PROBES OF MECHANISM AND TRANSITION-STATE STRUCTURE IN THE ALCOHOL DEHYDROGENASE REACTION

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I. INTRODUCTION

The recent confluence of high resolution X-ray crystallographic, spectroscopic, chemical modification, and kinetic studies on alcohol dehydrogenases suggests that we are now in a position to understand the detailed mechanism of this enzyme system. In fact, while the available structural data provide the number and nature of active site residues, together with considerable insight into the mode of coenzyme and substrate binding, a completely satisfactory integration of structure with chemical mechanism has not yet been achieved. It is the objective of this review to describe the questions and tentative answers available from probes of mechanism and transition state structure in horse liver and yeast alcohol dehydrogenases. Structural and chemical modification studies are presented both to provide a frame of reference for the interpretation of kinetic data and to indicate the extent of homology among alcohol dehydrogenases from divergent evolutionary sources. Early investigators pointed out that as zinc-metallo enzymes, alcohol dehydrogenases were unique among NAD(P)-dependent enzymes. A second distinguishing feature of these enzymes concerns the successful kinetic isolation of bond rearrangement from protein isomerization and product desorption steps. This latter feature has greatly facilitated detailed studies of mechanism and transition state structure.

II. MECHANISTIC SCAFFOLDING: THE X-RAY STRUCTURE OF HORSE LIVER ALCOHOL DEHYDROGENASE

Horse liver alcohol dehydrogenase is a dimer, comprised of identical 40,000 mol wt subunits. Apoenzyme crystallizes in an orthorhombic space group containing one subunit per asymmetric unit. The X-ray structure of apoenzyme has been refined to 2.4 Å.[1] These data, together with the completed primary structure,[2] have led to a positioning of the 374 amino acids per polypeptide chain.

A. Coenzyme-Binding Domain

Each subunit is divided into two domains, separated by a cleft with a deep pocket (Figure 1). The smaller coenzyme binding domain consists of residues 176 to 318.[1] In analogy with previously described structures for dehydrogenases and kinases (e.g., lactate and malate dehydrogenase,[3,4] and adenyl and phosphoglycerate kinase[5,6]), the main chain of this domain folds to generate a series of six, parallel, pleated sheets flanked by helices. Although the high degree of conservation of secondary structure in the coenzyme binding domains of kinases and dehydrogenases has been interpreted

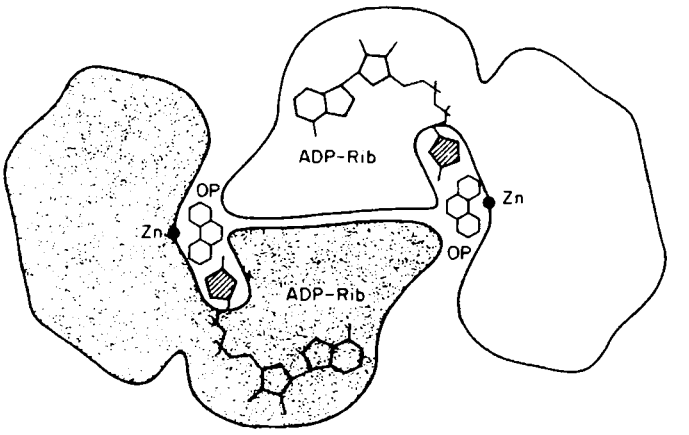


FIGURE 1. A schematic representation of the horse liver alcohol dehydrogenase dimer, illustrating the region of intersubunit contact and the active site cleft. ADP-Rib is ADP-ribose, and OP is orthophenanthroline.

to indicate a unique means of generating an enzymatic NAD^+ or ATP binding site, an increasing number of unrelated proteins (e.g., flavodoxin,[7] subtilisin,[8] superoxide dismutase,[9] and immunoglobulins[10]) have been demonstrated to contain similar structures. Thus a number of investigators have suggested that these structures may be more relevant to the number of thermodynamically stable states available to a folded protein than coenzyme binding and catalysis.[11,12]

Enzyme-coenzyme complexes crystallize in a unique (triclinic) space group containing one molecule of dimeric enzyme per asymmetric unit; and high resolution structures of such complexes are not yet published. The difference Fourier map of the complex of the competitive inhibitor ADP-ribose with orthorhombic enzyme has been reported at 2.4 Å. The details of the binding of this fragment include: (1) placement of the adenine ring into a hydrophobic pocket, (2) hydrogen bonding between the 2′ hydroxyl of the adenine ribose and the carboxylate of Asp 223, and (3) a salt bridge between Arg 47 and the pyrophosphate of ADP-ribose. The terminal ribose of ADP-ribose points toward the bottom of the cleft separating domains with possible hydrogen bonds from the 2′ and 3′ hydroxyl groups to the main chain carbonyl oxygens of Gly 293 and Ile 269. Interestingly, the side chain Lys 228, which when chemically modified leads to an enhanced rate of coenzyme dissociation, is found in the region of the adenine ribose. Crystals of liver alcohol dehydrogenase, activated by modification of amino groups, have been prepared which are suitable for detailed X-ray analysis.[13]

B. Catalytic Domain

The catalytic domain of horse liver alcohol dehydrogenase consists of residues 1 to 175 and 319 to 374.[1] Two zinc atoms are bound to this domain, confirming the presence of two metals per subunit. One zinc is bound at the bottom of the cleft between domains, 20 Å from the protein surface. This metal is liganded to 3 protein ligands — Cys 46, Cys 174, and His 67; a fourth ligand is water or hydroxide ion, depending on the pH. A second zinc is contained in a stretch of polypeptide chain (residues 95 to 113) which forms a loop projecting from the catalytic domain; this metal is liganded to four cysteines — Cys 97, Cys 100, Cys 103, and Cys 111. The protein ligands to zinc are summarized in Figure 2. The bond angles to the first zinc atom are given in Table 1; the inter- and intrasubunit distances between zinc atoms are summarized in Table 2.

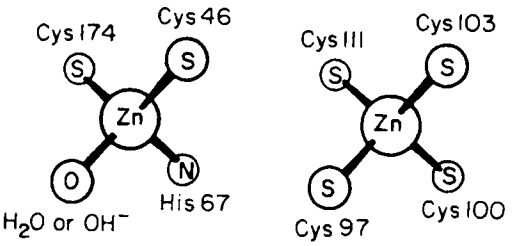


FIGURE 2. Ligands to the two zinc atoms per subunit of horse liver alcohol dehydrogenase.

Table 1
BOND ANGLES BETWEEN LIGANDS TO THE FIRST ZINC ATOM[14]

Atoms	Angle (°)
S(Cys 46)-Zn-N(His 67)	122
S(Cys 46)-Zn-S(Cys 174)	115
S(Cys 174)-Zn-N(His 67)	108
O(H,O)-Zn-S(Cys 46)	86
O(H,O)-Zn-N(His 67)	125
O(H,O)-Zn-S(Cys 174)	98

Table 2
INTRA- AND INTER-SUBUNIT DISTANCES BETWEEN ZINC ATOMS AND FLUORESCENT TRYPTOPHANS[1]

Distance	Å
Zn(c),1 → Zn(s),1	20.6
Zn(c),1 → Zn(c),2	44.6
Zn(c),1 → Zn(s),2	43.8
Zn(s),1 → Zn(s),2	42.2
Trp 15,1 → Trp 314,1	41
Zn(c),1 → Trp 314,1	21
Zn(c),1 → Trp 314,2	24
Trp 314,1 → Trp 314,2	6
Zn(c),1 → Trp 15,1	24
Zn(c),1 → Trp 15,2	68
Trp 15,1 → Trp 15,2	84

Binary complexes of alcohol dehydrogenase, with the inhibitors imidazole and 1,10-phenanthroline, form crystals isomorphous with apoenzyme.[14] Kinetic studies of imidazole inhibition support binding at the substrate site.[110] A difference Fourier pattern at 2.9-Å resolution indicates that imidazole binds at the bottom of the deep pocket between domains; its density partly overlaps with that originally attributed to zinc-bound water.[14] These results corroborated the assignment of the first zinc atom in Figure 2 as the catalytic site zinc. The larger 1,10-phenanthroline behaves as a competitive inhibitor toward both substrate and coenzyme, forming ternary complexes with the coenzyme fragment of ADP-ribose.[110] A 4.5-Å resolution difference Fourier map of the enzyme-1,10-phenanthroline complex is consistent with direct coordination of the nitrogens at positions 1 and 10 to the active site zinc.[14]

The two subunits of the dimer are related by a crystallographic twofold axis; the majority of the intersubunit interactions occur within the coenzyme binding domains (Figure 1).[14] The residues in the area of contact between subunits are largely hydrophobic, consistent with the inability to prepare enzyme subunits in the absence of protein denaturation. Both subunits contribute residues to the active site pocket, the majority of which are hydrophobic.

A detailed picture of the substrate binding pocket of horse liver alcohol dehydrogenase is given in Figure 3, illustrating the hydrophobic barrel through which substrate must pass to reach the catalytic site zinc residing at the bottom of the barrel.[15] The only hydrophilic residues, other than $ZnOH_2/ZnOH$, near the active site are Ser 48

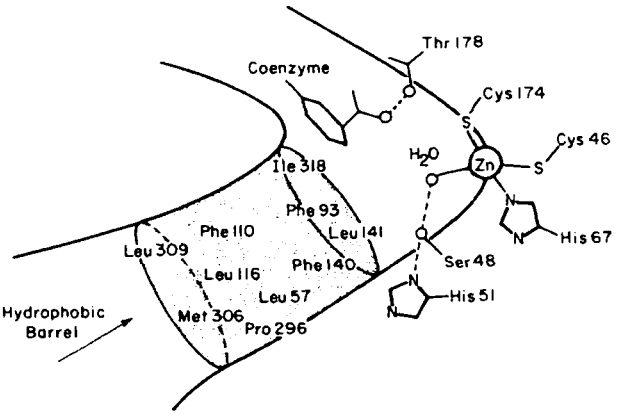


FIGURE 3. A schematic representation of the hydrophobic barrel constituting the active site cleft of horse liver alcohol dehydrogenase.

and His 51; these residues have been proposed to form a proton relay from the bottom of the barrel to solvent, facilitating proton cycling in and out of the active site as required by the pH dependence of the overall equilibrium, Equation 1:

$$NAD^+ + RCH_2OH \rightleftharpoons NADH + H^+ + RCHO \qquad (1)$$

C. Mechanistic Implications

Our ability to infer mechanism from X-ray data rests on the important assumption that the structure of crystalline protein resembles that in solution. Although this question has not been addressed in detail, triclinic crystals of liver alcohol dehydrogenase have been demonstrated to catalyze substrate turnover slowly.[16] In this context, it is of interest to consider difference Fourier studies of the complex of enzyme and 3-iodopyridine adenine dinucleotide, a coenzyme for liver alcohol dehydrogenase.[17] In contrast to NADH or NAD^+, 3-iodopyridine adenine dinucleotide forms complexes isomorphous with apoenzyme. Despite the low resolution (4.5 Å), the ADP-ribose portion of this coenzyme can be positioned in a manner analogous to the ADP-ribose fragment. Disorder in the nicotinamide orientation precludes a unique positioning of this portion of the molecule; however, it is clear that the pyridine ring is at the surface of the molecule, 15 Å away from the active site pocket. Thus, while apparently a competent substrate in solution,[113] the preferred conformation of 3-iodopyridine adenine dinucleotide bound to crystalline alcohol dehydrogenase is concluded to be nonproductive. Conversely, orthorhombic crystals cannot accommodate 3-iodopyridine adenine dinucleotide in a catalytically competent manner, and caution must be exercised in attempting to deduce mechanistic details from information of the structure of apoenzyme. More recent studies indicate that a variety of coenzyme analogs undergo similar nonproductive binding to the orthorhombic form of alcohol dehydrogenase; in the case of the tetrahydro form of NAD^+, H_2NADH, coenzyme binds to the active site region, but is unable to trigger the conformational change observed in the presence of either NADH or NAD^+.[18]

Regarding the mechanistically relevant structures of binary and ternary complexes of horse liver alcohol dehydrogenase, a low resolution (4.5 Å) map of triclinic E·NADH·dimethylsulfoxide is published.[19] The structure demonstrates that the interaction of the ADP portion of NADH is analogous to ADP-ribose binding to apoenzyme, and that the nicotinamide ring of NADH is close to the active site zinc. In

contrast to the close similarity of electron density maps in the coenzyme binding domain for apoenzyme and E·NADH·dimethylsulfoxide, the catalytic domain has undergone a large conformation change upon formation of (abortive) ternary complex. This conformational change is postulated to reduce the size of the active site cleft between domains; importantly, no new residues have been introduced into the active site. In addition, *preliminary higher resolution data indicate several important features which include (1) an interaction between the 2′ hydroxyl of the nicotinamide ring of NADH and His 51, and (2) direct coordination of the oxygen of both dimethylsulfoxide and alcohols to the active site zinc of E·NADH.*[20]

Employing the 4.5-Å map of the E·NADH·dimethylsulfoxide structure, efforts had been made by Plapp et al. to obtain difference maps for *productive ternary complexes of enzyme with NAD⁺ and a variety of alcohols.*

A major objective of these investigators was to characterize the mode of interaction of the oxygen of alcohol with the catalytic zinc (via direct coordination as an alcoholate or in a second sphere complex).[21] From measured constants for the conversion of

$$E\cdot NADH\cdot \phi CHO \underset{k_o=12s^{-1}}{\overset{k_f=340s^{-1}}{\rightleftharpoons}} E\cdot NAD^+\cdot \phi CH_2OH$$

it was reasoned that complexes of E·NAD⁺·*p*BrϕCH_2OH would lie almost exclusively in the direction of oxidized coenzyme. A difference Fourier map for [E·NAD⁺·*p*BrϕCH_2OH] − [E·NADH·dimethylsulfoxide] failed to locate the hydroxymethyl group of *p*-BrϕCH_2OH, however, presumably due to partial overlap between this group and dimethylsulfoxide. Efforts were made to obtain crystals of E·NAD⁺, which crystallized with the crystallizing agent (methylpentanediol). Nonetheless, a comparison of E·NAD⁺·*p*BrϕCH_2OH to E·NAD⁺·methylpentanediol did indicate some positive density for the hydroxymethyl group of *p*-BrϕCH_2OH; presumably a consequence of only partial occupancy of the substrate site in the E·NAD⁺ · methylpentanediol crystals. Using the 2.4-Å structure of apoenzyme as a guide, a model of the active site has been published, involving an inner sphere complex between the oxygen of alcohol and the catalytic zinc. In light of the large number of assumptions necessary for the construction of this model, it must be considered tentative. Hopefully, the availability of high-resolution data for the E·NAD⁺ form of liver alcohol dehydrogenase will permit an unambiguous answer to the mode of alcohol binding in productive ternary complexes.

A different approach to substrate binding was undertaken in an earlier study by Dutler.[22] On the assumptions (1) that the oxygen atom of alcohol substrate binds directly to zinc and (2) that the hydrogen to be transferred is 2.5 Å away from and pointing toward C-4 of the nicotinamide ring, cyclohexanol was placed into the available 2.4-Å structure of apoenzyme. Using this position of the cyclohexanol ring, the addition of substituents to positions on the ring which sterically overlapped with either the protein or coenzyme was found to correlate with decreased or abolished activity. The success of this correlation led Dutler to predict direct coordination of substrate to the active site zinc. Although these model building experiments failed to provide a quantitative assessment of alternative models of substrate binding, and were based on the available X-ray structure for apoenzyme, such an approach provides a valuable means of relating structural information derived from crystalline enzyme to kinetic activity in solution.

III. HOMOLOGY BETWEEN YEAST AND HORSE LIVER ALCOHOL DEHYDROGENASE

A. Primary Structure and Chemical Modification Studies

The two major systems subjected to extensive kinetic studies are yeast and horse liver alcohol dehydrogenases.[2,23,24] The oligomeric structure of the two proteins is quite different: the yeast enzyme is a tetramer comprised of identical 37,500-mol wt subunits in contrast to the liver enzyme which is a dimer comprised of identical 40,000-mol wt subunits. Both proteins are zinc-metallo enzymes, although the total number of zinc atoms per subunit in the yeast enzyme is a question of some controversy. The determination of the primary structure of the yeast enzyme led to a plausible alignment of these two proteins.[25] From such an alignment, it is clear that the total number of identical residues is quite small, 25%, increasing to a maximum value of 40% for residues 25 to 85 in the yeast enzyme. This stretch of polypeptide chain contains Cys 43 and His 67, amino acids homologous to two of the three active-site zinc ligands (Cys 46 and His 67) in the horse liver enzyme. As summarized in Table 3, the second most highly conserved polypeptide in the yeast enzyme is residues 125 to 183, which contains Cys 178, homologous to the third active site ligand (Cys 174) in the horse liver enzyme.

The homology in peptides containing Cys 43/46 was established early through chemical modification employing iodoacetate.[26] In retrospect, it is surprising that these initial studies suggested a single reactive cysteine in alcohol dehydrogenase. Subsequent work (see Table 4) has shown that changes in either the structure of the alkylating agent or the experimental conditions can lead to modification of Cys 43/46 or Cys 178/174.[27,28,30,31] In the case of an aromatic epoxide, chemical modification of alcohol dehydrogenase has been shown to lead to the simultaneous alkylation of Cys 43/46 and Cys 178/174.[29]

The unusual observation that carboxymethylated horse liver alcohol dehydrogenase forms binary and ternary complexes, catalyzing substrate turnover at a reduced rate, provides an active enzyme form characterized by modified active site properties.[32] As will be discussed in subsequent sections, detailed studies on carboxymethylated enzyme have been carried out in an effort to clarify the role of zinc vs. zinc-water in acid-base catalysis and to assign pK values to amino acid side chains relevant to catalysis. Crystallographic and magnetic resonance studies of carboxymethylated enzyme provide a structural basis for the interpretation of these data. From difference Fourier maps, Zeppezauer et al. report that the carboxymethylated sulfur atom of Cys 46 is still liganded to the active site zinc atom. There appear to be no major conformational changes in the protein, with the exception of some movement of the side chain of Arg 369 to prevent steric interference. Interestingly, the carboxylate of the carboxymethyl group does not loop back to ligand the active site zinc. Rather, neutralization of the negative charge on one of the two sulfur ligands to zinc appears to generate a new anion binding site; and the iodide ion liberated during alkylation is observed to displace buffer-derived imidazole as the fourth ligand to zinc.[33] Recent X-ray structural data suggest that NADH binds to the carboxymethylated form of enzyme without the concommitant conformational change observed with native enzyme.[18]

Nuclear magnetic resonance studies on horse liver alcohol dehydrogenase, carboxymethylated with 90% [1-^{13}C]-bromoacetate, indicate a unique signal 2 ppm downfield from the carboxylate chemical shift in small, model 5-carboxymethyl compounds. Apparently, the movement of the chemical shift downfield cannot be attributed to coordination of the thioether sulfur of modified Cys 46 to zinc or direct zinc-carboxylate coordination, both of which are expected to lead to upfield shifts. The authors suggest that the electrostatic interactions between either Arg 369 or Glu 68 may be the origin of the unusual chemical shift.[34]

Table 3
SUMMARIZED HOMOLOGY BETWEEN YEAST AND HORSE LIVER ALCOHOL DEHYDROGENASES[25]

Region	No. of residues	Identities/similarities %	
Whole enzyme	347	25	36
Catalytic domain	210	25	36
Coenzyme-binding domain	137	25	37
β-Pleated sheet structure	38	29	47
Maximum similarity:			
Yeast 25—yeast 85	62	40	55
Yeast 125—yeast 183	59	34	46
Minimum similarity:			
Yeast 87—yeast 114	28	14	14
Yeast 272—yeast 305	34	9	18

Note: Identities denote identical residues at corresponding positions, and similarities the sum of identical residues and conservative exchanges (Val/Leu/Ile, Ser/Thr, Lys/Arg, Glu/Asp).

B. Metal Content and Exchange Properties

As emphasized above, both primary sequence data and extensive chemical modification studies support similar active site configurations in yeast and horse liver alcohol dehydrogenase despite the fact that these two proteins are more dissimilar than any other characterized pair of yeast and mammalian enzymes. One of two regions of minimum similarity, residues 87 to 114 in the yeast enzyme (Table 2), corresponds to the region of horse liver enzyme which binds the structural zinc. Further, the yeast enzyme is characterized by a deletion of 21 residues, corresponding to residues 119 to 139 in the horse liver enzyme. From the X-ray data, residues 93 to 139 constitute part of the structural metal binding loop extending away from the catalytic domain, and Jörnvall has suggested that deletion of this region in the yeast enzyme could affect subunit interaction and/or binding of a second zinc.[25]

A number of investigators have recently reexamined the zinc content of yeast dehydrogenase. The only point of agreement to date is that enzyme obtained from commercial sources contains ~6 zinc atoms/mol.[35-37] Two major points of disagreement concern (1) whether freshly prepared enzyme contains 4 or 6 to 8 zinc atoms/mol and (2) whether zinc in excess of 4 correlates with enzyme activity. Although a concommitant loss of enzyme activity upon removal of zinc in excess of 4 zinc atoms/mol has been observed in two laboratories,[35,36] Sytkowski reports a reduction in zinc content to 4/mol in the absence of a change in enzyme activity.[37] The discrepancy between laboratories may reflect loss of either catalytic or structural zinc depending on the experimental conditions, together with little or no loss of activity upon removal of structural zinc. Further studies are clearly needed to establish both the number of zinc atoms and properties of the exchange of zinc by cobalt in the yeast alcohol dehydrogenase system.[36,37,114]

Cobalt exchange has been actively investigated in the horse liver enzyme, in an effort to provide modified enzyme for both spectroscopic and kinetic studies. The interpretation of studies employing zinc/cobalt hybrids is dependent on whether the catalytic or structural zinc is more susceptible to replacement by cobalt. Kinetically, two classes

of metal exchange sites were originally described. Considerable uncertainty has existed concerning whether the more rapidly exchanging metal ions are at a structural or catalytic site. This question has been addressed under varying conditions of temperature, pH and buffer and metal concentration by a range of techniques, which include (1) sensitivity of enzyme activity to replacement of zinc by cobalt, (2) quenching of NADH fluorescence by cobalt in zinc/cobalt hybrids, (3) extinction coefficient changes at 340, 655, and 740 nm with the progressive increase in cobalt content from 0 to 4 g-atom/mol, (4) effects of 1,10-phenanthroline on cobalt content and spectroscopic properties, and (5) the increase in the relaxation rate of the protons of water due to the presence of enzyme-bound, paramagnetic cobalt.[38-43] Whereas magnetic resonance studies support a rapid exchange of catalytic zinc,[42,43] the majority of experimental probes support cobalt substitution initially at a structural site.[38-41]

In contrast to reports indicating the preferential exchange of either two structural or two catalytic metals, the findings of Harvey and Barry implicate a unique type of hybrid.[44-56] These investigators observe that zinc replacement by cobalt is a triphasic kinetic process, leading to exchange of 1, 2, and 4 atoms of zinc after 1, 8, and 140 hr, respectively. From the absorption spectra of Co(1)/Zn(3)ADH vs. Co(4)ADH in the presence of NADH or 1,10-phenanthroline, the authors argue that a rapid exchange of a structural site zinc is followed by replacement of a catalytic zinc — i.e., that the Co(2)/Zn(2) hybrid has undergone exchange at *both* a structural and catalytic metal binding site.

A recent paper by Maret et al.[111] reports the preparation of a previously undetected hybrid, by treatment of crystaline suspensions of horse liver alcohol dehydrogenase with chelating agents (preferably dipicolinic acid). This (blue) Co(2)/Zn(2) hybrid is characterized by an absorption spectrum which differs from prior (green) Co(2)/Zn(2) hybrids in the 450 to 800-nm wavelength region. Although the authors conclude that the blue hybrid has undergone metal exchange exclusively at the catalytic site, assignment rests on the method of preparation and absorption spectrum of the hybrid.

As discussed above, horse liver alcohol dehydrogenase, carboxymethylated at Cys 46 by [1-^{13}C]-bromoacetate, has been prepared and characterized. Future studies, examining the paramagnetic effects of bound cobalt on the C-13 line width of carboxymethylated enzyme may provide considerable insight into the nature of the rapidly exchanging metal sites under a variety of experimental conditions. *Until this issue is resolved, mechanistic deductions from cobalt exchanged enzyme are best restricted to studies involving fully exchanged protein, Co(4)ADH.*

C. Tertiary Structural Deductions from the Primary Structure of Yeast Alcohol Dehydrogenase

A tentative tertiary structure has been constructed for yeast alcohol dehydrogenase employing a sequence alignment for the horse liver and yeast enzymes which optimizes positional identities and minimizes gaps and the tertiary structure of the horse liver enzyme.[46] Despite the low overall sequence homology, such an operation suggests that the yeast and horse liver alcohol dehydrogenase contain highly conserved tertiary structures. Out of 93 internal hydrophobic residues in the horse liver enzyme, 90 positions in the yeast enzyme are identical or similar. Glycine residues are overrepresented in identical positions (18), and the changes to larger residues observed to occur in 16 positions have been compensated by a reduction in the size of interacting residues.

A comparison of residues in the horse liver enzyme that participate in coenzyme or substrate binding are of interest (Table 5). As already discussed, the ligands to the active site zinc atom are conserved. Note that one of the two residues proposed to catalyze proton exchange between the active site and solvent, Ser 48, has undergone a

Table 5
RESIDUES AT POSITIONS THAT PARTICIPATE IN ENZYMATIC FUNCTIONS[46]

Function	Horse liver*	Yeast
Ligands to active site zinc atom	46 Cys	Cys
	67 His	His
	174 Cys	Cys
Additional hydrophilic residues in the active site cleft	48 Ser	Thr
	51 His	His
Nicotinamide binding pocket	178 Thr	Thr
Pyrophosphate binding pocket	47 Arg	His
	269 Ile	Ser
Adenine binding pocket	198 Phe	Ser
	222 Val	Ile
	224 Ile	Gly
	243 Pro	Phe
	250 Ile	Val
	274Thr	Ala
	277 Thr	Ala
Substrate binding pocket	57 Leu	Trp
	93 Phe	Trp
	110 Phe	Asn
	116 Leu	Leu
	140 Phe	Tyr
	141 Leu	Thr
	296 Pro	Ala
	318 Ile	Ile

* The numbers refer to the sequence of the horse liver protein.

conservative replacement by threoinine. The residues lining the substrate binding pocket are more bulky for the yeast enzyme, consistent with the more limited substrate specificity of this enzyme. Regarding coenzyme binding, Thr 178 (proposed to hydrogen bond the carboxamide side chain of the nicotinamide ring) is conserved; whereas Arg 47 (proposed to bind the pyrophosphate moiety of coenzyme) and the residues lining the adenine pocket are not conserved. Apparently, the further away one goes from the catalytically functional portion of the active site, the greater the change in residues.

Two residues proposed to stabilize the structural zinc binding loop in the horse liver enzyme have been changed in the yeast enzyme: Lys 323 and Ser 325, which appear to anchor the second zinc containing loop by hydrogen bonding to main chain carbonyls, have become a valine and glycine, respectively, in yeast alcohol dehydrogenase. The absence of these stabilizations in the yeast enzyme may lead to a more facile loss or exchange of zinc from the putative second zinc binding site.

IV. SOLUTION PROBES OF COENZYME AND SUBSTRATE BINDING

A. Coenzyme Binding

A number of investigators have determined the thermodynamic parameters for coenzyme binding to dehydrogenases, by both calorimetric techniques and from the temperature dependence of coenzyme binding. Although lactate, malate, and alcohol dehydrogenases are characterized by common tertiary structural features in their

coenzyme binding domains, these similarities do not necessarily extend to the energetics of coenzyme binding.[47] In Table 6, $\Delta G°$, $\Delta H°$, and $\Delta S°$ are summarized for the binding of NAD^+ to five dehydrogenases; *the notable feature of the interaction of NAD^+ with horse liver alcohol dehydrogenase is that this process is almost entirely entropy driven.* These studies have been extended to include a series of coenzyme analogs.[48] As summarized in Table 7, the binding of AMP, ADP, and ADP-ribose behave "normally", i.e., the dominant thermodynamic term for complex formation is $\Delta H°$. Analogous to NAD^+ binding, the addition of NADH to horse liver alcohol dehydrogenase is characterized by a large positive change in $\Delta S°$. These findings are in good agreement with an early study of Dalziel;[115] and corroborate the crystallographic data, which indicate a facile interaction of ADPR with apoenzyme, together with large protein conformational changes in the presence of NAD^+ or NADH complexes. It is of interest that yeast alcohol dehydrogenase more closely resembles malate and lactate than horse liver alcohol dehydrogenase, in the thermodynamic properties of coenzyme binding. This difference between yeast and horse liver alcohol dehydrogenase may be relevant to the apparent greater complexity in relating the pH dependence of substrate binding to catalysis for horse liver alcohol dehydrogenase.

Early investigations of the magnitude and pH dependence of coenzyme binding to horse liver alcohol dehydrogenase indicated (1) an $\sim$*500*-fold tighter binding of NADH (K_d = 0.27 μM) than NAD^+ (K_d = 138 μM) at pH 7.1, (2) opposite pH dependencies for NADH binding (decreases with increasing pH) vs. NAD^+ binding (increases with increasing pH), and (3) a higher pK value for a coenzyme linked functional group for E(pK = 9.6) than $E \cdot NAD^+$ (pK = 8.1) complexes.[49]

Recently, Danenberg et al. synthesized the coenzyme analog, phenyladenine-dinucleotide (PhAD, Figure 4), in an effort to determine the structural features responsible for protein conformational states facilitating alcohol vs. aldehyde binding.[50] More specifically, the authors addressed whether it is the planar aromatic ring or the net positive charge on the nicotinamide ring which distinguishes NAD^+ from NADH binding. PhAD was observed to be a competitive inhibitor of NADH, with K_{ii} increasing 150 fold over the pH range 6 to 9. A comparison of PhAD to the charged analog, pyridine adenine dinucleotide ($PyAD^+$, Figure 4), indicated a 500-fold greater binding of PhAD at pH 7. Both of these results suggest that PhAD more closely resembles NADH than NAD^+; and that *the net charge on the nicotinamide ring rather than its shape distinguishes NADH from NAD^+ binding.*

The differential effect of NADH and NAD^+ on the pK of an enzyme-bound functional group has been extensively investigated. Shore et al. monitored proton production for $E \cdot NAD^+$ and $E \cdot NAD^+ \cdot$ trifluoroethanol formation in an effort to determine the position in the enzyme reaction sequence involving proton release.[51] Consistent with the proposal of Dalziel that the pK of a functional group is perturbed from 9.6 in free enzyme to 8.1 in the binary complex of enzyme and NAD^+, the release of 0.5 protons per enzyme equivalent at pH = 7.6 $\pm$ 0.2 was observed in the presence of saturating NAD^+. Formation of an abortive, ternary complex with trifluoroethanol led to the release of an additional 0.5 protons implying further perturbation of a functional group pK below 7.6 upon addition of alcohol to $E \cdot NAD^+$. Subsequent studies revealed that both proton release and the quenching of protein fluorescence on NAD^+ binding were characterized by similar pH dependencies.[51-53] A comparison of rate constants for these two processes at pH indicated k = 250s^{-1} and 300 to 400s^{-1}, respectively, implicating a kinetic step (possibly a conformational isomerization) prior to NADH production from the $E \cdot NAD \cdot$ ethanol complex (k_o = 130s^{-1}).

Initial studies of the pH dependence of rate constants for the binding of NAD^+ vs. ADPR and NADH indicated pKs of 10 $\pm$ 0.2 and 9.0 $\pm$ 0.2, respectively; in all cases

Table 6
THERMODYNAMIC PARAMETERS FOR THE BINDING OF NAD⁺ TO DEHYDROGENASES, 25° AND pH 7.6[47]

Enzyme	−ΔG° kcal·mol⁻¹	−ΔH° kcal·mol⁻¹	−ΔS° cal·K⁻¹·mol⁻¹
Pig heart m-MDH	3.7	10.2 ± 0.7	21.7 ± 2.7
Beef heart LDH	4.3	8.5 ± 0.4	14.2 ± 1.4
Rabbit muscle LDH	3.8	6.3 ± 0.2	8.3 ± 1.0
Yeast ADH	4.3	8.9 ± 0.4	15.4 ± 1.5
Horse liver ADH	4.6	1.0 ± 0.2	−12.1 ± 1.0

Table 7[48]
THERMODYNAMIC PARAMETERS FOR THE BINDING OF COENZYME FRAGMENTS AND COENZYMES TO HORSE LIVER ALCOHOL DEHYDROGENASES, 25° AND pH 7.0[48]

Ligand	−ΔG° kcal·mol⁻¹	−ΔH° kcal·mol⁻¹	−ΔS° cal·K⁻¹,mol⁻¹
AMP	5.6	10.2 ± 0.8	15.5 ± 3.1
ADP	4.9	9.6 ± 0.6	15.9 ± 2.2
ADP-ribose	6.1	8.8 ± 0.3	9.1 ± 1.5
NAD⁺	5.2	0.86 ± 0.22	−14.4 ± 0.96
NADH	8.9	−0.60 ± 0.79	−27.7 ± 3.1

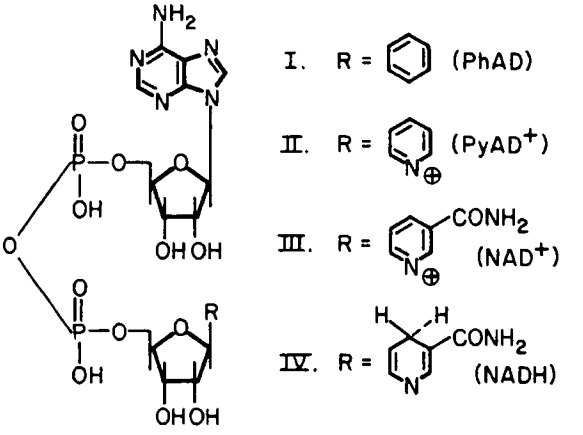


FIGURE 4. Structural comparison of phenyl dinucleotide and pyridine adenine dinucleotide to NAD⁺ and NADH.

the coenzyme was observed to bind faster to the protonated form of enzyme.[54] A recent investigation of this problem indicates that identical pKs control the binding of both NAD⁺ and NADH, pK = 9.2.[55] In contrast to k_{on}, the pH dependence of k_{off} is markedly different for oxidized vs. reduced coenzyme, Figure 5. It is the latter property which leads to the unique property of NAD⁺ to reduce the pK of an essential functional group.

The identity of this functional group, "X", has been the subject of considerable speculation. Since the overall chemical reaction requires proton release from the hy-

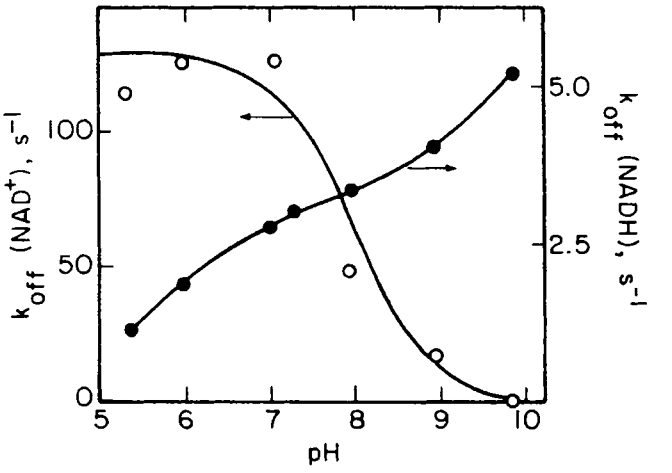


FIGURE 5. pH dependence of rate constants for the dissociation of NAD^+ (O—O) and NADH (●—●) from horse liver alcohol dehydrogenase.

droxyl group of alcohol to water, a logical candidate for "X" has been the active site zinc bound water. The ε-amino group of Lys 228, implicated in coenzyme binding, has also been considered as a candidate for "X". In an effort to identify "X", Parker et al. compared the effects of NAD^+ and trifluoroethanol on the pH dependence of fluorescence quenching in native enzyme (NAT) to enzyme carboxymethylated at Cys 46 (CM) or acetamidylated at Lys 228 (AI).[56] Carboxymethylation of one of the sulfur ligands to the active site zinc is expected to increase the net positive charge on zinc thereby reducing the pK of $ZnOH_2$; and acetamidylation of Lys 228 is expected to raise its pK by 2 pH units. As summarized in Scheme I, all three proteins indicate similar pKs for apoenzyme. Although the CM and AI enzymes appear unable to undergo the coupled pK perturbation-conformation change(s) observed on NAD^+ binding to native enzyme, addition of trifluoroethanol reduces the pK of "X" far below 7.6 in all cases. These studies initially led Parker et al. to suggest that neither $ZnOH_2$ nor Lys 228 is the pK = 9.2 functional group in apoenzyme.

The analysis of fluorescence quenching in horse liver alcohol dehydrogenase is considerably simplified by the fact that there are only two tryptophan residues per subunit of defined spatial relationship: Trp 15, close to the protein surface and Trp 314, buried in a hydrophobic milieu at the interface of subunits (cf. Table 2). By examination of excitation and emission spectra in the presence of either potassium iodide or NAD^+ and alcohol, several laboratories report the resolution of Trp 15 from Trp 314 fluorescence.[57,58] For example, excitation at 280 nm is reported to lead to a preferential quench (of Trp 15) by potassium iodide, whereas excitation at 295 nm leads to a preferential quench (of Trp 314) by NAD^+ and trifluoroethanol. Since the emission spectrum of Trp 314 and the absorption spectrum of NAD^+ do not overlap significantly, quenching of the fluorescence of buried Trp 314 by NAD^+ is concluded to require some protein conformation changes in the $E \cdot NAD^+$ complex. The large degree of quenching of Trp 314 upon ternary complex formation, and identity of the spectrum of the quenched species with that due to alkaline pH, led Laws and Shore to propose ionization of tyrosine(s) coupled to a protein conformational change as a factor in the quenching of protein fluorescence. The observation of a 242 nm shoulder in the difference spectrum, [$E \cdot NAD^+ \cdot$ trifluoroethanol] − [E], was presented as confirmatory evidence.[59]

Although it was originally suggested that the elusive "X" was Tyr 286, subsequent studies demonstrated the same spectral shoulder at 242 nm for both the E·NADH·isobutyramide and E·NAD⁺·trifluoroethanol complexes.[60] This observation necessitates that proton release coupled to NAD⁺ (but not NADH) binding occurs from a group other than Tyr 286. In addition, the studies of Parker et al. on chemically modified enzyme require reexamination, in light of a role for tyrosine ionization. For example, the similar pH dependencies for native, CM, and AI apoenzyme fluorescence (pK = 9.8, Scheme 1) may reflect tyrosine ionization as opposed to a conformational change linked to ionization of "X". Interpretation of the differences among pKs in the E·NAD⁺ complexes is difficult; since these differences may arise either from an uncoupling of pK perturbation from NAD⁺ binding, as originally proposed, or from intrinsic differences in pK among native, CM, and AI enzyme. *Although the use of chemically modified enzyme to assign pKs to catalytically functional groups is a valuable experimental approach, the available data for horse liver alcohol dehydrogenase have not yet permitted an unambiguous identification of "X".* Recent studies by Dunn et al. suggest that the pK of tyrosine can be successfully separated from the pK of "X" in 50% v/v DMSO/water;[61] extension of these studies to include chemically modified enzyme may provide valuable information concerning the critical functional group involved in coenzyme binding to horse liver alcohol dehydrogenase.

Early characterization of the interaction of coenzyme with yeast alcohol dehydrogenase focused on delineating the stoichiometry of coenzyme binding, with estimates ranging from 2 to 4 mol of coenzyme bound per tetrameric enzyme.[62,63] Discrepancies in the literature may be a function of heterogeneity of yeast alcohol dehydrogenase, especially with regard to total metal content. In this context it may be relevant that Grunow and Schöpp have reported on the presence of a proteolytic activity which copurifies with crystalline yeast alcohol dehydrogenase.[64]

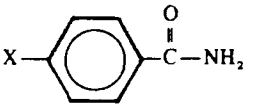
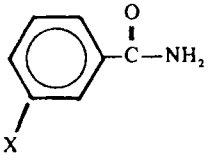
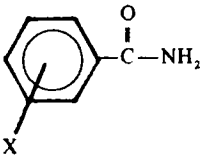
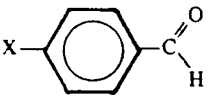
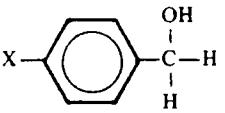
Sloan and Mildvan examined the conformation of NADH at the active site of yeast alcohol dehydrogenase, employing enzyme inactivated with a paramagnetic iodoacetamide analogy (I·).[65] Analogous with the horse liver enzyme, both substrates and coenzyme continue to bind with comparable affinity constants subsequent to alkylation. Further, metal analysis of enzyme carboxymethylated at pH 7 indicates little or no loss of zinc.[111] Examination of the effect of (I·) on the relaxation rates of six protons of NADH led to a proposed conformation for bound NADH which is analogous to the structure deduced for bound ADP-ribose from difference Fourier maps of the orthorhombic crystal form of horse liver alcohol dehydrogenase.

Although comparatively few studies have addressed the question of pK perturbations in coenzyme binding to yeast alcohol dehydrogenase, analysis of steady state kinetic parameters suggest (1) slightly faster binding and release of NAD⁺ to the protonated form of enzyme (pK ≃8) and (2) an increase in k_{on} vs. a decrease in k_{off} for NADH with decreasing pH.[66] Subsequent studies of the pH dependence of enzyme inactivation by the histidine reagent, diethylpyrocarbonate, indicate pKs of 7.1 for apoenzyme and the E·NAD⁺ complex, as opposed to a pK = 8.4 for inactivation of the E·NADH complex.[67] It appears (1) that a group of pK = 7.1 controls NADH but not NAD⁺ binding and (2) that this group undergoes a small perturbation to a higher pK in the presence of reduced coenzyme.

B. Substrate Binding

Both horse liver and yeast alcohol dehydrogenase are characterized by a preferred order of binding, in which the formation of enzyme-coenzyme precedes the addition of substrate. Consequently, investigations concerning substrate binding have largely involved substrate analogs or inhibitors. Mechanistically oriented studies have focused

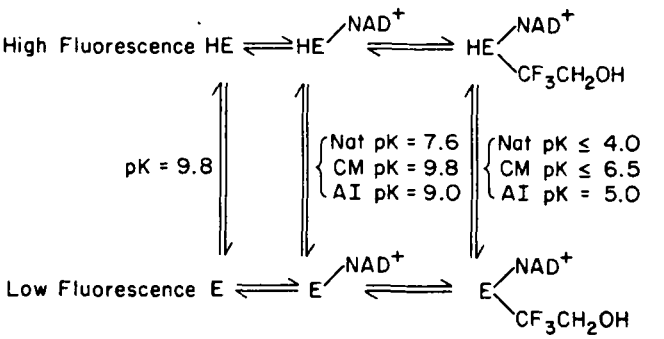
Table 8
ANALYSIS OF LIGAND BINDING TO ALCOHOL DEHYDROGENASE IN TERMS OF ELECTRONIC, HYDROPHOBIC AND STERIC FACTORS

Ligand	Best correlation[a]	Ref.
Horse liver ADH		
E · NADH + 	$\log K = -0.986\pi + 2.45$ $n = 6; r = 0.88$	68
E · NADH + 	$\log K = 0.921\sigma + 2.63$ $n = 7; r = 0.94$	68
E · NADH + 	$\log \frac{1}{K} = 0.453(\pm0.28)\pi - 0.804(\pm0.30)\sigma - 0.232(\pm0.17)E_s - 2.369(\pm0.20)$ $n = 14; r = 0.95$	70
Yeast ADH		
E · NADH + 	$\log \frac{1}{K} = 0.082(\pm0.13)\log P - 0.96(\pm0.20)\sigma^+ - 0.87(\pm0.12)$ $n = 6; r = 0.99$	71
E · NAD$^+$ + 	$\log \frac{1}{K} = 0.57(\pm0.16)\log P + 0.11(\pm0.12)R - 0.14(\pm0.26)$ $n = 6; r = 0.99$	71

[a] The substituent constants employed in the horse liver alcohol dehydrogenase study are the electronic Hammett constants σ, the hydrophobic constants Π_X, where $\Pi_X = \log P_X - \log P_H$, and the steric constants E_S. The constants employed in the yeast alcohol dehydrogenase study are the electronic Hammett constants σ^+, the hydrophobic constants P_X, and Van der Waals' radii, R. In the above equations, K is a dissociation constant.

hyde binding is unexpected, and suggests a variation in binding modes between aldehyde and alcohol substrates. Our ability to discern different ground state interactions for enzyme-bound aldehyde vs. alcohol may be relevant to a proposed role for intermediates in the alcohol dehydrogenase reaction (see Section V. B.).

By replacement of diamagnetic zinc by paramagnetic cobalt in horse liver alcohol dehydrogenase, Mildvan and co-workers have attempted to distinguish inner from second sphere complexes between the active site metal and substrate.[72] As discussed earlier, considerable uncertainty exists concerning the relative exchange rates of zinc by cobalt at the catalytic and structural metal binding sites. This ambiguity can be circumvented by employing fully cobalt substituted enzyme; and measurements of the longitudinal relaxation rates ($1/T_1$) of (1) the C_2 and $C_{4,5}$ protons of imidazole and (2) the



Scheme 1. A comparison of pK values for native, carboxymethylated, and acetamidated horse liver alcohol dehydrogenase, derived from fluorescence quenching studies.

on elucidating the factors that contribute to substrate binding, and relating these factors to catalysis. In addition, the central, long-standing issue regarding the mode of interaction of the carbonyl/carbinol oxygen of aldehyde/alcohol to the active site zinc has been addressed.

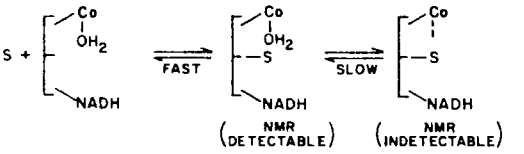
The broad substrate specificity of alcohol dehydrogenase has facilitated an investigation of structure-activity relationships. Sarma and Woronick measured dissociation constants for amide binding to E·NADH.[68] For substituted benzamides, these authors concluded that the position of the substituent is a major factor in determining whether electronic (meta substituents) or hydrophobic (para substituents) factors predominate (Table 8). On the premise that a single parameter correlation is inadequate in the analysis of ligands binding to macromolecules, Hansch and co-workers pioneered the application of multiple linear regression analysis:[69]

$$\delta x \log k(K) = \delta x \Delta G \text{ hydrophobic} + \delta x \Delta G \text{ electronic} + \delta x \Delta G \text{ steric} \quad (2)$$

According to Equation 2, the effect of substituents on the log of a rate or equilibrium constant can be factored into a linear combination of hydrophobic, electronic, and steric contributions. A collaborative effort between Hansch and Sarma led to a reexamination of benzamide binding according to Equation 2. It is encouraging that the best three parameter equations, summarized in Table 8,[70] indicate electronic and hydrophobic contributions similar to the original deductions of Sarma and Woronick.[68] *The observation that electron-releasing substituents facilitate amide binding is consistent with an interaction of the oxygen of the carbonyl functional group with an active site electrophile.* Hydrophobic interactions are of the greatest importance, as expected from the structure of the substrate binding pocket of horse liver alcohol dehydrogenase, deduced from X-ray crystallographic studies subsequent to the described inhibitor binding studies.

As considered in greater detail in the next section of this review, kinetic studies of yeast alcohol dehydrogenase indicate that substrate binding and release is rapid, relative to the interconversion of ternary complex, for aromatic substrates. Consequently, kinetic data have been used directly to analyze binding constants for a series of benzaldehydes and benzyl alcohols. The results of multiple linear regression analysis (Table 8) indicate the importance of electronic and hydrophobic contributions to benzaldehyde and benzyl alcohol binding, respectively.[71] The magnitude of these effects is in close agreement with the results for benzamide binding to horse liver alcohol dehydrogenase. The failure to observe a significant hydrophobic contribution to benzalde-

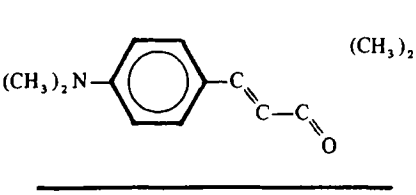
CH_3 and $-CH-$ protons of isobutyramide in the presence of the binary complex of Co(4)ADH and NADH have been reported.[42] Examination of the frequency dependence of $1/T_1$ supports a rapid exchange of bound and free ligand. On the assumption that only the catalytic cobalt affects the relaxation rate of the protons of imidazole and isobutyramide, calculated proton-to-cobalt distances are too long for direct coordination. In addition, correction for an additional paramagnetic contribution from a structural cobalt leads to an increase rather than decrease in the estimated distances. *Thus nuclear magnetic resonance studies of inhibitor binding support second sphere complexes, in marked contrast to X-ray studies of alcohol dehydrogenase which support direct coordination to the active site zinc.* As discussed earlier, a detailed assessment of the mode of interaction of substrate with the binary complex of the triclinic crystal form of enzyme and coenzyme requires further structural refinement. Nonetheless, the apparent discrepancy between X-ray crystallographic and nuclear magnetic resonance data poses the persistant question in enzymology: to what extent can crystal-packing forces stabilize an active site configuration which is different from the most stabile structure in solution? Sloan and Mildvan have suggested that the discrepancy between X-ray crystallographic and nuclear magnetic resonance data might reflect the presence of both second and inner sphere complexes in solution, together with a slow exchange of substrate from the inner coordination sphere of metal (Equation 3).

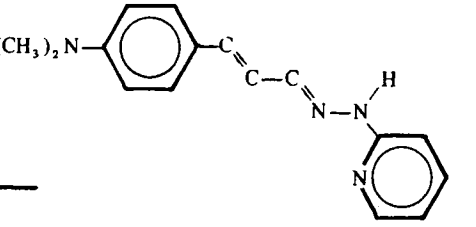


(3)

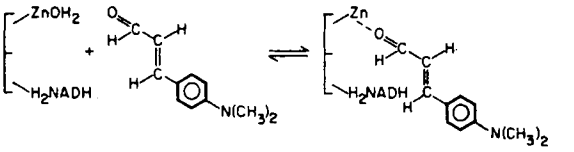
Dunn and co-workers have carefully studied the interaction of the chromophoric N,N-dimethylaminocinnaminaldehyde to E·NADH demonstrating the formation of a red-shifted spectroscopic intermediate; the properties of this intermediate have been explored with regard to (1) cofactor requirements for intermediate formation, (2) the kinetic properties of intermediate breakdown, and (3) the spectral properties of model divalent metal complexes which mimic the absorption properties of the enzyme bound intermediate. Substitution of NADH by 1,4,5,6-tetrahydronicotinamide adenine dinucleotide (H_2NADH) leads to a chromophoric species (λ_{max} = 468 nm; $\varepsilon_{max} = 5.8 \times 10^4 M^{-1}cm^{-1}$) which does not break down further to product.[73] For example, Dunn et al. report that the E·H_2NADH·I complex is stable for more than a month at 4°C (pH 5.98 and 8.72); in contrast to E·NADH·I species which has a half-life of only 0.5 and 23 sec. (at pH 5.98 and 8.72, respectively). Analogous to intermediate formation with E·NADH, the formation of the 468-nm chromophore in the presence of E·H_2NADH is independent of pH in the range of 6 to 11. Model studies employing M^{2+} complexes of both N,N-dimethylaminocinnaminaldehyde and N,N-dimethylaminocinnaminaldehyde-2′-pyridinohydrazone indicate red-shift spectral bands of 39 to 110 nm, depending on the nature of M^{2+} and solvent.[74] A comparison of the spectral properties of N,N-dimethylaminocinnaminaldehyde and N,N-dimethylaminocinnaminaldehyde-2′-pyridinohydrazone to their metal complexes is summarized in Table 9. The similarity of λ_{max} and ε_{max} for the Lewis acid complexes of these chromophores to the spectroscopic properties of E·NADH·I and E·H_2NADH·I, together with the pH independence of the formation of the enzyme bound chromophore, has led to the suggestion that N,N-dimethylaminocinnaminaldehyde coordinates to the active site zinc of alcohol dehydrogenase (Equation 4).

Table 9
SPECTRAL PROPERTIES OF N,N-DIMETHYLAMINOCINNAMINALDEHYDE AND N,N-DIMETHYLCINNAMINALDEHYDE-2′-PYRIDINOHYDRAZONE AND THEIR LEWIS ACID COMPLEXES[74]



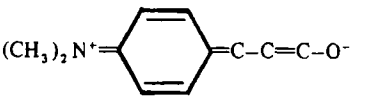


Conditions	λmax (nm)	εmax (M^{-1}, cm^{-1})×10^{-4}	λmax (nm)	εmax (M^{-1}, cm^{-1})×10^{-4}
$(C_2H_5)_2O$, —	366	3.4	371	5.6
$(C_2H_5)_2O$, $ZnCl_2$	431	~4.5	—	—
$(C_2H_5)_2O$, $SnCl_4$	452	—	—	—
H_2O, LADH · H_2NADH	468	5.8		
CH_3CN, —	377	3.3	374	5.1
CH_3CN, ZnI_2	—	—	428	4.5
CH_3CN, $CoCl_2$	—	—	430	3.6
CH_3CN, $MnCl_2$	—	—	415	—



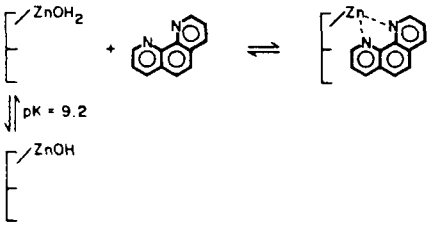
(4)

Although Equation 4 is consistent with the presence of zinc at the enzyme active site, it should be kept in mind that factors other than direct coordination of the carbonyl oxygen to zinc could cause a red shift in λ_{max} for the enzyme bound intermediate (e.g., stabilization of the positively rather than negatively charged end of the exited state dipole of N,N-dimethylaminocinnaminaldehyde,



by functional group(s) in the binding pocket).

The interaction of 1,10-phenanthroline with apoenzyme is well-defined from difference Fourier maps; as described earlier, the nitrogens of 1,10-phenanthroline appear to ligand to the active site zinc, displacing water. Originally De Traglia et al. measured the pH dependence for the addition of 1,10-phenanthroline to apoenzyme, observing a pK = 8.1; dissociation of 1,10-phenanthroline was found to be pH independent.[75] Reexamination of both 1,10-phenanthroline and 2,2-bipyridine association rate constants indicates faster binding to the protonated forms of enzyme, pK = 9.2, analogous to coenzyme.[76] These results are consistent with a mechanism in which ligand displacement can only occur from the acidic form (e.g., $ZnOH_2$) of enzyme, Equation 5.

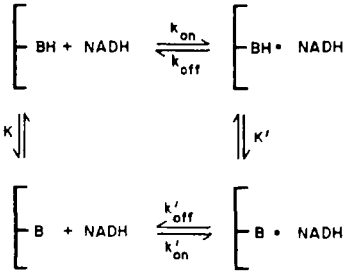


(5)

The pH dependence of 2,2-bipyridine binding has also been determined in the presence of imidazole and NAD⁺; whereas NAD⁺ leads to a pK = 7.6, the presence of imidazole eliminates the pH dependence of 2,2-bipyridine binding.[76] These results support the assignment of a pK = 9.2 to the active site $ZnOH_2$ in apoenzyme. Although further studies of the pH dependence of ligand binding to enzyme carboxymethylated at Cys 46 and E·NADH would be of value in corroborating the assignment of pKs in the horse liver alcohol dehydrogenase, the pH independence of N,N-dimethylaminocinnaminaldehyde binding up to pH 11 requires reexamination in light of the properties of 1,10-phenanthroline and 2,2-bipyridine binding. If Equation 5 is correct, displacement of water by N,N-dimethylaminocinnaminaldehyde should also reflect the pK of $ZnOH_2$. The absence of a pH dependence suggests either that the spectral properties of N,N-dimethylaminocinnaminaldehyde are not a consequence of inner sphere complexation to M^{2+}, coupled to water loss; or that the pK of $ZnOH_2$ in the E·NADH and E·H_2NADH complexes has been perturbed from a pK = 9.2 to a pK greater than the experimentally accessible pH range (pK > 11).

A potentially important distinction between 1,10-phenanthroline and N,N-dimethylaminocinnaminaldehyde binding is the increase in the coordination number of zinc from four to five in the 1,10-phenanthroline complex. A similar mechanism for N,N-dimethylaminocinnaminaldehyde binding would lead to pentacoordinate zinc. Although formation of five coordinate zinc is expected to be less sensitive to the ionization state of $ZnOH_2$ than a mechanism involving water displacement, complexation to zinc should still reflect the different electrostatic properties of metal in the $ZnOH_2$ vs. ZnOH complexes.

The most likely explanation for the pH independence of N,n-dimethylaminocinnaminaldehyde binding is the perturbation of the pK of the active site residue ($ZnOH_2$) in the presence of reduced coenzyme. As discussed earlier, the protonated form of an enzyme functional group (pK = 9.2) is proposed to control the rate of addition of both oxidized and reduced coenzyme to horse liver alcohol dehydrogenase. In contrast to the pH dependence of K_{off} for NAD⁺ release, which indicates faster dissociation from the protonated form of an enzyme functional group, pK = 7.6, the release of NADH increases with increasing pH. According to Equation 6, the magnitude of the pK perturbation due to NADH binding is a function of the relative magnitude of binding and dissociation rate constants:



(6)

where $pK' = pK + \log k_{on}/k_{on}' + \log k_{off}'/k_{off}$. Since k_{on}/k_{on}' and k_{off}'/k_{off} are both greater than one, $pK' > pK$. A quantitative assessment of pK would be possible in the event that precise measurements of k_{on}/k_{on}' and k_{off}'/k_{off} were available.

As discussed in the context of coenzyme binding, proton release studies by Shore and co-workers indicate that addition of trifluoroethanol to complexes of horse liver alcohol dehydrogenase and NAD^+ leads to the perturbation of a functional group from $pK = 7.6$ to $pK < 5$.[51] In a recent study, Kvassman and Pettersson measured the pH dependence of trifluoroethanol dissociation, observing a pK of 4.3; *importantly, trifluoroethanol is observed to dissociate faster from the protonated form of enzyme.*[77] This result may simply reflect a preferred pathway for binary and ternary complex formation through the protonated form of enzyme, together with the stepwise perturbation of an enzyme-bound functional group from $pK = 9.2$ (E) to $pK = 7.6$ ($E \cdot NAD^+$) to $pK = 4.3$ ($E'NAD^+ \cdot CF_3CH_2OH$), as illustrated in Scheme 2 for E–BH = $ZnOH_2$. As an alternative to Scheme 2, Kvassman and Pettersson have proposed that the pK controlling trifluoroethanol binding is the ionization of metal-bound alcohol, subsequent to displacement of metal-bound water (Scheme 3).

V. KINETIC INVESTIGATIONS OF MECHANISM AND TRANSITION STATE STRUCTURE

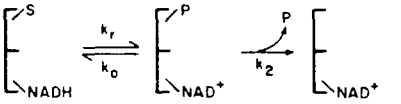
A. Liver Alcohol Dehydrogenase

The kinetic properties of alcohol dehydrogenase have been extensively characterized over a period of more than two decades. Early studies were largely concerned with demonstrating that the preferred kinetic pathway involves the addition of coenzyme followed by substrate, and that coenzyme release is rate determining in the steady state interconversion of acetaldehyde-ethanol[78] (Scheme 4).

The demonstration by Bernhard and co-workers of biphasic kinetics in the transient state reduction of aromatic aldehydes represents the beginning of intensive kinetic investigations focused on the elucidation of chemical mechanism.[79] In addition, the observation that each of the two kinetic processes (subsequently shown to reflect a rapid aldehyde reduction, followed by slow desorption of alcohol) was characterized by equal optical density changes led Bernhard et al. to propose "half of the sites" reactivity for horse liver alcohol dehydrogenase.[79]

The latter phenomenon has been pursued by a number of investigators, with conflicting results and interpretation.[80-83] Although it is generally agreed that pre-steady-state amplitudes approach the concentration of enzyme activity sites for the acetaldehyde-ethanol interconversion and benzyl alcohol oxidation, the reported magnitudes of amplitudes for *para*-substituted benzaldehyde reduction vary from $\sim$0.5 to $\sim$1.0. A persistent problem in evaluating these amplitudes is that benzaldehyde reduction occurs largely within the dead time of the stopped flow instrumentation under conditions of saturating substrate concentration. Attempting to circumvent this problem, Dunn et al. reinvestigated the relative magnitude of pre-steady state amplitudes for the reduction of benzaldehydes by [4-^{2}H] NADH (where $k_{NADH}/k_{[4\text{-}^2H]NADH} = 2.3$); these authors report persistent, less than stoichiometric optical density changes, which vary from 0.5 (*p*-NO_2) to 0.65 – 0.70 (*p*-H and *p*-Cl).[84]

Among the mechanisms proposed to explain these observations are (1) incorrectly assigned extinction coefficients for ternary complexes, (2) equilibria constants near unity for the interconversion of ternary complex, and (3) partitioning of the $E \cdot NAD^+ \cdot$ alcohol complexes between $E \cdot NADH \cdot$ aldehyde (k_o) and $E \cdot NAD^+$ (k_2), where $k_r = k_o + k_2$.

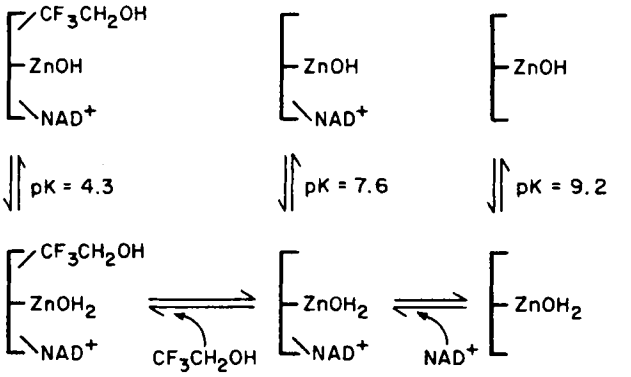


(7)

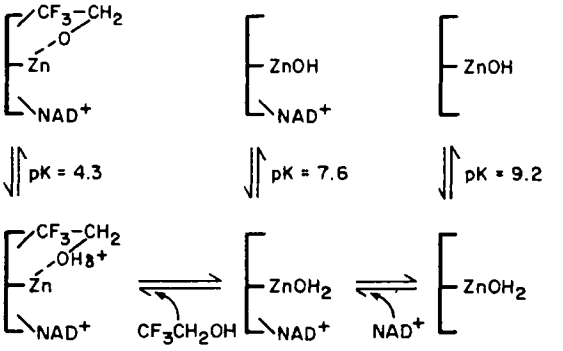
As will be discussed, large substituent effects on k_r/k_o have been demonstrated for both the horse liver and yeast alcohol dehydrogenase catalyzed interconversion of aromatic substrates, suggesting that the observation of marked biphasicity for a range of *para*-substituted benzaldehydes is not due to mechanisms, (2) and (3) above.[84] One critical factor in determining the magnitude of burst amplitudes appears to be the correct assignment of extinction coefficients to the relevant enzyme bound intermediates. The data of Dunn et al. for unsubstituted benzaldehyde are essentially identical to those of Weidig et al.[82] and Kvassman and Pettersson;[83] it is the application of a correction factor for absorption due to the abortive E·NAD⁺·pyrazole complex at 330 nm which reduces the magnitude of burst amplitudes for benzaldehyde reduction from ~0.90 (Weidig et al., no correction) and 0.87 (Kvassman and Pettersson, no correction) to 0.65 (Dunn et al., after a 13.3% correction to the total optical density change). It should be noted that early molar extinction studies failed to indicate significant absorbance due to E·NAD⁺·pyrazole at 330 nm.[116,117] In addition, the extinction coefficient correction by Dunn et al. was determined by conversion of E·NADH to E·NAD⁺·pyrazole at a wavelength above the isosbestic point for bound vs. free NADH, so that the observed increase in absorbance contains a contribution from free NADH. The situation is somewhat more complicated in the case of *p*-NO_2 benzaldehyde reduction, which even in the absence of a correction factor is characterized by a burst amplitude considerably less than 100%. Further characterization of this substrate appears necessary. In the event that the data of Dunn et al. are corroborated, it will be necessary to explain a "half of the sites" mechanism which involves burst amplitudes greater than 50% of the enzyme active sites.

Shore and Gutfreund were the first to demonstrate that the pre-steady state oxidation of ethanol vs. ethanol-d_5 is characterized by a large primary hydrogen isotope effect.[85] Using the criterion of primary hydrogen isotope effects (in the range of 3 to 6) as evidence for a rate determining hydrogen transfer between coenzyme and substrate, the effect of variations in substrate structure and pH on the chemical transformation step(s) has been investigated. Initial studies of structure reactivity correlations in the horse liver alcohol dehydrogenase reaction focused on the transient state reduction of aromatic aldehydes. The misleading conclusion that aldehyde reduction is insensitive to electronic substituent[86] may reflect the kinetic complexity of the measured parameters as well as the limited range of substrates studied. Hardman et al. examined benzyl alcohol oxidation in the transient state, under conditions where the oxidation of *p*-CH_3O benzyl alcohol is characterized by a hydrogen isotope effect of 4.3. Rate constants measured at saturation levels of alcohol were obtained by correction of the burst rate constant for a contribution from the steady-state rate constant. As summarized in Table 10, electronic substituents have a relatively small effect on the rate of hydride transfer within a series of alcohols.[87] Analogous structure-reactivity correlations were first observed in the yeast alcohol dehydrogenase reaction. As discussed in Section V.B., multiple linear regression analyses of data in the yeast alcohol dehydrogenase system have allowed a separation of the relative contributions of electronic, steric, and hydrophobic factors to k_{cat}.

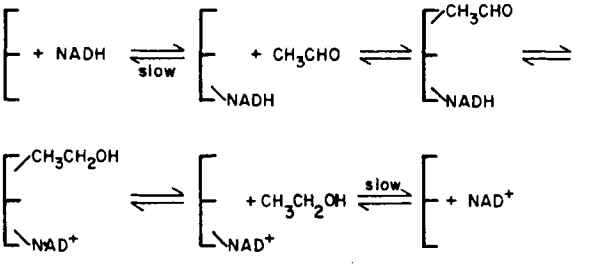
Numerous investigators have examined pH effects under pre-steady state kinetic conditions. The early observation of a group of pK = 6.4 controlling the pre-steady-state oxidation of ethanol[88] did not appear to extend to aromatic alcohol oxidation.[89] A recent reinvestigation of benzyl alcohol oxidation over a wider pH range (pH 5 to



Scheme 2. pK values controlling coenzyme and inhibitor binding to horse liver alcohol dehydrogenase. The ionizing group has been attributed to $ZnOH_2$ in all cases.

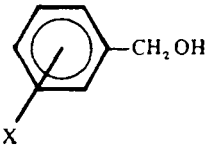


Scheme 3. pK values controlling coenzyme and inhibitor binding to horse liver alcohol dehydrogenase. The ionizing group has been attributed to $ZnOH_2$, with the important exception of the pK = 4.3 group suggested to reflect inhibitor ionization (77).



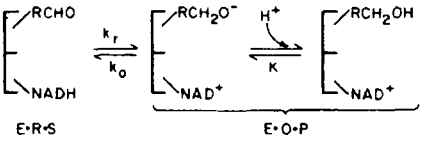
Scheme 4. Ordered kinetic scheme, involving rate limiting coenzyme dissociation for the interconversion of acetaldehyde/ethanol, catalyzed by horse liver alcohol dehydrogenase.

Table 10
EFFECT OF SUBSTRATE STRUCTURE ON THE RATE CONSTANT FOR HYDROGEN TRANSFER FROM ALCOHOL TO NAD^+, CATALYZED BY HORSE LIVER ALCOHOL DEHYDROGENASE[87]

Substrate		k_{ox}, s^{-1}
	X = *p*-CH_3O	19.6
	p-CH_3	16.3
	p-H	13.8
	m-CH_3	7.5
	m-Cl	10.9
	p-Cl	10.0
	m-NO_2	3.5

9) indicates that *in addition to aliphatic alcohols, the oxidation of aromatic alcohols requires the free base form of a functional group, pK = 6.4,*[90] Figure 6. In contrast to alcohol oxidation, *the pre-steady-state reduction of benzaldehyde by [4-²H]NADH and β-naphthaldehyde by NADH undergoes no more than a two fold change in rate from pH 6.0 to 9.0*(Table 11).[89,90]

The marked asymmetry in the pH dependence of pre-steady-state rate constants for aldehyde reduction vs. alcohol oxidation has received considerable attention in the literature. As Kvassman and Pettersson point out, the principle of microscopic reversibility requires hydrogen transfer from coenzyme to substrate to be catalyzed in the direction of aldehyde reduction in the event that alcohol oxidation is base catalyzed.[90] These authors argue that proton loss from alcohol to solvent must precede substrate oxidation, Equation 8.



(8)

According to Equation 8, the pK controlling catalysis in the direction of alcohol oxidation reflects the perturbed pK of alcohol, complexed to the active site zinc. As discussed earlier, a number of experiments (e.g., the pH dependence of rate constants for NADH binding and release; and the pH independence of N,N-dimethylaminocinnaminaldehyde binding to E·NADH) support the perturbation of the pK of a critical enzyme functional group (e.g., $ZnOH_2$) above the experimentally accessible pH range; and the pH independence of benzaldehyde reduction almost certainly reflects a similar perturbation. *Importantly, as long as the potential for pK perturbation in the enzyme-substrate complex exists, asymmetry of pH dependencies of pre-steady-state rate constants does not indicate the position in the reaction sequence involving loss of the alcohol derived proton to solvent.*

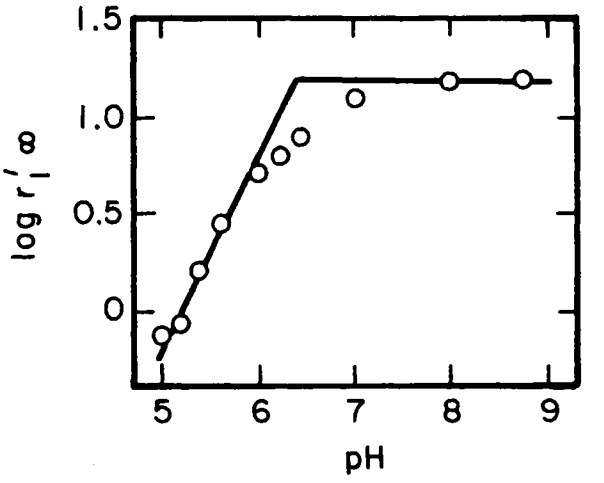


FIGURE 6. pH dependence of first order rate constants at saturating substrate ($r_i'^{\infty}$) for the transient state oxidation of benzyl alcohol, catalyzed by horse liver alcohol dehydrogenase.

Table 11
pH DEPENDENCE OF RATE CONSTANTS FOR THE PRE-STEADY-STATE REDUCTION OF AROMATIC ALDEHYDES, CATALYZED BY HORSE LIVER ALCOHOL DEHYDROGENASE[89,90]

Substrate	pH	k_{cat} s^{-1}
β-naphthaldehyde, NADH	6.0	133
	7.0	143
	7.7	173
	8.2	180
	8.2	170
	8.75	300
	9.9	245
Benzaldehyde, [4-^{2}H]NADH	7.9	150
	8.75	150
	10.0	130

A more compelling argument for the mechanism illustrated in Equation 8 is the large difference in pK observed for trifluoroethanol dissociation (pK = 4.3) vs. ethanol and benzyl alcohol oxidation (pK = 6.4). The observed trend in pKs is consistent with the ionization of alcohol, as opposed to an enzyme functional group.[77] Extension of these studies to include a range of substrate alcohols would be extremely valuable in attempting to identify the ionizing group in $E \cdot NAD^+ \cdot RCH_2OH$ complexes. Unless a clear-cut correlation can be made between the intrinsic pK of the alcohol functional group and the pH dependence for alcohol oxidation, however, it may be difficult to distinguish direct effects of variations in alcohol structure on bound alcohol ionization from indirect effects on the perturbation of an enzyme functional group.

Our ability to distinguish stepwise from concerted modes of acid-base catalysis is intimately related to the mode of substrate interaction with the active site zinc. Despite the difficulties inherent in solvent isotope effect measurements in enzyme reactions,

the magnitude of these effects can provide insight into the extent of coupling of proton transfer to heavy atom rearrangements. Recent studies of Schmidt et al. indicate an isotope effect of 1.0 ± 0.1 for the transient reflecting aromatic aldehyde reduction. In the case of transients reflecting benzyl alcohol dissociation or benzyl alcohol oxidation, which decrease and increase with increasing pH, respectively, isotope effects are observed to be pH dependent. For alcohol dissociation, k_{H_2O}/k_{D_2O} decreased from 2.57 to 0.87 in the pH range 10.2 to 6.0, consistent with a normal isotope effect on the pK and little or no isotope effect on the limiting rate constant; whereas solvent isotope effects for benzyl alcohol oxidation remain slightly inverse at all pHs: 0.68, 0.95, and 0.86 at pH 6.0, 7.0, and 8.75, respectively.[91] Analogous to earlier studies on yeast alcohol dehydrogenase, *these data rule out a mechanism invoving concerted acid-base catalysis of hydride transfer*. The fact that the isotope effect for alcohol oxidation is essentially pH invariant suggests little or no effect on D_2O on pK. Since the same functional group has been concluded to control both alcohol release and oxidation from the E·NAD alcohol complex, the lack of correspondence between the solvent isotope effect on pK for these two processes is unexpected. Until this discrepancy is resolved, solvent isotope effects on pK cannot provide any information concerning the mode of interaction of substrate with metal bound water (see Section V.B.).

Unlike reaction mechanism studies in solution, studies on enzymes are limited by our ability to alter the nature and concentration of the catalytically relevant functional groups. The availability of cobalt-substituted and carboxymethylated alcohol dehydrogenase provides catalytic species with altered electrostatic properties at the active site metal. Kinetic studies on fully substituted cobalt alcohol dehydrogenase were carried out by Shore and Santiago, who observed that Co(4)ADH and Zn(4)ADH catalyze hydrogen transfer from substrate to coenzyme at comparable rates (Table 12). In light of the well-documented pH dependence of alcohol oxidation with Zn(4)ADH, the agreement between rate constants for zinc and cobalt enzyme may be fortuitous. Thus a distinction between effects of metal substitution on pK vs. limiting rate constants is essential before meaningful mechanistic deductions can be made. Although a similar difficulty pertains to a comparison of rate constants for ethanol oxidation at pH 8.0, catalyzed by native and carboxymethylated enzyme[32] (Table 12), Hardman and co-workers have examined rate constants for a series of aliphatic alcohols[92,93] (Table 13). In contrast to native enzyme, where rate constants for the chemical step can only be obtained from transient state kinetics, a comparison of ethanol to ethanol-d_5 indicates an isotope effect of 3 to 4; and the data summarized in Table 13 for carboxymethylated enzyme were collected under steady state conditions. The choice of aliphatic alcohols for the comparison increases the potential for hydrophobic and steric contributions to the observed rate constants. In addition, isotope effects have only been measured for a single alcohol in the studies of carboxymethylated enzyme, raising the possibility that steps other than hydrogen transfer contribute to V_{max}. Nonetheless, as discussed by Hardman, plots of log k vs. the Taft substituent constant, σ^*, indicate straight lines of comparable slope for both native and carboxymethylated enzyme. As discussed earlier, neutralization of charge on one of the ligands to the active site zinc is expected to alter the electrostatic properties of the active site metal, and this alteration should be reflected in the sensitivity of rate to the electronic structure of the substrate. *Thus the observation of similar structure-reactivity correlations for native and carboxymethylated enzyme is contrary to our expectations and must be accounted for in any complete description of chemical mechanism in the horse liver alcohol dehydrogenase reaction.* Additional data for a series of substituted benzyl alcohols with carboxymethylated enzyme would be quite valuable in this regard.

Plapp and co-workers have explored a different approach toward the preparation

Table 12
KINETIC PARAMETERS FOR MODIFIED LIVER ALCOHOL DEHYDROGENASE

Parameter	Native	Modified enzyme: Co(4)ADH	Modified enzyme: CM-ADH	Ref.
V_{max}/E, s^{-1}[a]				
pH 7.0	3.1	3.1	—	39
10.0	4.1	3.1	0.15	32
k_0, s^{-1}[a]				
pH 7.0	138	92	—	39
8.0	140	—	0.19	93
k_{off}(NADH), s^{-1}				
pH 7.0	3.7	3.4	—	39
8.0	5	—	3.2	93

[a] Turnover number for oxidation of ethanol.
[b] Rate constant for the hydrogen transfer step, ethanol to NAD^+ within the ternary complex.

Table 13
EFFECT OF SUBSTRATE STRUCTURE ON RATE CONSTANTS FOR ALCOHOL OXIDATION, CATALYZED BY NATIVE VS. CARBOXYMETHYLATED HORSE LIVER ALCOHOL DEHYDROGENASE[92,93]

Substrate	Enzyme: Native, k_{cat}, s^{-1}	Enzyme: Carboxymethyl, V_{max}/E, s^{-1}
Propanol	360	0.32
Cyclohexanol	160	—
Cyclohexylmethanol	—	0.13
Ethanol	140	0.19
Benzyl alcohol	49	0.16
2-Methoxyethanol	7.2	0.009

and characterization of modified enzyme, demonstrating that alkylation of amino groups of liver alcohol dehydrogenase leads to a change in the rate determining step. Using either picolinimidylated or hydroxybutyrimidylated enzyme, these investigators observe deuterium isotope effects of 4.8 and 2 to 4 for ethanol[94] and benzyl alcohol[95] oxidation, respectively, under conditions of the steady state. Extension of these studies to a series of *para*-substituted aromatic substrates indicated $\rho^+ \geq 1.1$ for benzaldehyde reduction and $\rho^+ = -0.2$ for benzyl alcohol oxidation;[95] thus steady state studies on chemically modified enzyme corroborate previous finding from transient state studies of native enzyme.

pH dependencies were also found to be similar to transient state studies — i.e., no effect of pH on aldehyde reduction, together with a faster oxidation of alcohol at high pH, pK = 8.4. The increase in the pK controlling k_{cat} for alcohol oxidation from 6.4 (native) to 8.4 (hydroxybutyrimidylated) enzyme has been discssed by Dworschack and Plapp; as pointed out by these authors, the contribution of a pH independent step preceding or following the hydrogen transfer step is expected to reduce the apparent

pK relative to an intrinsic value.[95] The disparity between native and hydroxybutyrimidylated enzyme may reflect chemical differences, as well as differential abilities of the two enzyme forms to undergo pK perturbations upon coenzyme and substrate binding (see Scheme 1).

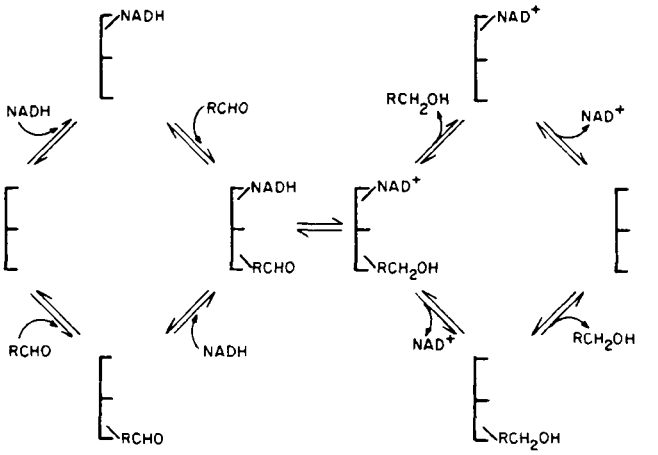
The discussion of kinetic probes of chemical mechanism has focused on experimental efforts to isolate and characterize the hydrogen transfer step. Considerable attention has also been given to the pH dependence of the product release step in the catalysis of aldehyde reduction. As discussed earlier, the interaction of N,N-dimethylaminocinnaminaldehyde with $E \cdot H_2NADH$ leads to a red-shifted intermediate, attributed to a Lewis acid complex between aldehyde and the active site zinc. Dunn and co-workers have examined the pH dependence for the disappearance of the intermediate in the presence of either NADH or [4-^{2}H]NADH.[96] Their data indicate a change in rate determining step from hydrogen transfer (at low pH) to alcohol release (at high pH). Similar results have also been observed by Cleland for the steady state reduction of cyclohexanone, where the V/K isotope effect is observed to change from 2.2 at low pH to an inverse value of 0.85 at pH 11.[97] Direct measurements of the primary, equilibrium isotope effect for the interconversion of aldehydes and ketones by NADH and [4-^{4}H]NADH to alcohol and NAD^+ indicate inverse isotope effects of 0.85 to 0.89.[98] Thus the data of Cleland are in accord with a rapid equilibration of ternary complex followed by slow product release at high pH. The pH dependence of isotope effects for aldehyde and ketone reduction, together with the pH dependence of alcohol desorption, are frequently attributed to a protonation of enzyme-bound alcoholate. An alternative, equally plausible mechanism involves a pH dependent protonation of an enzyme functional group controlling alcohol dissociation (see Scheme 3 vs. Scheme 2).

B. Yeast Alcohol Dehydrogenase

The turnover number of the yeast enzyme is several orders of magnitude faster than horse liver alcohol dehydrogenase. Early steady state kinetic studies indicated small but significant primary hydrogen isotope effects both for acetaldehyde reduction and ethanol oxidation, indicating that the hydrogen transfer step is partially rate limiting under these conditions.[99-101] It is curious that the horse liver enzyme has evolved in such a manner to bind coenzyme much more tightly, and consequently to turn over more slowly; this property may be relevant to the, as yet, poorly defined physiologic function of mammalian alcohol dehydrogenases.

Although the substrate specificity is more narrow with the yeast than horse liver enzyme, the kinetic properties of aliphatic and aromatic substrates have been investigated.[71,102-104] Regarding the kinetic mechanism, it can be concluded that alcohol dehydrogenase is characterized by a preferred rather than obligatory order of binding in which coenzyme precedes substrate. Analysis of steady state parameters for aliphatic substrates supports a random component in the direction of alcohol oxidation;[102] whereas analysis of isotope effects on V_{max}/K_m for aromatic substrates indicates a random component in both directions[104] (Scheme 5).

Substitution of the methyl group of ethanol by a phenyl group has been found to reduce k_{cat} by at least an order of magnitude. Importantly, from the point of view of determining chemical mechanism, large primary deuterium isotope ffects have been observed for the reduction of a series of *para*-substituted benzaldehydes by NADH and [4-^{2}H] NADH.[104] Subsequent investigation of isotope effects in the oxidation of a series of [1R,S-$^2H_2$] benzyl alcohols indicated comparably large primary deuterium isotope effects, Table 14.[71] The magnitude of isotope effects for alcohol oxidation are expected to be related to those for aldehyde reduction by the overall equilibrium iso-



Scheme 5. Random kinetic scheme for the reaction catalyzed by yeast alcohol dehydrogenase; for the majority of substrates the upper pathway is preferred.

Table 14
PRIMARY DEUTERIUM ISOTOPE EFFECTS FOR THE INTERCONVERSION OF AROMATIC SUBSTRATES CATALYZED BY YEAST ALCOHOL DEHYDROGENASE[71,104,105,108]

Substrate	$k_{r,H}/k_{r,D}$	$k_{o,H}/k_{o,D}$	$\left(\frac{k_{r,H}/k_{r,D}}{k_{o,H}/k_{o,D}}\text{ OBS}\right)$	$\left(\frac{k_{r,H}/k_{r,D}}{k_{o,H}/k_{o,D}}\text{ CALC}\right)$
p-Br	3.5	4.8	0.73	
p-Cl	3.3	4.2	0.78	
p-H	3.0	3.4	0.88	0.74
p-CH_3	5.4	4.2	1.21	
p-CH_3O	3.4	3.2	1.06	

tope effect, $K_{eq,H}/K_{eq,D} = 0.89$ in the direction of NADH oxidation.[105] Since dideuterated rather than monodeuterated alcohols were studied, correction must also be made for a secondary kinetic deuterium isotope effect ($k_H/k_D = 1.2$) in the direction of alcohol oxidation:

$$(k_{r,H}/k_{r,D})/(k_{o,H}/k_{o,D}) = (K_{eq,H}/K_{eq,D})\,(k_{\alpha D}/K_{\alpha H}) \qquad (9)$$

As summarized in Table 14, observed values for $K_{r,H}/k_{r,D}/k_{o,H}/k_{o,D}$ indicate reasonably good agreement with the value calculated from Equation 9; and, with the possible exception of *p*-CH_3 substrate, the data summarized in Table 14 have been concluded to support a single rate determining hydrogen transfer step for the interconversion of aromatic substrates under conditions of the steady state.

This feature of the yeast alcohol dehydrogenase reaction has greatly facilitated investigations of the pH dependence and magnitude of solvent isotope effects on the hydrogen transfer step.[106] The effects of pH on k_{cat} for benzyl alcohol oxidation and k_{cat}/K_m for acetaldehyde reduction are reproduced in Figure 7. Constant isotope effects of 4.1 and 2.6 were observed for benzyl alcohol and acetaldehyde, respectively. Importantly, the data in Figure 4 indicate opposite titration curves characterized by the same pK = 8.25. *Thus, in marked contrast to the asymmetric pH dependencies of horse liver alcohol dehydrogenase, pH data for the yeast enzyme are consistent with a cata-*

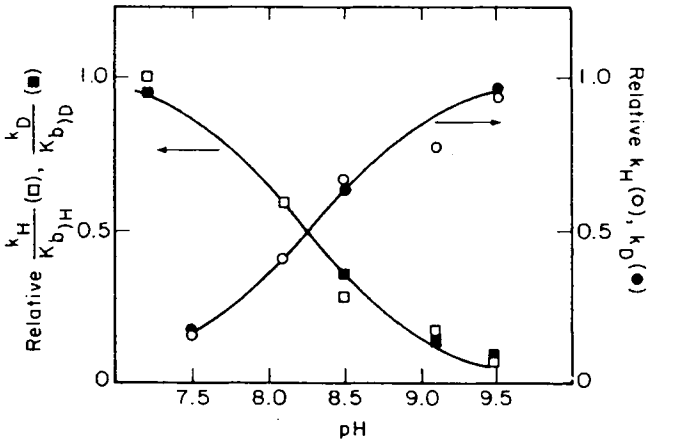


FIGURE 7. pH dependence of the reduction of acetaldehyde by NADH (□ to □) and oxidation of *p*-CH_3-benzyl alcohol by NAD^+ (○ to ○), catalyzed by yeast alcohol dehydrogenase.

lytic role for a single functional group which must exist in different ionization states for alcohol oxidation (E-B) vs. aldehyde reduction (E-BH). This important difference between the horse liver and yeast systems almost certainly derives from the marked pK perturbations observed upon coenzyme binding in the former enzyme. As pointed out earlier, NAD^+ binds and dissociates from horse liver alcohol dehydrogenase with different pKs (9.2 and 7.6, respectively), whereas a single residue of pK = 8 appears to play a (minor) role in NAD^+ binding and release to yeast alcohol dehydrogenase.

Originally, it was suggested that the pH dependencies of yeast alcohol dehydrogenase were consistent with concerted acid base catalysis of the hydrogen transfer step. Solvent isotope effects have failed to confirm this mechanism, however.[107] Measurement of catalytic constants as a function of pH for *p*-CH_3O-benzaldehyde reduction by both NADH and [4-^{2}H]NADH and *p*-CH_3O benzyl alcohol oxidation have allowed a separation of isotope effects on pK from those on limiting kf8f1values. An unusually small effect of D_2O on pK was observed under all circumstances, Table 15; this result contrasts with the commonly observed $\Delta pK = pK_D - pK_H \cong 0.4$ to 0.6. This effect of H_2O vs. D_2O on the pK of most oxygen and nitrogen acids results primarily from the difference in force constants for the O-H(D) bonds in H_3O^+ and D_3O^+. Thus Klinman et al. have proposed that the small ΔpK in yeast alcohol dehydrogenase reflects the ionization of an H_3O^+-like species, e.g.,

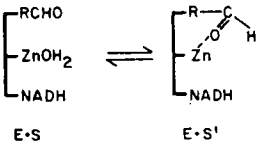
$$\overset{\delta^+}{Zn}\overset{\delta^+}{OH_2}: \qquad \overset{\delta^+}{Zn}\overset{\delta^+}{OH_2} + H_2O \rightleftharpoons \overset{\delta^+}{Zn}\overset{\delta^+}{OH} + H_3O^+ \qquad (10)$$

The effects of D_2O on k_{cat} are also summarized in Table 15, indicating a small solvent isotope effect, $k_{H_2O}/k_{D_2O} = 1.20$, for alcohol oxidation. In contrast, aldehyde reduction is characterized by a large inverse solvent isotope effect, $k_{H_2O}/k_{D_2O} = 0.50$ and 0.58 for reduction by NADH. Since concerted acid-base catalysis is expected to give rise to primary isotope effects ≥ 2, the observed solvent isotope effects rule out such a mechanism. The inverse isotope effects on k_{cat} in the direction of aldehyde reduction has been attributed to the loss of the hydronium ionlike properties of an active site functional group, prior to the rate determining hydrogen transfer step. Of the two mechanisms previously proposed to account for these inverse kinetic isotope effects,[105] preequilibrium proton transfer from an active site residue vs. displacement of metal-

Table 15
SOLVENT ISOTOPE EFFECTS ON pK AND k_{cat} FOR THE REDUCTION OF *p*-CH_3O BENZALDEHYDE AND OXIDATION OF *p*-CH_3O BENZYL ALCOHOL CATALYZED BY YEAST ALCOHOL DEHYDROGENASE[107]

Substrate	pK			k_{cat}, s^{-1}		
	H_2O	D_2O	ΔpK	H_2O	D_2O	$\frac{k_{H_2O}}{k_{D_2O}}$
Aldehyde reduction	8.27	8.29	0.02	0.14	0.28	0.50
Alcohol reduction	8.14	8.35	0.21	0.78	0.65	1.20

bound water by substrate, the latter mechanism is more consistent with the crystallographic and spectroscopic data implicating inner sphere complexes in the horse liver system.



(11)

Although the magnitude of solvent isotope effects on k_{cat} for aldehyde reduction supports the formation of an inner-sphere complex, an important distinction between the yeast and horse liver alcohol dehydrogenase is that this complex is proposed to occur subsequent to the E·S complex in the yeast system, i.e., the inner sphere complex, E·S', does not reflect the most stable ground state in the direction of aldehyde reduction.

Detailed analyses of structure-reactivity correlations have been carried out for the interconversion of aromatic substrates catalyzed by yeast alcohol dehydrogenase.[71] Following Hansch (see Equation 2), linear regression analysis of the available kinetic constants permitted the separation of electronic, hydrophobic, and steric contributions to k_{cat} (Table 16). A similar analysis of substrate binding from kinetically determined K, has already been summarized in Table 8. The best equation (among seven) for benzyl alcohol oxidation is very poorly correlated; and k_o was concluded to be independent of electronic, steric, and in all probability, hydrophobic effects. The data for benzaldehyde reduction were almost equally well fit by both one- and two-parameter equations, characterized by similar electronic contribution; the distinction concerns the positive hydrophobic contribution to the second equation. From the latter equation, it appears that hydrophobic factors contribute to k_{cat} for benzaldehyde reduction (Table 16) as opposed to K, for benzyl alcohol oxidation (Table 8). This observation may reflect either (1) large changes in the interaction of protein with bound aldehyde upon formation of inner sphere complex (Equation 11) or (2) the formation of a chemical intermediate resembling alcohol in its charge properties (see Scheme 9).

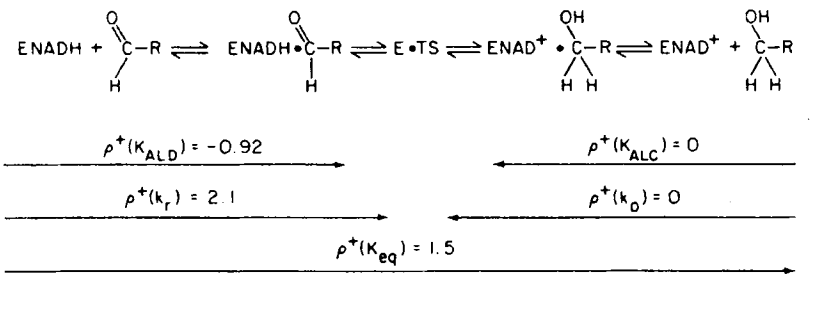
The interrelationships of electronic substituent effects on k_{cat} and K, has been represented in Scheme 6. The observation that $\rho^+ = 2.1$ for aldehyde reduction, together with $\rho^+ = -0.92 \pm 0.18$ for aldehyde binding, indicated a rho value of 1.2 for V/K in the direction of aldehyde reduction. This value is within experimental error of the measured $\rho^+ = 1.5$ for the overall interconversion of aldehydes and alcohols. In addition, $\rho^+ = 0$ for alcohol oxidation, as required by the principle of microscopic reversibility for a single rate determining hydrogen transfer step. Thus *structure-reactivity*

Table 16
MULTIPLE LINEAR REGRESSION ANALYSIS OF k_{CAT} FOR THE YEAST ALCOHOL DEHYDROGENASE REACTION TO DETERMINE THE CONTRIBUTION OF ELECTRONIC, HYDROPHOBIC AND STERIC FACTORS [71]

Reaction	Best correlation[a]
E · NADH + X–C(=O)H	$\log k_R = (2.1 \pm 0.37)\sigma^+ - (0.093 \pm 0.14)$ $n = 12, F^b = 31.7, F(0.99) = 10.0$
	$\log k_R = (1.9 \pm 0.33)\sigma^+ + (0.46 \pm 0.20) \log P_x - (0.46 \pm 0.20)$ $n = 12, F = 24.8, F(0.99) = 8.02$
E · NAD$^+$ + X–C(OH)H–H	$\log k_o = (-0.073 \pm 0.25) \log P_x - (0.65 \pm 0.19)$ $n = 11, F = 0.085, F(0.99) = 10.6$

[a] The substituents constants employed in this study were the electronic Hammett σ^+ constants, the hydrophobic constants P_x, and Van der Waals' radii, R.

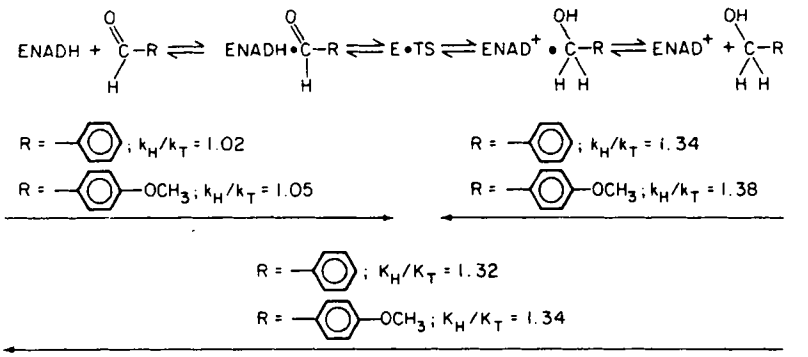
[b] F relates the variance of the null hypothesis to the variance of each correlation; and F(0.99), from statistics tables, represents a lower limit for F to be significant at the 99% level.



Scheme 6. Summary of electronic substituent effects in the yeast alcohol dehydrogenase reaction.

correlations indicate a distribution of charge at C-1 substrate in the transition state which is the same as alcohol in the ground state.

Increasingly, secondary isotope effects are being studied in enzyme systems as probes of changes in bond hybridization at the transition state. A recent study of secondary tritium isotope effects in the yeast alcohol dehydrogenase indicates little or no change in bond hybridization in the direction of aldehyde reduction, $k_T/k_H = 1$; and an isotope effect in the direction of alcohol oxidation, $k_H/k_T = 1.3$, which is equal to the equilibrium isotope effect for alcohol/aldehyde interconversion[108] (Scheme 7). It should be noted that large primary kinetic tritium isotope effects have been observed under the conditions of the secondary isotope effect experiments, confirming that the values summarized in Scheme 7 reflect hybridization changes in the hydrogen transfer



Scheme 7. Summary of secondary tritium isotope effects in the yeast alcohol dehydrogenase reaction.

step per se. Thus *secondary isotope effects indicate that with regard to bond hybridization changes at C-1 of substrate, the transition state is the same as aldehyde in the ground state.*

The large discrepancy between transition state structures derived from secondary isotope effects and structure-reactivity correlations requires elaboration. To the extent that changes in bond lengths and angles are coupled to changes in charge, it is not possible to rationalize the present results with a simple hydride transfer mechanism (Scheme 8). On the other hand, if one permits the movement of electrons to become (partially) uncoupled from the transfer of the hydrogen nucleus, a range of transition state structures varying in charge and bond hybridization at C-1 is possible. In the extreme, one can write a radical mechanism in which aldehyde reduction is a two-step process involving a one electron transfer from NADH to aldehyde followed by a rate determining hydrogen atom transfer (Scheme 9).

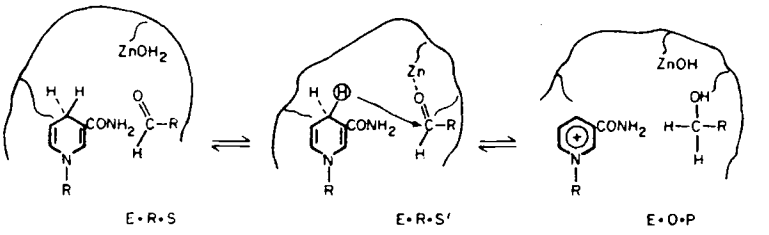
According to Scheme 9, the reaction proceeds from the most stable ground state ES, through a series of increasingly activated intermediates, E·S′ and E·S″. Hydrogen atom transfer from NADH ⊕ to the highly activated E·S″ is expected to occur through an early transition involving relatively little rehybridization of bonds at C-1. Thus the mechanism illustrated in Scheme 9 predicts a transition state structure which resembles aldehyde with regard to bond hybridization changes and alcohol with regard to charge properties.

A persistent problem in corroborating Scheme 9 is the failure to detect the presence of radical intermediates in NAD(P)H-dependent reactions by electron spin resonance. In addition, the premise that changes in bond reorganization and charge will be coupled in the transition state may be faulty. Funderburk and Jencks have recently discussed the imbalance in values of Bronsted αs and βs, rho values and secondary isotope effects for the acid catalyzed breakdown of carbinolamines to semicarbazones; as pointed out by these authors, secondary isotope effects indicate little or no bond rehybridization toward the sp^2 carbon atom in the transition state, in contrast to rho values, which support a large degree of carbon oxygen bond cleavage in the transition state.[109]

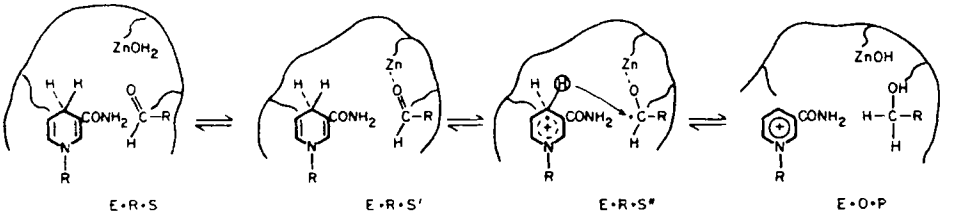
VI. A UNIFYING MECHANISM?

A. The Mode of Acid-Base Catalysis

Both X-ray crystallographic studies of abortive enzyme-coenzyme-substrate complexes on crystalline enzyme, and spectroscopic studies of complexes between enzyme



Scheme 8. Direct hydride transfer mechanism for the reduction of aldehydes by NADH.



Scheme 9. Two step mechanism, involving an uncoupling of electron from hydrogen atom transfer, in the reduction of aldehydes by NADH.

and chromophoric aldehydes in solution support direct coordination of substrate to the active site zinc atom of horse liver alcohol dehydrogenase; in addition, the high degree of active site homology between horse liver and yeast alcohol dehydrogenase is consistent with a similar active site conformation for the yeast enzyme. The failure to detect inner sphere complexes by examination of the paramagnetic effects of an active site cobalt [(in fully substituted horse liver alcohol dehydrogenase) Co(4)ADH] on bound substrate analogues may be a consequence of a dynamic equilibrium between second sphere and inner sphere complexes, together with a dependence of the relative energy of inner and outer sphere complexes on the physical state of the protein and the nature of the substrate. In the yeast alcohol dehydrogenase system, kinetic solvent isotope effects suggest displacement of zinc-bound water by aldehyde subsequent to the ES complex. This finding poses the important question of whether inner sphere complexes can be expected to contribute to k_{cat} or K_s in the case of productive ternary complexes in solution. A distinction between the horse liver and yeast systems may be the greater ease of formation of inner sphere complexes for the horse liver enzyme, a consequence of the presumed higher pK of metal-water in E·NADH·aldehyde complexes of horse liver (pK > 11) than yeast (pK ≃8.25) alcohol dehydrogenases.

Another mechanistic feature relevant to the mode of substrate coordination to the active site zinc atom concerns whether zinc undergoes an expansion in its coordination number from four to five in the presence of substrate. The X-ray crystallographic structure of the enzyme 1,10-phenanthroline complex clearly demonstrates the potential for pentacoordinate zinc. Formation of such a pentacoordinate complex with substrate could occur either as an intermediate on the way to water displacement via an associative mechanism or as the stable form of inner sphere complexes between enzyme and substrate. Detecting such complexes and relating them to the overall mechanism is a challenge for the future.

Employing the interaction of substrate with the active site zinc water as a central feature of the catalytic mechanism of both yeast and horse liver alcohol dehydrogenase one would like to relate the extensive pH studies on substrate binding and catalysis to unique ionizing functional groups. Although four distinct pKs operate in the formation

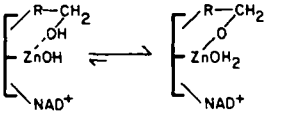
of binary and ternary complexes in the horse liver enzyme, a range of studies suggest that these pKs may reflect a single functional group. As summarized in Scheme 10, the ionization of metal-water has been proposed to undergo large perturbations in pK from 11 to 6.4, depending on the nature of bound coenzyme and substrate. It should be noted that there are, at present, no direct studies correlating the measured pKs with $ZnOH_2$, although such an assignment is consistent with the X-ray crystallographic structure and chemical intuition. If correct, Scheme 10 requires a structural basis for the wide range of pKs attributable to a single ionizing residue. The reduction in pKs observed upon NAD^+ and alcohol binding could be due in part to different electrostatic interactions between the charged nicotinamide ring of NAD^+ and the active site $ZnOH_2$ in binary and ternary complexes; whereas the increase in pK observed upon NADH bonding cannot yet be rationalized in a simple manner.

Recent work by Kvassman and Pettersson has led to the suggestion that the pK controlling k_{cat} in the direction of alcohol oxidation is the metal bound substrate (Scheme 11). The observation of different pKs controlling trifluoroethanol binding vs. benzyl alcohol and ethanol oxidation [possible evidence that the ionization of metal-bound substrate controls k_{cat}, Scheme 11)] must be weighed against the observation of different pKs controlling benzyl alcohol oxidation for native vs. acetimidylated enzyme [possible evidence for the ionization of an enzyme-bound functional group, Scheme 10]. Further studies employing a range of substrates, characterized by different intrinsic pKs for the hydroxyl functional group, together with studies of cobalt-substituted and carboxymethylated enzyme, should help to clarify the current ambiguity in pK assignment.

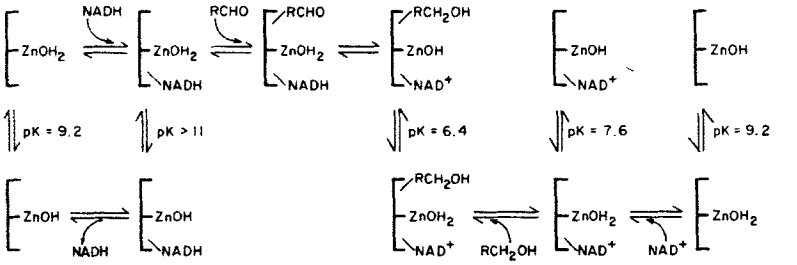
An interesting distinction between Schemes 10 and 11 concerns the absence of water from ternary complex in the latter. In the event that water is still present in $E \cdot NAD^+$ alcohol complexes, the assignment of pK reduces to a question of the relative pKs of metal-water vs. (metal-)alcohol, i.e., is the deeper potential energy well for the proton one in which the proton resides on $ZnOH_2$ vs. alcohol?

The pH dependencies of the yeast system appear to be less complex than those of horse liver alcohol dehydrogenase; and the observation of the same pK controlling acetaldehyde reduction and p-CH_3 benzyl alcohol oxidation is strong evidence for the participation of an enzyme functional group, pK = 8.25 (Scheme 12). Assignment of this pK to $ZnOH_2$ is more tenuous in the yeast system due to the absence of structural data, although the unusually small effect of D_2O on the kinetically determined pKs has been attributed to $ZnOH_2$ ionization. It should be noted that the "normal" ΔpK observed for alcohol oxidation in the horse liver system may reflect the ionization of bound substrate as opposed to metal-water.

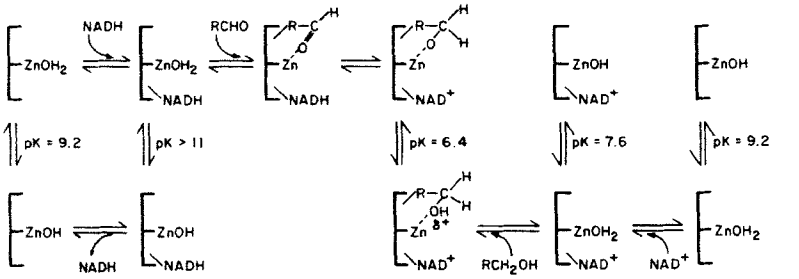
Although Scheme 10 (for horse liver alcohol dehydrogenase) and Scheme 12 (for yeast alcohol dehydrogenase) contain the correct prototropic forms of an active site residue for concerted acid-base catalysis of hydrogen transfer, kinetic solvent isotope effects clearly rule out such a mechanism in both systems. All of the currently proposed schemes involve a preequilibrium transfer of the alcohol proton either to an active site group (Schemes 10 and 12) or solvent (Scheme 11) prior to the hydrogen transfer step. Formation of the alcoholate in Schemes 10 and 12 is expected to occur in a higher energy complex along the reaction path from the most stable $E \cdot S$ complex to $E \cdot S‡$; although in the event that the pK of alcohol is less than the pK of $ZnOH_2$ in $E \cdot NAD^+ \cdot$ alcohol complexes, alcoholate would form directly:



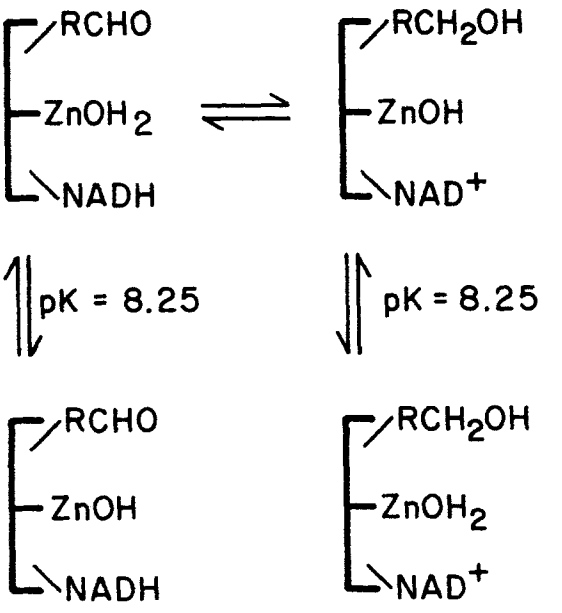
(12)



Scheme 10. pK values controlling substrate binding and catalysis in the horse liver alcohol dehydrogenase reaction. The ionizing group has been attributed to $ZnOH_2$ in all cases.



Scheme 11. pK values controlling substrate binding and catalysis in the horse liver alcohol dehydrogenase reaction. The ionizing group has been attributed to $ZnOH_2$, with the important exception of the pK = 6.4 group, suggested to reflect substrate ionization (90).



Scheme 12. pK values controlling catalysis in the yeast alcohol dehydrogenase reaction.

In such an instance, the distinction between Schemes 10 and 11 reduces to the question of the coordination number of zinc in ternary complex.

B. Mode of Hydrogen Activation

Although formally considered to be a hydride transfer reaction, the precise mode of hydrogen transfer between substrate and coenzyme in NAD(P)H-dependent reactions remains ambiguous. Among the mechanisms considered are those involving concerted hydride transfer (Scheme 8) vs. the step-wise transfer of one electron followed by a hydrogen atom (Scheme 9). Depending on the energy level of the putative radical intermediate illustrated in Scheme 9, direct demonstration of such an intermediate may not be experimentally feasible. It is thus of considerable interest that the discrepancy between the magnitude and direction of structure-reactivity correlations vs. secondary tritium isotope effects in the yeast alcohol dehydrogenase reaction are easily reconciled by Scheme 9. It should be borne in mind, however, that measurements of electronic substituent effects and secondary isotope effects in model reactions involving $sp^3 \rightleftharpoons sp^2$ interconversions at carbon suggest that charge redistribution and bond rehybridization need not be tightly coupled at the transition state. Careful studies of electronic substituent effects and secondary isotope effects in model dehydrogenase reactions would facilitate interpretation of the data in the enzymatic reaction.

A comparison of structure-reactivity correlations in the horse liver to the yeast system indicates a similar (possibly identical) distribution of charge at C-1 of substrate in the transition state. Although secondary isotope effects have not yet been reported for the horse liver system, measurement of these effects would provide a subtle test of the extent to which transition state structure has been conserved among enzymes from divergent evolutionary sources catalyzing the same reaction. In addition, detection of a closer correlation between the deduced charge distribution and bond hybridization at C-1 of substrate in the transition state of the horse liver alcohol dehydrogenase reaction could provide the necessary information to distinguish a concerted hydride transfer mechanism from the uncoupled, radical mechanism of hydrogen activation of Scheme 9.

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