg (Ovalbumin) (45,000)

Albumin, Bovine Plasma (66,000)

and high molecular weight standards containing (range of approximate molecular weight 29,000-205,000)

Carbonic Anhydrase, Bovine Erythrocytes (29,000)

Albumin, Egg (Ovalbumin) (45,000)

Albumin, Bovine Plasma (66,000)

Phosphorylase B, Rabbit Muscle (subunit 97,400)

β -Glactosidase, *Escherichia coli* (subunit 116,000)

Myosin, Rabbit Muscle (subunit 205,000)

The stock solutions were prepared as follows:

0.5 M Tris-HCl, pH 6.8

0.60 gm Tris base ~ 60 ml deionised water. The pH was then adjusted to 6.8 using HCl. It was then made upto 100 ml with deionised water and stored at 40 C.

10% SDS

1 gm of SDS was dissolved in water with gentle stirring and diluted till 10 ml with deionised water.

1% Bromophenol Blue

0.1 gm of bromophenol blue was dissolved in 10 ml deionised water with gentle stirring.

The sample buffer, running buffer, staining and destaining solutions were prepared as follows.

1. Sample buffer:

Deionised water 2.9 ml

0.5 M Tris-HCl, pH 6.8 1.0 ml

Glycerol 2.0 ml

10% (w/v) SDS 1.6 ml

β -mercaptoethanol 0.4 ml

1.0% bromophenol blue 0.1 ml

1 part sample was diluted with 2 parts sample buffer.

2. 10x Running buffer:

Tris-base 15.0 gm

Glycine 72.0 gm

SDS 5.0 gm

and made upto 500 ml with deionised water. Running buffer was at ~ pH 8.3. This pH was not adjusted. 30 ml of this buffer was diluted with 270 ml deionised water to give the final running buffer for one electrophoresis run.

3. Coomassie Blue R-250 Staining solution:

Methanol 400 ml (40%)

Acetic acid 100 ml (10%)

Coomassie blue R-250 1.0 gm (0.1%)

Deionised water 500 ml

4. Coomassie Blue R-250 Destaining Solution:

Methanol 400 ml (40%)

Acetic acid 100ml (10%)

Deionised water 500 ml

The β -mercaptoethanol was added to the sample buffer immediately before use. The samples and molecular weight standards were diluted 1:2 in sample buffer and heated in a water bath at 95°C for 5 to 10 minutes for reducing and denaturing the proteins. The gel was inserted in the Mini-PROTEAN II cell and the comb was removed. 10 μ L of standards and samples were loaded on the wells using a micropipette. Electrophoresis was run for 35 minutes at 200 volts at room temperature (current was 50 mA) using an Ortec 4100 power supply.

Before loading the gel it was made sure that at least 1 μ g of protein per band was present since the staining was done with Coomassie Blue R-250. The gel was removed from the gel cassette and placed on a tupperware tray and just covered with staining solution. It was then placed on a shaking platform for 12 hours preferably overnight.

After staining the gels were gently removed from the staining solution, rinsed with deionised water and placed in another tray containing destaining solution and placed on the shaking platform. The destaining solution was changed three times in 2 hours until the background was clear. The gel was then removed from the destaining solution and stored in deionised water to prevent drying.

The positions of purified enzyme bands and standards were measured. Subunit molecular weight of the two enzymes were extrapolated from a constructed plot of log molecular weight against mobility of standards.

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. The same ready gels were used. The stock solutions were also prepared as before. The standards used were:

α -Lactalbumin, Bovine Milk (14,200)

Carbonic Anhydrase, Bovine Erythrocytes (29,000)

Albumin, Chicken Egg (45,000)

Albumin, Bovine Serum (monomer: 66,000 and dimer: 132,000)

Urease, Jack Bean (trimer: 272,000 and hexamer: 545,000)

The unknown proteins were prepared so that the concentration is about 1 mg protein per ml in phosphate buffer. The sample was diluted with two parts of sample buffer. The protein standard vials were reconstituted with 1 ml of 50 mM NaCl-1 mM sodium phosphate, pH 7.00, except the vial containing Urease which was dissolved in 5 ml water. immediately before use the standards were diluted with two parts of sample buffer. Samples loaded in the wells were enzymatically active and the sample buffer and the running buffers were prepared as follows.

1. sample buffer:

Deionised water 4.9 ml

0.5 M Tris-HCl, pH 6.8 1.0 ml

Glycerol 2.0 ml

Bromophenol blue 1.0 ml

2. 10x Running Buffer:

Tris base 15.0 gm

Glycine 72.0 gm

to 500 ml deionised water. The pH was ~ 8.3 and it was not adjusted. 30 ml of this buffer was diluted with 270 ml deionised water to give the final running buffer for one electrophoresis run. The Electrophoresis was run at 200 volts (50 mA) for 35 minutes. The staining and destaining was done as in above. The molecular weight of the enzymes were found similarly by constructing a plot of log molecular weight against mobility of the standards.

Molecular Weight Determination of Alcohol dehydrogenase:

The native molecular weight was also determined *via* Sephacryl S-300 gel filtration with a column length of 45 cm and diameter 2.5 cm. The elution buffer (pH 7.00) consisted of 10 mM phosphate buffer (made from dilution of the stock phosphate buffer), 1.0 mM DTT and 1 mM EDTA. An 1 ml aliquot of the purified ADH (WADH and FADH at different times) was loaded onto the column and 2 ml fractions were collected at a flow rate of 40 ml/hr. The fractions were assayed for enzymic activity and the dehydrogenase active fractions were identified. The standard proteins used were Catalase (232,000 Da), bakers yeast AlcDH (150,000 Da), horse liver ADH (80,000 Da), Bovine Serum Albumin (132,000 Da) and Pepsin from Hog stomach (35,000). Their elution peaks were determined by either dehydrogenase assays at 340 nm, or absorbance at 280 nm for Catalase, Bovine Serum Albumin and Pepsin. The native molecular weight was determined from a plot of elution volume vs. log molecular weight. of the standard proteins.

Gel filtration by HPLC using BioSep-SEC-S3000 column was also performed. The exclusion range for native proteins through this column is 5,000-700,000. the flow rate was 1.0 ml/min. The standards used are as above. The native molecular weight was determined

by the same method as above. The native molecular weight was also determined by Non SDS-discontinuous gel electrophoresis. The molecular weight was determined by plotting the log molecular weight of the standard proteins against their mobility.

The above two procedures did not give good result, therefore the native molecular weight calculated from size exclusion chromatography from Sephacryl S-300 was considered.

Chromatofocussing of alcohol dehydrogenase:

The preparation of the gel and column packing were carried out as described in Chromatofocussing with polybufferTM and PBETM handbook, Pharmacia Fine Chemicals Inc.

A column (53 X 0.9 cm) was packed with poly buffer exchanger PBE 94 and equilibrated with starting buffer 0.025 M ethanolamine-CH₃COOH, pH 9.4. The sample was applied by first running on 5 ml of eluent (poly buffer 96-acetate, pH 6.00), followed by the sample and then switching back to the eluent again. The pH, protein adsorption and dehydrogenase activity of the fractions were monitored.

Stability of enzyme activity at different stages of purification:

Enzyme fractions were assayed as soon as they were obtained from each step. The enzyme was stored at 4°C. Wheat enzyme solutions were in 10 mM phosphate buffer, 1.0 mM DTT, 1.0 mM EDTA, pH 7.00. Fish liver enzyme was in 10 mM Tris-HCl buffer, 1.0 mM DTT, 1.0 mM EDTA, pH 7.5. Enzymes were then assayed for the indicated activities periodically.

Effect of pH on enzyme activity:

Investigations for the effect of change of pH on NAD⁺ linked activity of FADH and

WADH were performed. The pH profiles were generated from initial rate data determined at fixed substrate concentration over the pH range 5.0-11.0. For this wide range of pH different buffers were used. Sodium acetate buffer for 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 sodium bicarbonate buffer for pH 11.0. Buffer concentrations were at 50 mM.

Alcohol dehydrogenase activity dependence on pH. The effect of pH variation on velocity of the reaction was investigated. Experiments were performed at NAD^+ concentration fixed at 10 mM and ethanol concentration at 100 mM. Specific activity/activity was plotted against pH. This was performed with both the enzymes.

Esterase activity dependence on pH. The effect of pH variation on esterase activities of FADH was also investigated. Experiments were performed under assay conditions of varied pH. The concentration of *p*-nitrocaproate was fixed at 5.0 mM and initial rate data were taken at each pH.

Dismutase activity dependence on pH. The pH profile of dismutase activity was done only with FADH since it displayed multifunctionality which was absent in the case of WADH. The octanal concentration was fixed at 1 mM and NAD^+ fixed at 10 mM and assays were performed at different pH.

Steady state kinetics:

Kinetics. Initial rates of NAD^+ reduction were followed at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) in a Varian Cary spectrophotometer at room temperature (25°C). The reaction mixtures (1.0 ml) contained the purified enzyme, a varied concentration of ethanol and NAD^+ in 0.2 M Tris-HCl buffer pH=9.0. In case of FADH, NAD^+ linked reactions used lower enzyme concentrations (5.0 mg/ml) than the NAD^+ reaction of WADH (10.0 mg/ml). Assay mixtures contained varied concentrations of NAD^+ (0.8-10 mM for both wheat and fish)

and varied concentrations of ethanol (0.01-0.1 M for fish and 10-1.00 mM for wheat) as substrates.

Product inhibition studies. The products of the reaction of alcohol dehydrogenase with ethanol and NAD^+ (NADH and acetaldehyde) were used to investigate the kinetic mechanism of the enzyme action through inhibition studies. Alcohol dehydrogenase is a bisubstrate enzyme and according to the experimental protocol, one substrate was held at a constant nonsaturating level, and the other was varied in a concentration range around its Michaelis constant. The products were added one at a time at several fixed concentrations including zero. The concentrations of NADH and CH_3CHO are given in the graphs.

The inhibition patterns were obtained by constructing double-reciprocal plots of initial velocity (v) versus substrate concentrations. The patterns were further analyzed by replotting the slopes and intercepts of the reciprocal plots versus the inhibitor concentrations.

Chemical modifications:

There is a chemical basis for every enzyme action, and its information can be obtained by chemical modifications of specific amino acid in that enzyme. The enzyme activity is measured during the modifications of specific residues, preferably active site residues, to identify the change in enzymatic activity with the modification. Purified alcohol dehydrogenase from fish liver was subjected to amidation. The carboxyl groups of this protein can be converted to amides by reaction with different amines (taurine, propylamine, ethylenediamine) promoted by a water soluble carbodiimide. The reaction mixtures consisted of EDC, FADH and the amines. The reaction was carried out at 0°C . Aliquots were removed at various times for activity measurements.

Results

Purification of alcohol dehydrogenases. The procedure developed for wheat and fish liver were very similar. The enzyme required DTT for stabilisation. The fish alcohol dehydrogenase (FADH) was purified to homogeneity from liver of Rainbow trout with 90.4 ± 25.1 fold purification (specific activity of 2.4 ± 0.72 units/mg) and a total recovery of $16.4 \pm 4.9\%$. The wheat alcohol dehydrogenase (WADH) was partially purified since affinity chromatography failed to remove last trace of contaminant proteins. Most of the purification steps described above are similar to that which was used to purify pickerel liver alcohol dehydrogenase, rat liver alcohol dehydrogenase and alcohol dehydrogenase from wheat germ by Tsai, *et al.* (1987)³³. The purification steps for WADH are summarised in Table 4. It represents changes in some parts from Tsai's method, mainly by using carboxymethyl cellulose ion exchangers (CM-52) instead of anionic exchanger (DE-52) in case of wheat enzyme, and also using phosphate buffer for wheat enzyme instead of Tris-HCl buffer. After anion/cation exchange and size exclusion chromatography, the enzyme was concentrated from sucrose instead of precipitation by 75% ammonium sulphate. The procedure started with ammonium sulphate fractionation at 30-70% for wheat and 30-80% for fish to be followed by an anion exchange chromatography. Initially DE-52 (anion exchanger) was used for both enzymes. It was noted that for the wheat enzyme, the purification and resolution was not good. Therefore CM-52 (cation exchanger) was used instead which gave fairly good results. For the fish enzyme the column was at first eluted with Tris-HCl buffer, pH 7.5, till the A_{280} of the eluate was same as that of the buffer. Then the column was again eluted with a linear NaCl gradient (0.0 - 0.5 M) for any other isozymes, if present. For both the fish and wheat enzyme, sephadex G-200 was replaced by sephacryl S-300 for a better resolution in size exclusion chromatography. The final step was affinity chromatography. The affinity chromatography did not work for the wheat enzyme because of its instability. The fish enzyme was purified to 90.4 ± 25.1 fold by affinity (NAD^+) chromatography. The enzyme was bound to the column and finally released by the use of NAD^+

gradient (0-3 mM NAD^+). The purified fish enzyme was electrophoretically homogenous. This was confirmed by a single band in SDS-polyacrylamide gel electrophoresis. In case of wheat enzyme, SDS-polyacrylamide gel electrophoresis was also performed but a single band was not obtained because affinity chromatography did not work.

Table 4: Summary of Purification: FishLiver (Rainbow Trout) and Wheat alcohol dehydrogenase

Step	Volume (ml.)	Activity (Units per ml)	Protein (mg/ml)	Specific activity (Units per mg)	Total Activity (Units)	Yield (Relative)	Purification (fold)
Enzyme Extract	154±18	0.22±0.03	6.85±0.23	0.03±0.01	33.9±6.07	100	1.0
	176±23	0.5±0.13	6.75±0.33	0.09±0.02	88±7.2	100	1.0
30% $(\text{NH}_4)_2\text{SO}_4$ Supernatant	141±16.8	0.1±0.024	6.9±0.25	0.02±0.01	14.1±1.6	42.97±9.1	0.75±0.47
	170±18	0.2±0.02	6.85±0.3	0.03±0.01	34±2.3	38.6±8.6	0.33±0.12
70% $(\text{NH}_4)_2\text{SO}_4$ after dialysis (conc.)	53±8.2	0.4±0.074	6.8±0.21	0.06±0.03	21.2±5.15	64.6±19.7	2.25±1.4
	42±9.5	0.85±0.11	7.0±0.28	0.12±0.01	35.7±3.4	40.6±17.2	1.33±0.4
DE (after conc. from sucrose)	15±1.8	0.91±0.06	6.6±0.15	0.138±0.1	13.7±1.87	41.8±9.5	5.18±4.31
	12.5±2.1	0.9±0.09	7.2±0.18	0.13±0.07	11.3±1.2	12.8±4.3	1.44±0.31
Sephacryl S-300	40±7.0	0.29±0.07	0.14±0.03	2.2±0.7	11.6±3.4	35.35±8.1	82.5±3.9
	14.1±6.4	0.55±0.08	0.1±0.2	5.4±0.2	7.8±2.3	8.9±7.1	60.25±0.4
Affinity	12.8±1.9	0.41±0.08	0.18±0.04	2.4±0.72	5.25±1.3	16.0±4.9	90.4±25.1
	—	—	—	—	—	—	—

The first row of each purification step belongs to FADH and the second row belongs to WADH. Three sets of experiments were performed from three enzyme preparations.

FIGURE 10 Elution profile of FADH from DE-52 anion exchanger. The column was eluted with Tris-HCl buffer, pH 7.5. Fractions were collected at a flow rate of 32 ml/hr and 2 ml fractions were collected. The ADH activity was measured as detailed in the experiments.

FIGURE 11 Sephacryl S 300 gel filtration chromatography of FADH in 10 mm Tris-HCl buffer pH 7.5.
4 ml/fraction were collected at a flow rate of 40 ml/hr.
y = Est is the esterase activity.

FIGURE 12 Elution profile of FADH from NAD^+ affinity column with a linear gradient of 0-10 mM NAD^+ in 10 mM Tris-HCl buffer, pH 7.5. 2ml/fraction were collected at a flow rate of 10 ml/hr.

FIGURE 13 Elution profile of WADH from CM-52. The column was eluted with 10 mM phosphate buffer, pH 7.0. Fractions were collected at a flow rate of 32 ml/hr and 2 ml fractions were collected. The ADH activity was measured as detailed in the experiments.

FIGURE 14 Sephacryl S 300 gel filtration chromatography of WADH in 10 mm phosphate buffer pH 7.0.
4 ml/fraction were collected at a flow rate of 40 ml/hr.

Molecular weight determination. The native molecular weight of FADH and WADH was determined by three methods. The best result was obtained from Sephacryl S-300 gel filtration. By plotting the log molecular weight of five standard proteins against their elution volumes, the mean value was measured to be $80,530 \pm 1302$ Da for FADH (Figure 15) and $200,321 \pm 6477$ Da for WADH (Figure 15).

The relative mobility values (R_f) of the standard proteins from SDS-polyacrylamide gel electrophoresis were plotted vs their molecular weights. The subunit molecular weight of the purified FADH (Figure 16) was measured from the plot to be $40,263 \pm 1506$ Da from the molecular weight standards (Plate 1). The subunit molecular weight of wheat was a little difficult to decide since the enzyme was not completely purified. But since the protein concentration was high it was concluded that the highly stained band was the enzyme (Plate 2). The subunit molecular weight was found to be $50,630 \pm 1894$ Da (Figure 16).

From the above estimations it was deduced that FADH consists of two subunits and WADH consists of four (3.97) subunits.

Multifunctionality. Multifunctionality of FADH (rainbow trout) are given in Table 5. It is known that horse liver alcohol dehydrogenase catalyses dehydrogenation, dismutation and esterolysis. Table 5 shows that the purified FADH displayed all these three multifunctionality, but the dismutase activity was lower than what was observed in HLADH.

Table 5: Multifunctional activities of alcohol dehydrogenases

Alcohol Dehydrogenases	Specific activity (Units/mg)			
	DHase ^d		DM ^{e,f}	ES ^{e,f}
	NAD	NADP		
Horse liver ^a	1.78	—	13.4	3.56
Purified yeast ^b	2.57	—	—	—
Rainbow trout liver	2.4	—	0.05	18.69
Wheat	5.4	5.2 ^d	—	—

a Data taken from reference 12.

b Data taken from reference 12.

c Specific activities are measured in Units (mg protein)⁻¹.

d Specific activity ($\times 10^{-4}$) is expressed in Units (mg protein)⁻¹.

e DM denotes dismutase and ES denotes esterase.

f Specific activities ($\times 10^{-3}$) are expressed in Units. (mg protein)⁻¹.

Table 6: Multifunctionalities of Fish Liver (Rainbow Trout) at different stages of purification.

	ADH ^a		DM ^b	ES ^b	ΨP^a
	NAD	NADP			
Enzyme Extract	0.03 $\pm$ 0.01	—	0.7749	1.443	0.021
30% (NH ₄) ₂ SO ₄	0.02 $\pm$ 0.01	—	0.135	2.68	0.024
70% (NH ₄) ₂ SO ₄ after dialysis	0.06 $\pm$ 0.03	—	0.610	47.4	0.017

Table 6: Multifunctionalities of Fish Liver (Rainbow Trout) at different stages of purification.

	ADH ^a		DM ^b	ES ^b	ΨP ^a
	NAD	NADP			
AEDE (after concentrating from sucrose)	0.138±0.1	—	0.21	67.5	—
Sephacryl S-300 (after concentrating from sucrose)	2.2±0.7	—	0.152	6.9	—
Affinity (after concentrating from sucrose)	2.4±0.72	—	0.05	18.69	—

a Specific activities are expressed in Units.(mg protein)⁻¹.

b Specific activities ($\times 10^{-3}$) are expressed in Units.(mg protein)⁻¹.

Chromatofocussing. The chromatofocussing of crude extract of FADH (80% ammonium sulphate fraction) on PBE 94 exchanger in the pH range 9-6 (Figure 17), revealed several protein bands (5 bands). Among these only one dehydrogenase bands were detected unlike Pickerel liver alcohol dehydrogenase. The PI for this peak was 8.2 and was characterised by high NAD⁺ activity. The total NAD⁺ linked activity recovered from chromatofocussing was 12.5%. Because of the low activity recovered, Chromatofocussing was not employed as a purification step.

Plate 1 SDS-polyacrylamide gel electrophoresis of FADH from rainbow trout.

From left to right: Lane 1: Enzyme extract (crude); Lane 2: 30% ammonium sulphate (supernatant); Lane 3: 70% ammonium sulphate (after dialysis); Lane 4: Concentrated enzyme (from sucrose) after AEDE; Lane 5: Concentrated enzyme (from sucrose) after Sephacryl-S300; Lane 6: Purified FADH; Lane 7: High molecular weight standards; Lane 8: Low molecular weight standards.

Plate 2

SDS-polyacrylamide gel electrophoresis of wheat alcohol dehydrogenase (WADH).

From left to right: Lane 1: Enzyme extract (crude); Lane 2: Enzyme extract (after centrifugation); Lane 3: 30% ammonium sulphate (supernatant); Lane 4: 70% ammonium sulphate (after dialysis); Lane 5: After CM-52 (concentrated from sucrose); Lane 6: After Sephacryl S300 (concentrated from sucrose); lane 7: After affinity (it did not work); Lane 8: Low molecular weight standards; Lane 9: High molecular weight standards

FIGURE 15 Determination of the molecular weight of purified FADH and WADH by sephacryl S-300 gel filtration. Standard proteins are: Catalase (232,000 Da), bakers yeast AlcDH (150,000 Da), horse liver ADH (80,000 Da), Bovine Serum Albumin (132,000 Da) and Pepsin from Hog stomach (35,000). The flow rate was 40 ml/hr.

The eluting volume of FADH (rainbow trout) was 218 ml (average of three set of experiments).

The eluting volume of WADH was 126 ml (average of three set of experiments)

FIGURE 16 Determination of subunit molecular weight of FADH and WADH by SDS-polyacrylamide gel electrophoresis.

The Rf of FADH is 4.2 and that of WADH is 3.7. These are average values from three set of experiments.

FIGURE 17 Chromatofocussing of FADH on PBE 94 in the pH range 9-6.

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 enzyme³⁴. The active sites of the enzymes are composed of ionizable groups which must be in the proper ionic form to maintain the conformation of the active site, bind the substrate, or catalyse the reaction. The objective of the pH studies is to identify those groups that play important roles in the enzymic reaction³⁵.

The effect of pH on dehydrogenase activities of FADH and WADH were investigated in this study. Studies were investigated over the pH range of 5.0-11.0.

Effect of pH on NAD⁺ linked activity of FADH. Initial rate velocity assays of NAD⁺ linked activity of FADH were measured with the variation in pH from 5.0 to 11.0. Figure 18 shows the pH velocity profile of NAD⁺ linked activity of FADH (rainbow trout). A biphasic pH plot was obtained when specific activity is plotted against pH. This may indicate that more than one ionic form of the enzyme are catalytically active. Takahashi et al.³⁶ in their study on the pH velocity profiles 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 a group with $pK_{a2} = 9.5$. A charge relay system may function at this nonphysiological alkaline region. The groups involved can be a tyrosine, analogous to tyrosine 311 in glyceraldehyde phosphate dehydrogenase (GAPDH). At physiological pH, a general base (most probably histidine analogous to histidine 176 in GAPDH) catalysed 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. The biphasic pH velocity plot of NAD⁺ linked activity of FADH obtained suggests the independent presence of more than one catalytically active enzyme form. Two ionizable species can be iden-

tified from the pH plot, one with pK 7.0 and the other with pK 9.5.

FIGURE 18 The pH velocity profile of NAD^+ linked activity of FADH.

Effect of pH on the esterase activity of FADH. The pH dependence of the esterase activity was also studied by single point assays (Figure 19). Corrected initial rate data were obtained at 5 mM concentration of p-nitrophenylcaprylate. The pH dependence of esterase activity showed a maximum at pH 7.0 and enzyme activity of 0.003685 Units/mg. There was another maximum at pH 8.12 with activity of 0.034 Units/mg. pH-Velocity dependence could not be studied above pH 9.0 because initial rate data could not be obtained at that pH (above 9.0) due to high rates of spontaneous hydrolysis of the ester.

pH Dependence of the dismutase activity of FADH. The FADH from rainbow trout had very low activity and so detailed pH studies could not be performed. Initial rate data was obtained at substrate concentration of 1.0 mM at different pH values. In the pH velocity profile (Figure 20), a primary maximum was at acidic pH 5.5, with a very low enzyme activity of 0.54×10^{-3} Units/mg. The second maximum had a higher enzyme activity of 2.8×10^{-3} Units/mg and occurred at pH 8.91.

Effect of pH on NAD⁺-linked activity of WADH. The effect of pH on WADH was also studied over a pH range of 5.0 to 11.0. As the pH was increased, activity of the enzyme increased to a maximum between pH 8.5 to 9.5. When the pH was increased further, a decrease in enzyme activity was noted. the pK values could not be assigned with this data. The pH dependence of NAD⁺ activity of WADH was a bell shaped curve as shown in Figure 21.

FIGURE 19 pH velocity profile of esterase activity of FADH.

FIGURE 20 pH velocity profile of dismutase activity.

FIGURE 21 pH Velocity plot of WADH.

Steady state kinetics studies of FADH and WADH. Initial velocity experiments are performed to elucidate kinetic characteristics of enzyme mechanisms. Distinction between ping-pong and sequential mechanisms can be made by inspection of initial rate plots. In sequential class of bisubstrate enzyme catalysed reactions, substrates must bind in compulsory order prior to catalysis. For converging reciprocal plots (sequential mechanism), the general expression for the initial velocity is given by the bisubstrate equation (Cleland, 1963)³⁷ shown in:

$$v = \frac{V_{\max} [A] [B]}{[A] [B] + K_b [A] + K_a [B] + K_{ia} K_b} \quad (\text{Eq. 2})$$

A and B are substrates; $V_{\max}$ 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.

The inverse of the Equation 2 gives:

$$\frac{1}{v} = \left[1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ia} K_b}{[A] [B]} \right] \frac{1}{V_{\max}} \quad (\text{Eq. 3})$$

The plots, $1/v$ against $1/[NAD^+]$ at a fixed concentration of ethanol will be linear with

$$\text{slope} = \frac{1}{V_{\max}} \left(K_a + \frac{K_{ia} K_b}{[B]} \right) \quad (\text{Eq. 4})$$

and

$$\text{intercept} = \frac{1}{V_{\max}} \left(1 + \frac{K_b}{[B]} \right) \quad (\text{Eq. 5})$$

The secondary plots of the slopes and intercepts against $1/[\text{ethanol}]$ will give V , K_{ia} , K_a and K_b .

There are four different kind of bisubstrate mechanisms that gives the above steady state rate Equation 2. They are (i) Ordered, with one ternary complex, (ii) Ordered with two ternary complexes, (iii) Theorell-Chance mechanism and (iv) Rapid equilibrium random mechanism.

All of these above mechanisms have different product inhibition patterns. Initial velocities in the asymptotic region follow Michaelian kinetics and were analysed according to Equation 2. Explanation of the kinetic parameters and their determination are given in the discussion part of this thesis.

Initial velocities were measured at different substrate concentrations for the NAD^+ linked activities for both the enzymes. Initial rate data were analysed graphically by double-reciprocal plots. Secondary plots were used to obtain the kinetic parameters. The kinetic parameters obtained for both the enzymes are listed in Table 7.

Table 7: Kinetic parameters of different enzymes

ADH	$V_{\max}$ mM/min	K_a mM	K_b mM	K_{ia} mM	E_t μM	$V_{\max}/E_t$ min^{-1}
Horse Liver ^a	0.0813	0.335	0.0687	0.263	0.0436	1.8×10^3
Fish liver (Rainbow trout) ^b	0.514 ± 0.2	1.11 ± 0.25	0.09 ± 0.06	0.095 ± 0.04	7.8	65.8

Table 7: Kinetic parameters of different enzymes (Continued)

ADH	$V_{\max}$ mM/min	K_a mM	K_b mM	K_{ia} mM	E_t μM	$V_{\max}/E_t$ min^{-1}
Fish Liver ^a (Pickerel)	0.192	0.164	0.565	0.190	3.33	57.8
Wheat ^b	2.72 $\pm$ 1.1	66.68 $\pm$ 5.5	1.926 $\pm$ 0.3	98.77 $\pm$ 7.4	4.42	615
Ta. Brockii ^a	1.67	46.8	1.1	92.1	2.34	714

a Data taken from reference 12.

b Three sets of experiments were done from each of three enzyme preparations and the best fitting lines were taken.

Fish Liver Alcohol dehydrogenase (Rainbow trout). FADH used NAD^+ as the coenzyme and its mechanism of action was investigated kinetically. Curves for the velocity dependence on varied coenzyme concentrations at fixed ethanol concentration (100 mM) were prepared. The velocity plot followed a normal hyperbolic pattern. The maximum activity was achieved at a NAD^+ concentration of 0.5 mM. The activity plateau was maintained upto a NAD^+ concentration of 14.5 mM with 6.0% inhibition in the activity. When the NAD^+ concentration was increased to 20 mM, an inhibition of 20.9% was observed (Figure 22).

The kinetic studies of this two substrate reaction was studied over a broad range of ethanol and NAD^+ concentrations. Double reciprocal plots for NAD^+ linked activities are shown in Figure 24. It can be seen that these plots are non-linear. The plot passes through a minimum and curves up as it approaches the $1/v$ axis. The curvature of this plot however is not much. The asymptote part of this plot was taken and plotted again to obtain a best

fitting straight line. Figure 52 in Appendix 1 shows the error bars of Figure 24.

FIGURE 22 Saturation plots of NAD^+ linked activity of FADH.
The assays were done at pH 9.0.

From the nonlinear nature of the double reciprocal plot of NAD^+ it can be suggested that substrate inhibition of the enzyme is taking place when concentration of NAD^+ is high. Inhibition was 9.1% at 100.0 mM ethanol, 20.0 mM NAD^+ , compared to 25% inhibition with the same concentration of NAD^+ but 20 mM ethanol. It showed that even the highest concentration of coenzyme resulted in finite rate and partial inhibition. It can be concluded that high coenzyme concentrations are giving rise to a different 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 takes the general form of a rational polynomial as in Equation 6:

$$v = \frac{\sum_{i=1} \alpha_i S^i}{\sum_{i=1} \beta_i S^i} \quad (\text{Eq. 6})$$

Where S is NAD^+ concentration α_i and β_i are kinetic coefficients related to rate constants and alcohol concentration for NAD^+ of the i th degree in numerator and denominator respectively. According to Bardsley and Childs's³⁸ diagnostic plots (Figure 23), the rate equation was analyzed graphically for the $n:m$ function. The n and m depend on the maximum number of enzyme species reacting with the substrate, and represent the highest degree of substrate terms in the numerator and denominator. Plots of $v[\text{NAD}^+]^\lambda$ (λ =integer) versus $[\text{NAD}^+]$, gave three different kind of graphs. The plots were compared and it was seen that for $\lambda=0$ ($m=n$), the plot was a plateau and

$$\lim_{S \rightarrow \infty} (V_{m=n}) = \text{constant} \quad (\text{Eq. 7})$$

The intercepts and slopes of the double reciprocal plot (Figure 24) were next plotted against $1/\text{Ethanol}$ concentration to give the secondary plots (Figure 25) and these plots

yielded the apparent Michaelis constants listed in Table 7.

FIGURE 23 Diagnostic plots of NAD⁺ linked activities of FADH.

FIGURE 24 Double reciprocal plot of NAD⁺ linked activity of FADH in Tris-HCl buffer, pH 9.0.

Initial rates were measured at varied NAD⁺ concentration at fixed ethanol concentration. Ethanol concentration in mM are: 100 mM (line 1); 80 mM (line 2); 40 mM (line 3); 20 mM (line 4).

Three sets of experiments were done from each of three enzyme preparations.

FIGURE 25 Secondary plots of intercepts and slopes vs $1/[\text{EtOH}]$, pH 9.0.

FIGURE 26 Saturation plots of NAD^+ linked activity of WADH.
The assays were done at pH 9.0.

FIGURE 27 Diagnostic plots of NAD⁺ linked activities of WADH.

FIGURE 28 Double reciprocal plot of NAD^+ linked activity of WADH in phosphate buffer, pH 7.4.

Initial rates were measured at varied NAD^+ concentration at fixed ethanol concentration. Ethanol concentration in mM are: 10.0 mM (line 1); 8.0 mM (line 2); 6.0 mM (line 3); 4.0 mM (line 4), 1.0 mM (line 5).

Three sets of experiments were done from each of three enzyme preparations.

FIGURE 29 Secondary plots of intercepts and slopes vs $1/[EtOH]$, pH 9.0.
 slope line: $y = 69.992 x + 24.525$
 intercept line: $y = 0.7086 x + 0.3678$

Wheat alcohol dehydrogenase.

Wheat alcohol dehydrogenase on the other hand used both NAD^+ and NADP^+ as the coenzyme. The rate with NADP^+ being very small it was ignored and the kinetics analysis was done mainly taking NAD^+ as the coenzyme. In this case however, a very small inhibition was observed in the velocity plot of NAD^+ linked activity (Figure 26) against varying NAD^+ concentration at fixed ethanol concentration of 100 mM, at very low NAD^+ concentration. However at high NAD^+ concentration no inhibition was observed. This indicates that the rate is highly dependent on NAD^+ concentration. The NAD^+ linked reaction with ethanol of WADH displayed sequential mechanism represented by linear converging lines at low $1/\text{NAD}^+$ values. The double reciprocal plots obtained with NAD^+ as coenzyme with ethanol as the second substrate was linear with increasing $1/v$ values at low $1/\text{NAD}^+$ (from 0 to 0.5 mM^{-1}), then $1/v$ values decreased linearly and finally $1/v$ values increased linearly at high $1/\text{NAD}^+$ values (Figure 28). This shows that there was an inhibition when NAD^+ concentration varied from 0.8 mM to 2 mM. This is also evident in Figure 26 which shows that at NAD^+ concentration of around 0.5 mM to 2 mM the plot is a small plateau. The plot was linearized at low $1/\text{NAD}^+$ (high NAD^+) and also at high NAD^+ . All the calculations were done with the asymptote part of the plots (the linear part at low $1/\text{NAD}^+$) by obtaining best fitting straight lines through the points (Figure 29). The linear part at high $1/\text{NAD}^+$ concentration was not considered for obtaining the kinetic parameters since it did not give desirable results. Figure 53 in Appendix 1 shows the error bars of the double reciprocal plots of NAD^+ linked activity of WADH (Figure 28). The Bardsley and Childs³⁸ diagnostic plots for NAD^+ -linked activity of WADH is given in Figure 27. In this plot a positive horizontal asymptote was not observed as $[\text{NAD}^+]$ was increased. This indicates that the n:m function is 1:1 and random mechanism is not involved in this enzyme.

Product inhibition. Originally product inhibition was investigated by Alberty³⁹ to differentiate between various two substrate mechanisms⁴⁰. Rudolph⁴¹ described product inhibition for four basic bi-substrate mechanism. In general there are three different kind of inhibitions 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 gives competitive, uncompetitive or non-competitive inhibition. The inhibition patterns are predicted by rate equations of two substrate systems and are given in Table 8.

Table 8: Product inhibition patterns for Bireactant Mechanisms

Mechanism	Product	Varied Substrate			
		A		B	
		Unsaturated with B	Saturated with B	Unsaturated with A	Saturated 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 ordered BiBi	P	.(U)	-	.(C)	.(C)
	Q	C(C)	.(C)	C(N)	-
Rapid Equilibrium random BiBi	P	C(N)	-	C(C)	.(C)
	Q	C(C)	.(C)	C(N)	-

Table 8: Product inhibition patterns for Bireactant Mechanisms (Continued)

Mechanism	Product	Varied Substrate			
		A		B	
		Unsaturated with B	Saturated with B	Unsaturated with A	Saturated with A
Steady state Random BiBi	P	N ^f	N ^g	N ^f	N ^g
	Q	N ^f	N ^g	N ^f	N ^g

Table adapted from Rudolph⁴¹ and Segel⁴².

a. The abbreviations are: C, competitive; N, noncompetitive; U, uncompetitive; and NL, nonlinear. The 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 intercepts replots are hyperbolic functions of product inhibitor concentration, while slopes are more complex functions of inhibitor concentration.

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

Fish liver Alcohol dehydrogenase (Rainbow trout). The nonlinearity in the Lineweaver-Burk plot of NAD⁺ linked activity of FADH indicates a randomness in the NAD⁺ activity. Product inhibition studies support the proposed mechanism of a Steady State Random BiBi mechanism. From Bradsley and Childs diagnostic plot it was suggested that NAD⁺ activity might operate through a steady state random mechanism. The steady state random BiBi system yield a very complicated velocity equation with thirtyseven new

terms in the denominator, plus those of the ordered BiBi equation.

The results obtained for the product inhibition studies are represented in Figure 30, Figure 31, Figure 32 and Figure 33. Inhibition of the NAD^+ linked reaction by NADH was noncompetitive with respect to both NAD^+ and ethanol. Acetaldehyde was also a noncompetitive inhibitor with respect to NAD^+ and ethanol. The double reciprocal plots (Figure 30, Figure 31, Figure 32 and Figure 33) yielded linear plots but slopes and intercept replots were hyperbolic (Figure 34 and Figure 35).

FIGURE 30 (Primary plot 1) Double reciprocal plot of product (NADH) inhibition of NAD⁺ linked activity of FADH, at unsaturated ethanol (5.5 mM).

NAD⁺ concentration was varied in presence of NADH as an inhibitor. The NADH concentrations were: 0 μM (line 1); 1.0 μM (line 2); 2.5 μM (line 3); 4.0 μM (line 4); 8.0 μM (line 5). Assays were at room temperature, pH 9.0 in Tris-HCl buffer.

One set of experiment was done from each of three enzyme preparations.

FIGURE 31 (Primary plot 2) Double reciprocal plot of product (NADH) inhibition of NAD^+ linked activity of FADH, at unsaturated NAD^+ (5.5 mM). Ethanol concentration was varied in presence of NADH as an inhibitor. The NADH concentrations were: 0 μ M (line 1); 1.0 μ M (line 2); 2.5 μ M (line 3); 4.0 μ M (line 4); 8.0 μ M (line 5). Assays were at room temperature, pH 9.0 in Tris-HCl buffer.

One set of experiment was done from each of three enzyme preparations.

FIGURE 32 (Primary plot 3) Double reciprocal plot of product (acetaldehyde) inhibition of NAD^+ linked activity of FADH, at unsaturated ethanol (5.5 mM).

NAD^+ concentration was varied in presence of acetaldehyde as an inhibitor. The acetaldehyde concentrations were: 0 mM (line 1); 0.015 mM (line 2); 0.025 mM (line 3); 0.05 mM (line 4); 0.075 mM (line 5). Assays were at room temperature, pH 9.0 in Tris-HCl buffer.

One set of experiment was done from each of three enzyme preparations.

FIGURE 33 (Primary plot 4) Double reciprocal plot of product (acetaldehyde) inhibition of NAD^+ linked activity of FADH, at unsaturated NAD^+ (5.5 mM).

Ethanol concentration was varied in presence of acetaldehyde as an inhibitor. The acetaldehyde concentrations were: 0 mM (line 1); 0.015 mM (line 2); 0.025 mM (line 3); 0.05 mM (line 4); 0.075 mM (line 5). Assays were at room temperature, pH 9.0 in Tris-HCl buffer.

One set of experiment was done from each of three enzyme preparations.

FIGURE 34 Secondary plots for product inhibition studies of NAD^+ linked activity of FADH.
Plots of slopes and intercepts of graphs in Figure 30 (Figure 34a) and Figure 31 (Figure 34b)

FIGURE 35 Secondary plots for product inhibition studies of NAD^+ linked activity of FADH.

Plots of slopes and intercepts of graphs in Figure 32 (Figure 35a) and Figure 33 (Figure 35b)

This type of inhibition is known as “slope-hyperbolic, intercept-hyperbolic non-competitive inhibition”. This can be represented by Scheme 8.

SCHEME 8 Hyperbolic non-competitive inhibition

Here α and β are factors which influence binding and reaction rates.

A plot of $1/\Delta_{\text{slope}}$ versus $1/[I]$ and $1/\Delta_{\text{intercept}}$ versus $1/[I]$ converts the relationship to a straight line⁴³, where $\Delta_{\text{slope}} = \text{slope}_I - \text{slope}_0$ and slope_I is the slope at inhibitor concentration I , and slope_0 is the slope when $I=0$, and the same for the intercept (Figure 36, Figure 37, Figure 38 and Figure 39).

The curves are given by Equation 8 and Equation 9.

$$1/\Delta_{\text{intercept}} = \frac{\alpha K_i V_{\text{max}}}{(1-\beta)} \cdot \frac{1}{[I]} + \frac{\beta V_{\text{max}}}{(1-\beta)} \quad (\text{Eq. 8})$$

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

$$1/\Delta_{\text{slope}} = \frac{\alpha K_i' V_{\text{max}}}{K_s(\alpha - \beta)} \cdot \frac{1}{[I]} + \frac{\beta V_{\text{max}}}{K_s(\alpha - \beta)} \quad (\text{Eq. 9})$$

This plot is linear with a slope of $\alpha K_i' V_{\text{max}}/K_s(\alpha - \beta)$ and an intercept of $\beta V_{\text{max}}/K_s(\alpha - \beta)$.

These plots are shown in Figure 36, Figure 37, Figure 38 and Figure 39. α , β , K_i and K_i' were calculated from the plots and are given in Table 9.

It was seen that in the inhibition by NADH with respect to ethanol and by acetaldehyde with respect to NAD^+ , α values were less than unity. This shows that the inhibitor converts the enzyme to an EI form with an increased affinity for the substrate. It also shows that 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 an α value greater than unity. This indicates that these products as inhibitors decrease the affinity of the enzyme for the substrates. The β values in all cases are less than unity indicating that these products as inhibitors decreased the V_{max} of the reaction.

Table 9: Product inhibition constants of NAD^+ - linked activity of FADH

Product	Varied substrate							
	NAD^{+a}				Ethanol ^b			
	K_i	K_i'	α	β	K_i	K_i'	α	β
NADH	2.23	9.38	1.7	0.04	3.6	0.19	0.6	0.11
Acetaldehyde	22.0	0.05	0.19	0.09	0.02	0.33	2.06	0.06

In the above table inhibition constants are in μM . All gave intercept hyperbolic,

slope-hyperbolic non-competitive inhibition. K_i , K_i' , α and β were determined from $1/\Delta_{intercept}$ and $1/\Delta_{slope}$ plots. One set of experiment was done from each of three enzyme preparations and best fitting lines were taken.

a Here NAD^+ was varied and ethanol concentration was fixed.

b Here ethanol was the varied substrate and NAD^+ was fixed.

FIGURE 36 Product inhibition studies of NAD^+ linked activity of FADH. The $1/\Delta_{intercepts}$ and $1/\Delta_{slopes}$ obtained from primary plot 1 (Figure 30) are plotted versus $1/[NADH]$.

FIGURE 37 Product inhibition studies of NAD^+ linked activity of FADH. The $1/\Delta_{\text{intercepts}}$ and $1/\Delta_{\text{slopes}}$ obtained from primary plot 2 (Figure 31) are plotted versus $1/[NADH]$.

FIGURE 38 Product inhibition studies of NAD^+ linked activity of FADH. The $1/\Delta_{\text{intercepts}}$ and $1/\Delta_{\text{slopes}}$ obtained from primary plot 3 (Figure 32) are plotted versus $1/[\text{acetaldehyde}]$.

FIGURE 39 Product inhibition studies of NAD^+ linked activity of FADH. The $1/\Delta_{\text{intercepts}}$ and $1/\Delta_{\text{slopes}}$ obtained from primary plot 4 (Figure 33) are plotted versus $1/[\text{acetaldehyde}]$.

FIGURE 40 Double reciprocal plot of product (NADH) inhibition of NAD⁺ linked activity of WADH, at unsaturated ethanol (10 mM). NAD⁺ concentration was varied in presence of NADH as an inhibitor. The NADH concentrations were: 0 μ M (line 1); 1.0 μ M (line 2); 2.5 μ M (line 3); 4.0 μ M (line 4); 8.0 μ M (line 5). Assays were at room temperature, pH 9.0 in Tris-HCl buffer.

One set of experiment was done from each of three enzyme preparations.

FIGURE 41 Double reciprocal plot of product (NADH) inhibition of NAD^+ linked activity of WADH, at unsaturated NAD^+ (0.1 mM). Ethanol concentration was varied in presence of NADH as an inhibitor. The NADH concentrations were: 0 μ M (line 1); 1.0 μ M (line 2); 2.5 μ M (line 3); 4.0 μ M (line 4); 8.0 μ M (line 5). Assays were at room temperature, pH 7.4 in phosphate buffer.

FIGURE 42 Double reciprocal plot of product (acetaldehyde) inhibition of NAD^+ linked activity of WADH, at unsaturated ethanol (10 mM).

NAD^+ concentration was varied in presence of acetaldehyde as an inhibitor. The acetaldehyde concentrations were: 0 mM (line 1); 0.015 mM (line 2); 0.025 mM (line 3); 0.05 mM (line 4); 0.075 mM (line 5). Assays were at room temperature, pH 7.4 in phosphate buffer.

One set of experiment was done from each of three enzyme preparations.

FIGURE 43 Double reciprocal plot of product (acetaldehyde) inhibition of NAD^+ linked activity of WADH, at unsaturated fixed NAD^+ (0.1 mM). Ethanol concentration was varied in presence of acetaldehyde as an inhibitor. The acetaldehyde concentrations were: 0 mM (line 1); 0.015 mM (line 2); 0.025 mM (line 3); 0.05 mM (line 4); 0.075 mM (line 5). Assays were at room temperature, pH 7.4 in phosphate buffer.

One set of experiment was done from each of three enzyme preparations.

FIGURE 44 Secondary plots for product inhibition studies of NAD^+ linked activity of WADH.
Plots of slopes and intercepts of Figure 40 (a) and Figure 41 (b).

FIGURE 45 Secondary plots for product inhibition studies of NAD^+ linked activity of WADH.
Plots of slopes and intercepts of Figure 42 (a) and Figure 43 (b).

Wheat alcohol dehydrogenase.

The product inhibition patterns obtained for NAD⁺ linked activity of wheat alcohol dehydrogenase is consistent with an Ordered BiBi mechanism. Different forms of double reciprocal bisubstrate rate equation can be obtained depending on the variable substrate, fixed substrate and the product that is acting as an inhibitor. The rate equation in presence of product P, [Q] = 0, variable A concentration, and fixed B concentration is given by Equation 10:

$$\frac{1}{v} = \frac{K_a}{V_{\max}} \left[1 + \frac{K_{ia}K_b}{K_a[B]} \left(1 + \frac{K_q[P]}{K_{iq}K_p} \right) \right] \frac{1}{[A]} + \frac{1}{V_{\max}} \left[1 - \frac{K_b}{[B]} \left(1 - \frac{K_q[P]}{K_{iq}K_p} \right) \frac{[P]}{K_{ip}} \right] \quad (\text{Eq. 10})$$

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.

When [B] is varied and A is fixed, the equation is as follows:

$$\frac{1}{v} = \frac{K_b}{V_{\max}} \left[\left(1 + \frac{K_{ia}}{[A]} \right) \left(1 - \frac{K_q[P]}{K_{iq}K_p} \right) \right] \frac{1}{[B]} + \frac{1}{V_{\max}} \left(1 + \frac{K_a}{[A]} + \frac{[P]}{K_{ip}} \right) \quad (\text{Eq. 11})$$

Here P behaves as 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:

$$\frac{1}{v} = \frac{K_a}{V_{\max}} \left[\left(1 + \frac{[Q]}{K_{iq}} \right) \left(1 + \frac{K_{ia}K_b}{K_a[B]} \right) \right] \frac{1}{[A]} + \frac{1}{V_{\max}} \left(1 + \frac{K_b}{[B]} \right) \quad (\text{Eq. 12})$$

From Equation 12 it can be seen that Q acts as a competitive inhibitor with respect to A at all B concentrations. However when [B] is the varied substrate and [A] is fixed, the equation becomes as in Equation 13:

$$\frac{1}{v} = \frac{K_b}{V_{\max}} \left[1 + \frac{K_{ia}}{[A]} \left(1 + \frac{[Q]}{K_{iq}} \right) \right] \frac{1}{[B]} + \frac{1}{V_{\max}} \left[1 - \frac{K_a}{[A]} + \left(1 + \frac{[Q]}{K_{iq}} \right) \right] \quad (\text{Eq. 13})$$

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, but [A] must be large enough compared to $K_{ia}[Q]/K_{iq}$, and $K_a[Q]/K_{iq}$ for these Q terms to disappear from the slope and intercept factors.

The product inhibition patterns as summarized in Table 8 would permit the establishment of the order of substrate addition and product release.

Three simple types of enzyme inhibitors are known. Assuming that only a single substrate is involved in the reaction, and only one type of inhibition is present at any time, the effects of these inhibitors are shown below.

The inhibitor I seems to have two possible sites of interaction with the enzyme. The velocity equation accommodating these two sites in reciprocal form is:

$$\frac{1}{v} = \frac{K_s (1 + [I]/K_{is}) + [S] (1 + [I]/K_{ii})}{V_{\max} [S]} \quad (\text{Eq. 14})$$

where I, S, K_{is} and K_{ii} are the inhibitor, varying substrate, slope effect of the formation of dead-end enzyme inhibitor complex, and K_{ii} is the intercept effect.

The plot of $1/v$ versus $1/[S]$ yields a straight line with

$$\text{intercept} = \frac{1}{V_{\max}} + \frac{[I]}{K_{ii} V_{\max}} \quad (\text{Eq. 15})$$

and

$$\text{slope} = \frac{K_s}{V_{\max}} + \frac{K_s [I]}{K_{is} V_{\max}} \quad (\text{Eq. 16})$$

A secondary plot of intercepts versus the concentration of inhibitor ($[I]$) gives K_{ii} and the secondary plot of slope versus $[I]$ gives K_{is} .

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 K_{is} is obtained. Uncompetitive inhibition occurs when the inhibitor interacts with the binary complex only, so only the intercept is affected, and K_{ii} is obtained. Noncompetitive 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 intercept being affected. In this case convergence to the $1/v$ axis is not expected. The product inhibition patterns as summarized in Table 8 would permit to establish the order of substrate addition and product release. Product inhibition

studies that were performed for the NAD^+ linked activity of WADH are presented in Figure 40, Figure 41, Figure 42 and Figure 43. The corresponding figure legends give the details concerning concentration of the fixed substrate, varied substrate and product under investigation. Secondary plots of the above plots are given in Figure 44 and Figure 45. Inhibition constants are listed in Table 10. NADH was a competitive inhibitor with respect to NAD^+ (Figure 40) and noncompetitive with respect to ethanol (Figure 41). Linear noncompetitive inhibition was observed by acetaldehyde when both NAD^+ and ethanol was the varied substrate (Figure 42 and Figure 43).

Table 10: Inhibition Constants of WADH

Product	Varied substrate			
	NAD^{+a}		EtOH^b	
	K_{ii}	K_{is}	K_{ii}	K_{is}
NADH	—	0.01625	0.28	0.05
Acetaldehyde	0.55	0.32	0.34	0.175

Inhibition constants are given in mM. When both constants are obtained a noncompetitive inhibition is observed. If only K_{ii} is obtained then uncompetitive inhibition is observed. Only K_{is} is obtained if the inhibition is competitive. Here only one set of experiment was done from three enzyme preparations. Average error calculated was $K_{ii} \pm 30\%$ and $K_{is} = 34\%$.

a At varied NAD^+ concentration, ethanol concentration was fixed at 10 mM.

b when CH_3CHO was varied, NAD^+ concentration was fixed at 0.1 mM.

Enzyme concentration in all experiments were 0.18 mg/ml. Product concentration for NADH were 0-0.1 mM, and for CH_3CHO it was 0-1.0 mM.

FIGURE 46 Absorption spectrum of purified FADH from rainbow trout at 25°C. Enzyme concentration was 20 $\mu\text{g/ml}$.

FIGURE 47 Absorption spectrum of partially purified WADH from rainbow trout at 25°C.
Enzyme concentration was 25 $\mu\text{g/ml}$.

Absorption spectra of the two enzymes. The absorption spectra of the two enzymes were obtained. Both the FADH (Figure 46) and the WADH (Figure 47) had an absorption maximum at 280 nm.

Chemical modifications. Asp-223 is an important residue in alcohol dehydrogenase. One of the carboxyl oxygens of Asp-223 is involved in hydrogen bond to O-2' hydroxyl of the adenosine ribose. The charge repulsion between the anionic 2' phosphate group of NADP⁺ and the carboxyl oxygen of Asp-223 weakens the binding of this coenzyme and explains the high degree of NAD⁺ specificity in these enzymes.

The interaction of the coenzymes, NAD⁺ or NADP⁺ with the carboxyl residue of Asp-223 in alcohol dehydrogenases, can be studied by means of chemical modification of the carboxyl groups with resultant changes in coenzyme specificity. Water soluble carbodiimide (WSC) reagents were first used for this kind of modification. WSC forms an O-acylisourea intermediate. This intermediate is highly reactive and there can be two possible pathways. First in the presence of an appropriate nucleophile, O-acylisourea condenses to form the corresponding amides and urea. The second possibility is that the O-acylisourea rearranges to an N-acylurea via an intramolecular acyl transfer as shown in Scheme 9.

SCHEME 9 The two possible pathways of the dissociation of the O-acylisourea intermediate.

In this study all reactions were performed in the presence of 1-ethyl, N'-(3-dimethylaminopropyl)-carbodiimide (EDC):

Just as the WSC can be varied, the amine component can also be varied. The character of the product will vary considerably depending on the amine employed. Hence the ionic character of the protein can be altered as shown in the following equations (Scheme 10):

SCHEME 10 Reaction of the carboxyl group of protein with different amines in present of EDC.

Another amine nucleophile, methylamine was also used.

With the various nucleophiles, the negative charge on the targeted carboxyl groups can be kept, removed or replaced by a positive charge. Any change in the $\text{NAD}^+/\text{NADP}^+$ specificity of enzymes studied can then be monitored. In this manner it becomes possible to determine the importance of carboxyls to the biological activity by monitoring the changes in the enzymic activity.

HLADH and YADH was used as a model in preliminary experiments in this laboratory. These results were compared with the observed result for fish liver alcohol dehydrogenase and wheat alcohol dehydrogenase.

In modification of FADH, EDC was used and the nucleophilic agent was also var-

ied. Hence the nucleophile groups varied in size and charge, which resulted in different influences on the enzyme activity. The effect of four different nucleophiles under comparable conditions on NAD⁺ linked activity of FADH is given in Figure 48. There was a large initial inactivation for all the reactions which stabilised after fifteen minutes. Among these five reactions, highest inactivation was observed in the presence of ethylenediamine cationic amine. The activity dropped to 3.4% of original activity after 30 minutes of reaction in presence of this amine. Anionic amine, taurine had the least inactivation, which was followed by methylamine and propylamine.

Effect of the same nucleophiles on NADP⁺ linked activity of FADH is presented in Figure 49. All the amines used enhanced NADP⁺ linked activity. The enhancement in the NADP⁺ linked activity by the amines differed in the extent but not in trend of activation. The greatest activation was achieved by methyl amine. A 12.6 fold increase in activity was observed after 6 minutes of modification, which was followed by a decline in activity and a final 9.6 fold activation at 45 minutes. The increase in activity of n-propylamine was lower. A 9.6 fold increase in activity was observed in presence of propylamine which was followed by a sudden decrease in activity to 0.13 fold. Taurine also induced an increase in NADP⁺ linked activity which is unlikely. A 9.6 fold increase was observed at 6 minutes which was followed by a decline in enhanced activity. Ethylenediamine enhanced the NADP⁺ linked activity by 3.9 fold at 6 minutes. The activity was continuously increased to reach a 7.7 fold enhancement in 45 minutes. An increase in the NADP⁺ linked activity was also observed by EDC alone. In the absence of any amine, a 1.4 fold increase in NADP⁺ activity by EDC was observed.

FIGURE 48 Amidation of NAD^+ linked activity of FADH in presence of various nucleophiles.

FIGURE 49 Amidation of NADP⁺ linked activity of FADH in presence of various nucleophiles.

Discussion

The substrate and coenzyme specificity of different alcohol dehydrogenases vary from one source to another. As mentioned earlier ADH occurs in a variety of organisms. In higher organisms, isozymes frequently have different distributions⁴⁴. Mammalian ADH is found mainly in liver, but it also occurs in other organs outside the liver^{45,46}.

In this study, alcohol dehydrogenase from two sources (plant and fish liver) has been partially purified and characterized to various degrees of purity.

The purification procedure in this study was developed initially in this laboratory (Tsai et. al.³³) for rat liver alcohol dehydrogenase. A few modifications to this method were made while purifying these two enzymes. The procedure for both wheat and fish alcohol dehydrogenase consists of ammonium sulphate fractionation, anion exchange chromatography, gel filtration and affinity chromatography. Bauermeister and Sargent⁵ isolated alcohol dehydrogenase from the liver and intestinal caecum of rainbow trout (*Salmo gairdnerii*). Their isolation procedures consisted of sequential chromatographies over DEAE cellulose and on Blue Sepharose CL-6B. The FADH in this study was isolated from rainbow trout livers. It was obtained as homogenous preparation (plate 1) only when all the purification steps were performed. The first chromatographic step was DE-52 anion exchange (Figure 10). The pH of the purification buffer (7.5) was lower than the pI of the enzyme (~8.2), thus no binding of the enzyme protein to the column would be expected. Despite the nonbinding of the enzyme to the exchanger, reasonable purification was obtained, because contaminating acidic proteins were retained on the column. The column was first eluted with Tris-HCl buffer till the A_{280} of the eluate was same as the A_{280} of the buffer. Then the column was eluted with a linear NaCl gradient (0.00-0.5 M) to elute other isozymes (if present). After chromatofocusing (Figure 17) was performed, it was concluded that the enzyme had no other isozyme. The use of affinity chromatography resulted in elution of many impurities. The NAD^+ linked activity of FADH was eluted in one peak on

addition of 10 mM NAD^+ (Figure 12). Dialysis in 1 litre of Tris-HCl buffer pH 7.5 removed the NAD^+ . A low esterase activity was coeluted with the NAD^+ linked activity (not shown in the graph). From this result, the possible existence of other esterases or isozymes can be suggested. The esterase activity measured in ammonium sulphate fraction was more than that in the crude extract. This may be due to low molecular weight inhibitors contained in crude extract that inhibited the activity. The esterase activities were also high in the anion exchange step. This is because anion exchangers removes acidic contaminants. A sharp drop in esterase activity at the sephacryl S-300 step resulted from removal of non-ADH esterases. There was no significant purification or yield in dismutase activity as a result of its instability.

Total recovery of NAD^+ activity from chromatofocusing was only 12.5%. This may be due to the exposure of the enzyme to extremes of pH in this step. this low recovery was the reason for rejecting chromatofocusing as the purification step.

Alcohol dehydrogenase from wheat was active with both NAD^+ and NADP^+ . However the NADP^+ activity was very low compared to the NAD^+ linked activity and was not considered further. The procedures developed for purification, including affinity chromatography were not sufficient to purify the enzyme to homogeneity. Various kinds of affinity (Matrex red gel, AEDE-blue, βNAD^+ coenzyme resins, 5' AMP etc.) were tried with no appreciable results. Just as other plant alcohol dehydrogenases, this enzyme was more acidic than the fish enzyme. The enzyme being very unstable, was difficult to handle. The activity of the enzyme died in three normal working days. Various experimental procedures including chromatofocusing could not be performed. The pI of the enzyme was taken to be approximately 5.5 (from the work of Langston et. al.⁷). The buffers for cation exchange chromatography was selected accordingly. The purification procedures were similar to that of FADH, with a few modification. CM-52 (Figure 13) was used as cation exchanger and it was eluted with phosphate buffer of pH 7.0. In this case the enzyme did not elute with the

buffer. The pI of the enzyme being less than the pH of the buffer, the enzyme was bound to the anion exchanger. It was eluted with a salt gradient of 0-1.0 M. The NADP⁺ activity was present, but very low. Both the NAD⁺ and NADP⁺ activities with ethanol coeluted from both the anion exchange and gel filtration (Sephacryl S-300) media (Figure 14). This suggests that these activities are associated with the same protein.

The affinity for a given enzyme for different affinity columns are due to the permitted binding of that particular enzyme. The affinity columns contain group specific adsorbents for separating enzymes which require certain coenzymes (NAD⁺ and NADP⁺). The ligands responsible for binding the enzyme to the matrix are structural analogues of the natural cofactors required by enzymes which bind to these adsorbents. For each individual enzyme a different specific ligand has to be chosen and bound to the matrix, at a certain distance to permit binding of the enzyme. The ligands NAD⁺ or NADP⁺ are covalently attached to an insoluble support either through adenine base^{47,48}, hydroxyl group of ribose moiety^{49,50} or the phosphate group⁵¹ with proper carbon chain spacers. Ability of affinity columns to retain certain enzymes may depend on whether the geometrical orientations of the modified ligands properly fit the binding sites of these enzymes. WADH showed nearly no affinity to NAD⁺ coenzyme resins, 5'-AMP and other affinity matrices. Hence the enzyme was not homogenous (plate 2).

Molecular weight of native FADH is $80,530 \pm 1302$ Da (from Figure 15). The subunit molecular weight is $40,263 \pm 1506$ Da (from Figure 16). This implies a dimeric structure for this enzyme similar to HLADH. The WADH resembles YADH and other plant alcohol dehydrogenases in its physical properties and nonmultifunctionality. Since the final enzyme (WADH) was not homogenous, a small esterase activity was present. But it lacked dismutase, phosphatase and glycosidase activity. It is not sure that if a suitable affinity chromatography was performed, whether the esterase would have coeluted with the enzyme or not. The native molecular weight of WADH was $200,321 \pm 6477$ Da (from Figure 15) and

the subunit molecular weight was $50,630 \pm 1894$ Da (from Figure 16). This shows that WADH have a tetrameric structure like YADH. The general idea is that plant alcohol dehydrogenases are tetrameric whereas animal alcohol dehydrogenases are dimeric³.

Both the FADH and the WADH exhibited the absorption spectrum of a typical protein with an absorption maximum at 280 nm in Tris-HCl, pH 7.5 and phosphate buffer, pH 7.0 respectively.

Steady state kinetic analysis and product inhibition studies of NAD^+ linked reactions of FADH and WADH revealed differences in their kinetic mechanisms. The kinetic mechanism can be mainly classified into two classes (Alberty 1953)³⁹: (i) Sequential and (ii) Ping Pong. These two types can be differentiated by inspection of the double-reciprocal plots of initial velocity versus substrate concentrations. The cardinal feature of Sequential mechanism is that the family of lines in double-reciprocal plots intersect at a common point, either to the left of the $1/v$ axis or on the axis. This implicates that all substrates must be present simultaneously at the enzyme's active site before product formation can occur. Initial velocity studies with FADH showed that this enzyme catalysed via sequential mechanism. The curves were nonlinear, but the asymptote part was considered for further calculations. Product inhibition patterns at non-saturating concentration of nonvaried substrate were all linear. Both NADH and acetaldehyde were noncompetitive inhibitors with respect to NAD^+ and ethanol. The slope and intercept replots were all hyperbolic. Inhibition patterns obtained (Table 9) distinguish the steady-state random mechanism according to Table 8. The curvature observed near the ordinate in the initial velocity plots (Figure 24) supports this steady-state random mechanism. The Bardsley and Childs diagnostic plots (Figure 23) for NAD^+ -linked activity agrees with $\lambda = 0$ ($m = n > 1$). This function also agrees with random addition of NAD^+ . The kinetic studies and product inhibition studies shows that the mechanism for NAD^+ -linked activity is via steady-state random mechanism as shown in Scheme 11.

SCHEME 11 Proposed kinetic mechanism of NAD^+ linked activity of FADH.

The NAD^+ -linked reaction with ethanol of WADH also displayed sequential mechanism represented by linear converging lines (Figure 28). The linear converging lines are at high NAD^+ concentration. The Bardsley and Childs plot did not show a positive horizontal asymptote as $[\text{NAD}^+]$ concentration was increased unlike FADH, which showed a plateau at $\lambda = 0$. this shows that the mechanism for NAD^+ -linked activity of WADH is not complex and has no randomness. Product inhibition studies using NADH and acetaldehyde, are consistent with an ordered mechanism with oxidised coenzyme being bound first and reduced coenzyme released last.

Horse liver alcohol dehydrogenase (HLADH) operates by an ordered ternary-com-

plex mechanism (Scheme 12), where the coenzyme binds first.

SCHEME 12 The ordered ternary-complex mechanism for HLADH. E, R and O stands for enzyme NADH and NAD⁺ respectively. Alc and Ald are alcohol and the corresponding aldehyde.

While investigating the effect of pH on the catalytic steps of alcohol binding^{54,55} and ternary-complex interconversion^{56,57} it was seen that the enzyme-NAD⁺ alcohol complex participates in a proton dissociation equilibrium which regulates the reactivity of reaction intermediate.

In case of HLADH, it was seen that affinity of the enzyme for coenzyme NAD⁺ increases when pH is raised from 7 to 10. The affinity of the enzyme for NADH remains constant upto pH 9.0 and then decreases⁵⁸. According to Theorell⁵⁹, this differential effect of pH on binding of oxidized and reduced coenzyme is because of electrostatic interactions of the NAD⁺ nicotinamide ring charge with an ionizing water molecule at the active site zinc ion of the enzyme subunit.

When enzyme activity is plotted against pH, the shape of the pH profile can be a broad plateau (Figure 50)⁶⁰. In the plateau region the enzyme is all in its active form. In this plot the two ionizations are apparent, one group with pK of 3.0 being required in the depro-

tonated state and another with a pK of 10.0 required in the protonated state. If, the pK 's of the essential groups are closer, a "bell-shaped" profile may be obtained. This type of curve is obtained for WADH (Figure 21). This can be explained by the fact that with increasing pH, before one group has been fully deprotonated to give its active form, another group required in the protonated form has started to lose its proton.

FIGURE 50 pH dependence of the rates of enzyme catalysed reactions.

For NAD^+ -linked activity of FADH, a biphasic pH velocity plot was obtained (Figure 18). This may be due to the deprotonation of two ionizable groups in a proton relay system. It also indicates that more than one ionic form of the enzyme is catalytically active. Two active deprotonated states in the liver enzyme and in the yeast enzyme has been identified previously. The pH profile obtained for NAD^+ -linked activity of FADH suggests a two-functional group model, and two ionizable species can be identified. One functional group has $pK = 9.5$ and the other has a $pK = 7.2$. The pK values obtained for the mitochondrial ($pI = 5.0$) isozyme of horse liver aldehyde dehydrogenase HLALDH by Takahashi et al.³⁶ are similar to these pK values (obtained for rainbow trout liver alcohol dehydrogenase). These pK_a 's were suggested to be involved in the rate determining step. The groups with these pK_a 's are: general base B in structure I and the electron rich group A: in structure II (Figure 51). In structure I, N (nucleophilic side chain of an amino acid) can be sulphur of cysteine for papain⁶¹ or GAPDH⁶². The general base can be histidine as in chymot-

rypsin, papain and GAPDH⁶³. In all the three enzymes, the histidine is in a close proximity to an electron sink so that a "charge relay" system can function, as shown in structure II. A (the electron sink) can be aspartate-carboxylate as in chymotrypsin, asparagine-carboxyl oxygen as in papain⁶³ and tyrosine-phenol as in GAPDH⁶².

FIGURE 51 Structure I: B is general base and N is a nucleophilic side chain of an amino acid.
Structure II: A is the electron sink such that a "charge relay" system can function.

In HLALDH (the mitochondrial isozyme with pI 5.0), only the group with lower pK_a , most probably a histidine would be involved (structure I), at physiological pH. The charge relay system function in the nonphysiological pH (structure II). Tyrosine, analogous to tyrosine 311 in GAPDH could be the group involved here³⁶. An acidic species can also be involved in this "charge relay" system. The action of charge relay system is not required for the hydrolysis of acyl intermediate at physiological pHs. Only a 3 fold increase in turnover number per active site of HLALDH was observed with the second ionization, which allows the "charge relay" system to function³⁶. In hydrolysis of acyl intermediate, the role of this "charge relay" system is doubtful in HLALDH. Here the biphasic pH profile obtained for FADH suggests the involvement of a general base with $pK_a = 7.2$, a histidine res-

idue. Any other acidic group, tyrosine etc. with $pK_a = 9.5$ can be involved in the “charge relay” system at alkaline pH. In FADH, a 30.2 fold increase in activity was noticed at alkaline pH.

Fourteen histidine residues or seven per subunit are present in HLADH⁶⁴. Two of them: His-51 and His-67 are strategically situated within the catalytic domain. Histidine-67 serves as a ligand to catalytic zinc, while His-51 serves as a proton sink during enzymatic catalysis^{65,66}. Amino acid sequences of cod liver alcohol dehydrogenase and horse liver alcohol dehydrogenases were aligned (Alignment 1 in Appendix 2). This along with the pH-velocity profile lend support to the involvement of histidine in “charge relay” system in FADH as described in structure I and II.

The pH dependence of velocity in NAD^+ -linked esterase (Figure 19) and dismutase (Figure 20) activities had one common feature. There is the double maxima in these two activity versus pH plots. The pH values at which these maxima occurred however differed from one activity to another.

Chemical modification (amidation) was performed to investigate the preference of NAD^+ over $NADP^+$ by FADH. Due to the charge repulsion between the anionic 2' phosphate group of $NADP^+$ and carboxyl oxygen of Asp 223 weakens the binding of $NADP^+$ to HLADH. This residue Asp223 is responsible for the high NAD^+ specificity in HLADH, YADH and fourteen other dehydrogenases. Amino acid sequences of known fish enzyme (cod - chain B) and wheat were compared (Appendix 2).

WADH showed a very weak $NADP^+$ activity. This may be due to the presence of acidic groups within the active site of this enzyme. Amidation was carried on with FADH, and the effect of acidic species loss on NAD^+ (Figure 48) and $NADP^+$ (Figure 49) activities were determined. The data for HLADH was taken from previous experiments performed in

this laboratory. Both the HLADH and YADH are NAD^+ specific due to the involvement of Asp 223 residue. Amidation of HLADH and YADH with molar excess of ammonium chloride in the presence of EDC inactivated NAD^+ -linked activity slightly. 80% of activity remained in both the enzymes after one hour. It can be assumed that loss of acidic species in the enzyme by amidation would increase the NADP^+ -linked activity by removing the ability of the enzyme to distinguish between NAD^+ and NADP^+ . There was a slight activation in NADP^+ activity of HLADH, but no effect was observed on this activity of YADH. This may be because ammonium chloride is not a strong nucleophile.

Amino acid sequences of HLADH and cod alcohol dehydrogenases and wheat alcohol dehydrogenase were aligned (Alignment 1 and Alignment 2 in Appendix 2). This verifies the presence of Asp-223 in FADH which explains the NAD^+ specificity of the enzymes.

Four different amines with different size and charges were used for amidation of FADH in the presence of EDC. These nucleophiles induced similar responses in both the NAD^+ and NADP^+ linked activities. A reduction in the negative charges on acidic moieties is caused by methylamine, and hence the presence of methylamine inactivated the NAD^+ -linked activity of FADH. n-Propylamine is uncharged and it influenced the NAD^+ activity in a similar way. n-Propylamine would have no effect on the NAD^+ linked activity of FADH if charge attraction by an acidic group was responsible for binding of NAD^+ to FADH. Since this was not observed, the idea that charge attraction by an acidic group was responsible for binding of NAD^+ to the enzyme was ruled out. Theoretically positively charged ethylene diamine would maximize charge repulsion between NAD^+ and acidic groups, leading to inactivation. It was seen that in presence of ethylenediamine NAD^+ -linked activity was deactivated more rapidly than in the presence of the other two amines (methylamine and n-propylamine). Negatively charged taurine should enhance the NAD^+ -

linked activity due to negative charges introduced on acidic groups. It was seen that the NAD^+ -linked activity with taurine was higher than the NAD^+ -linked activity without any amines, but there was a slight decrease in activity with time in presence of taurine.

All the nucleophiles activated the NADP^+ -linked activity of FADH in presence of EDC. Theoretically, taurine should lead to inactivation of NADP^+ -linked activity because negative charges on it still exists. But that was not observed. In presence of EDC only, the NADP^+ -linked activity increased as well. This indicates that EDC alone is sufficient enough to cause an increase in NADP^+ -linked activity. The possibility of polymerization can be considered.

In a study carried out in this laboratory⁶⁷, the role of nucleophile in the amidation reaction was monitored by incorporation of radiolabelled amidating reagent in FADH (pickrel liver) and YADH. Among the four nucleophiles methylamine, ethylenediamine, n-propylamine and taurine, it was found that methylamine was the nucleophile that induced the greatest increase in the $\text{NADP}^+/\text{NAD}^+$ ratio. Methylamine available in ^{14}C labelled form was used for the amidating nucleophile in this study. No incorporation of methylamine in FADH 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 FADH and YADH respectively. These results obtained confirm the involvement of EDC in the activation of NADP^+ linked activity in FADH. This suggests that EDC is affecting the structure of enzyme in such a manner that steric hindrances to extra phosphate group of NADP^+ diminish.

Summary

The purification procedure for fish liver alcohol dehydrogenase and wheat alcohol dehydrogenase represents a considerable modification of Tsai's³³ method for the purification of alcohol dehydrogenase from various sources. It achieves about 90 fold purification for fish and 60 fold purification for wheat. Alcohol dehydrogenase isolated from fish (Rainbow trout) liver (FADH) is a dimer like HLADH. Wheat alcohol dehydrogenase is a tetramer. A partial characterization of physical properties of the enzymes are given and compared with those of HLADH. The two enzymes differ in multifunctional activities. FADH is NAD^+ specific whereas WADH is both NAD^+ and NADP^+ specific. The NADP^+ activity of WADH being very small, was ignored here. No information was available about the activity, distribution and the role of FADH in rainbow trout and wheat. Kinetic studies provided an understanding of the mechanisms of NAD^+ linked activities. It was seen that EDC alone is sufficient enough to cause an increase in NADP^+ linked activity in FADH.

Detailed structural studies of FADH and WADH will enable us to understand the structure function relationship and the structural requisites for these two enzymes which enable them to use NAD^+ or NADP^+ or both and is suggested for future work.

Appendix 1

The error bars (standard deviation) of double reciprocal plots for NAD^+ linked activities of fish liver alcohol dehydrogenase (Figure 24) are shown in Figure 52.

FIGURE 52 Error bars of double reciprocal plot of NAD^+ linked activity of FADH (Figure 24).

The error bars (standard deviation) of double reciprocal plots for NAD^+ linked activities of wheat alcohol dehydrogenase (Figure 28) are shown in Figure 53.

FIGURE 53 Error bars of double reciprocal plot of NAD^+ linked activity of WADH (Figure 28).

Appendix 2

To discuss the likely presence of amino acid residues responsible for the observed pH effects and chemical modifications, known fish and plant alcohol dehydrogenase sequences were aligned. The alcohol dehydrogenases were from:

Cod liver Chain B, Alcohol Dehydrogenase (EE Isozyme)

Wheat Alcohol Dehydrogenase

Yeast Alcohol Dehydrogenase, Adh 1 p [*Saccharomyces cerevisiae*]

Horse Liver Alcohol Dehydrogenase, S Chain

Chicken Alcohol Dehydrogenase, ADH1_CHICK

Fish Liver Alcohol Dehydrogenase from Gilthead Sea Bream

The first four alignments are given below and the discussions are included in the previous section.

The alignments were done using the program <http://www.toulouse.inra.fr/multalin.html>⁶⁸. This is a multiple alignment tool. The amino acid sequences were obtained from Entrez⁶⁹ in Fasta format.

In all the alignments the High consensus colour is black, Low consensus colour is blue and Neutral colour is brown.

```

1      10      20      30      40      50      60      65
|-----|
N|G|T|A|G|K|V|I|K|C|K|A|V|L|E|Q|K|K|P|F|S|I|E|E|V|E|V|A|P|P|K|A|H|E|Y|R|I|K|H|V|R|A|G|I|C|R|S|D|-D|H|V|V|S|G|T|L|V|A|P|L|P|V|
A|T|V|G|K|V|I|K|C|K|A|V|L|E|Q|K|K|P|L|V|I|E|E|V|E|V|D|V|P|H|A|N|E|I|R|I|K|I|A|T|G|Y|C|H|T|D|L|Y|H|L|F|E|G|K|H|K|D|G|F|P|V|

66     75     85     95     105    115    125    130
|-----|
I|G|H|E|R|A|G|I|V|E|S|I|G|E|G|Y|T|T|V|R|P|G|D|K|V|I|P|L|F|I|P|Q|C|G|K|C|S|V|C|K|H|P|E|G|N|L|C|L|K|N|L|S|H|P|R|-G|T|H|Q|D|G|T|S|
V|L|G|H|E|R|A|G|I|V|E|S|Y|G|P|G|Y|T|E|F|Q|P|G|E|K|V|I|P|L|F|I|S|Q|C|G|E|C|R|F|C|Q|S|P|K|T|N|Q|C|Y|K|G|H|A|N|E|S|P|D|V|H|S|P|K|E|T|

131    140    150    160    170    180    190    195
|-----|
H|G|T|C|R|G|K|P|I|H|H|F|L|G|T|S|T|F|S|Q|Y|T|V|Y|D|E|I|S|V|A|K|I|D|A|R|S|P|L|E|K|V|C|L|Y|G|C|G|F|S|T|G|Y|G|S|A|Y|K|Y|A|K|Y|T|G|G|S|
R|F|T|C|K|G|R|K|V|L|Q|F|L|G|T|S|T|F|S|Q|Y|T|V|Y|N|Q|I|A|V|A|K|I|D|P|S|A|P|L|D|T|V|C|L|L|G|C|G|Y|S|T|G|F|G|A|Y|N|T|A|K|Y|E|P|G|S|

196    205    215    225    235    245    255    260
|-----|
T|G|A|Y|F|G|L|G|G|V|G|L|S|V|I|N|G|C|K|A|R|G|A|R|I|G|V|D|I|N|K|D|K|F|A|K|A|K|E|V|G|A|T|E|C|Y|N|P|Q|Q|Y|K|K|P|I|Q|E|V|L|T|E|H|S|
T|C|A|V|F|G|L|G|A|Y|G|L|A|A|V|H|G|C|H|S|A|G|A|K|R|I|A|V|D|L|N|P|D|K|F|E|K|A|K|Y|F|G|A|T|D|F|Y|N|P|N|H|S|E|P|I|S|Q|V|L|S|K|H|T|

261    270    280    290    300    310    320    325
|-----|
N|G|G|Y|D|F|S|F|E|Y|I|G|R|L|D|T|H|Y|R|A|L|S|C|C|Q|E|R|Y|G|V|S|V|I|V|G|Y|P|P|D|S|Q|N|L|S|H|N|P|M|L|L|S|G|R|T|H|K|G|A|I|F|G|G|F|K|
N|G|G|Y|D|F|S|L|E|C|Y|G|N|Y|G|V|H|R|N|A|L|E|S|C|L|K|G|H|G|V|S|V|L|V|G|H|T|-D|L|H|D|V|A|T|R|P|I|Q|L|I|A|G|R|T|H|K|G|S|H|F|G|G|F|K|

326    335    345    355    365    376
|-----|
I|S|H|D|S|Y|P|K|L|Y|A|D|F|H|A|K|K|F|A|L|D|P|L|I|T|H|V|L|P|F|E|K|I|N|E|G|F|D|L|L|R|S|G|K|S|I|R|T|I|L|T|F|
G|K|D|G|Y|P|K|H|Y|K|A|Y|L|D|K|K|Y|K|L|D|E|F|I|T|H|A|N|P|L|E|S|Y|N|D|A|I|D|L|N|K|H|G|K|C|I|R|T|V|L|S|L|

```

Alignment 1 Alignment of horse liver alcohol dehydrogenase and cod liver alcohol dehydrogenase.

The first sequence in each block is horse liver alcohol dehydrogenase.
The second sequence in each block is cod liver alcohol dehydrogenase.

```

1      10      20      30      40      50      60  65
|-----|
MGTAGKVIKCKAAVLMEQKKPFSIEEVEVAPPKAHEVRIKMVAAGICRSDDHVVSGLVAPL-PV
MATAGKVIECKAAVAMEAGKPLSIEEVEVAPPHAHEVRYKILYTALCHTDVYFWEAKGQTPVFPR

66      75      85      95      105     115     125  130
|-----|
IAGHEAAGIVESIGEGVTVRPGDKVIPLFIPQCCKCSYCKHPEGMLC-LKNLSMPRGTH-QDGT
ILGHEAGGIYESVGEVTELYPGDHVLPVFTGECKDCANCKSEESNLCOLLINVDORGVMIGDQ

131     140     150     160     170     180     190  195
|-----|
SFTCRGKPIHHFLGTSTFSQYTVVDEISVAKIDAAASPLEKYCLVGC GFSTGYGSAYKVAKVTQG
SRFTINGKPIHFVGTSTFSEYTVIHVGLAKINPEAPLDKYCVLSCGISTGLGATLNVAKPKKG

196     205     215     225     235     245     255  260
|-----|
SFCAYFGLGGVGLSVINGCKAAGAAARIIGVDINKDKFAKAKKEYGATECVNPQDYKKPIQEVLTEN
STVAIFGLGAYGLAAMEGARMAGASRIIGVDLNPAYEQAKKFGCTDFVNPKDHTKPVQEVLYEM

261     270     280     290     300     310     320  325
|-----|
ISNGGVDFSFEVIGRLDTMYAALSCCQEARVGVSVIYGVPDQSMLSMNPALLSGRTHKGAIFGGF
TNGGVDRARECTGHIDANIAAFECVHDGNGVAVLVGVPHKERVFKTYPHNLFNERTLKGTFFGNY

326     335     345     355     365     375  379
|-----|
MKGDSYPKLVADFMAKKFALDPLITHVLPFEKINEGFOLLRSGKSIRTILTF
KPRDLPVVENYMRKELELEKFITHSVFSSQINTAFDLNLKGEGLRCINRMDQ

```

Alignment 2 Alignment of horse liver alcohol dehydrogenase and wheat alcohol dehydrogenase.

The first sequence in each block is horse liver alcohol dehydrogenase.

The second sequence in each block is wheat alcohol dehydrogenase.

```

1      10      20      30      40      50      60      65
|-----|
MATAGKVI ECKAAVAMEAGKPLSIEEEVAPPHAMEVRVKILY TALCHT D VYFWEAKGQTPV-FP
MSIPETQKGVIFYESHGKLEYKDIPVPKPKANELLINVKYSGVCHTDLNHNHGDMLPVKLP

66      75      85      95      105     115     125    130
|-----|
RILGHEAGGIVESVGEGYTELVPGDNV-LPVFTGECKDCANCKSEESNLCOLLRINVD RGVHIGD
VGGHEGAGVYVGMGENYKGNKIGDYAGIKMLNGSCHACEYCELGNESNCPHADL-----

131     140     150     160     170     180     190    195
|-----|
GQSRFTINGKPIFHFYGTSTFSEYTYIHVGLAKINPERPLDKVCYLSCGISTGLGATLMVAKPK
ASGYTHDG-----SFQQYATADAVQAAHIPQGTOLAQVAPILCAGITYYKA-LKSANLM

196     205     215     225     235     245     255    260
|-----|
KGSTVAIFGL-GAVGLAAMEGARMAGASRIIGVDLNPAYEQAKKFGCTDFVNP KDHTKPVQEV
AGHNVAISGAGGLGSLAYQYAKANGY-RVLGIDGGEGKEELFRSIGGEVFIDFTKE-KDIVGAV

261     270     280     290     300     310     320    325
|-----|
VENTNGGVDRAVECTGHIDAMIAAFECVHOGWGYAVLVGVPHKEAVFKTYPNNFLNERTLKGTFF
KATDGGAGHYINVSYSARIEASTRYVRAN-GTTVLVGNPAGAKCCSDVFNQVYKSISIVGSYY

326     335     345     355     365     375     383
|-----|
GNYKPRIDLPEYVENYMRKELELEKFITHSVPFQINTAFDLMLKGEGL-RCINRNDQ
GN---RADTREALDFFARG---LVKSPIKYVGLSTLPEIYEKMEKGQIVGRYVVDTSK

```

Alignment 3 Alignment of wheat alcohol dehydrogenase and yeast alcohol dehydrogenase.

The first sequence in each block is wheat alcohol dehydrogenase.

The second sequence in each block is yeast alcohol dehydrogenase.

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