LIVER ALCOHOL DEHYDROGENASE

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I. INTRODUCTION

The structure and function of alcohol dehydrogenase (E.C. 1.1.1.1) was last reviewed in this journal by Klinman in 1981.[1] Since that time, our understanding of the mechanism of action of the alcohol dehydrogenase occurring in liver has been significantly advanced by a series of crystallographic, kinetic, spectroscopic, and metal-substitution studies, and therefore a new summing up at this stage appears justified. General background information on liver alcohol dehydrogenase can be found in the above review and that of Brändén et al.[2] The structural properties of the enzyme have been thoroughly discussed in three recent survey articles,[3-5] for which reason the emphasis of the present review will be placed on kinetic and mechanistic aspects.

II. PROTEIN STRUCTURE

Liver alcohol dehydrogenase may be present in multiple forms showing slight differences in amino acid composition.[2] Major forms isolated from mammals are dimers comprised of two identical 40,000 mol wt subunits and contain two firmly bound zinc ions per subunit. Such species have been purified to a crystalline state from horse, human, and rat liver. Their primary structures appear to be 80 to 90% identical and are highly homologous in all regions of particular interest with regard to polypeptide folding and substrate or coenzyme binding. Basic structural research during the last 5 years has focused on the isozymes isolated from the liver of primates.[6-17] The most extensive mechanistic and physicochemical studies, however, have been performed with the so-called "ethanol-active" major form of the horse liver enzyme. The sequence of the 374 amino acids in the single polypeptide chain constituting the subunit of this isozyme was determined by Jörnvall in 1970,[18] and detailed information on the three-dimensional structure of the isozyme is now available from X-ray crystallographic data presented by Brändén and co-workers.[2-5]

A. General Fold

Native horse liver alcohol dehydrogenase crystallizes in an orthorhombic space group. The corresponding enzyme structure has been determined at a resolution of 2.4 Å and used to deduce the structures of a number of isomorphous enzyme-inhibitor complexes by difference Fourier techniques (Table 1). Coenzyme-containing complexes usually give monoclinic or triclinic crystals depending on preparation conditions. Structures of several ternary enzymic complexes crystallizing in triclinic space have been determined to 2.9 Å resolution (Table 1). They correspond to a more densely packed conformational state (closed form) than that of orthorhombically crystallizing enzyme species (open form).

Table 1
REPRESENTATIVE CRYSTALLOGRAPHIC INVESTIGATIONS OF COMPLEXES FORMED WITH LIVER ALCOHOL DEHYDROGENASE

Ligands	Form	Resolution (A)	Ref.
2-Methyl-2,4-pentanediol	Orthorhombic	2.4	19
1,10-Phenanthroline	Orthorhombic	4.5	20
Imidazole	Orthorhombic	2.9	20,21
Pyrazole	Orthorhomic	3.2	21
ADP-ribose + methylpentanediol	Orthorhombic	2.9	22
H_2NADH + methylpentanediol	Orthorhombic	2.9	23
NADH + imidazole	Orthorhombic	2.4	24
NADH + dimethylsulfoxide	Triclinic	2.9	25
H_2NADH + dimethylaminocinnamaldehyde	Triclinic	2.9	23
NAD^+ + pyrazole	Triclinic	2.9	21
NAD^+ + trifluoroethanol	Triclinic	4.5	26
NAD^+ + bromobenzylalcohol	Triclinic	2.9	27

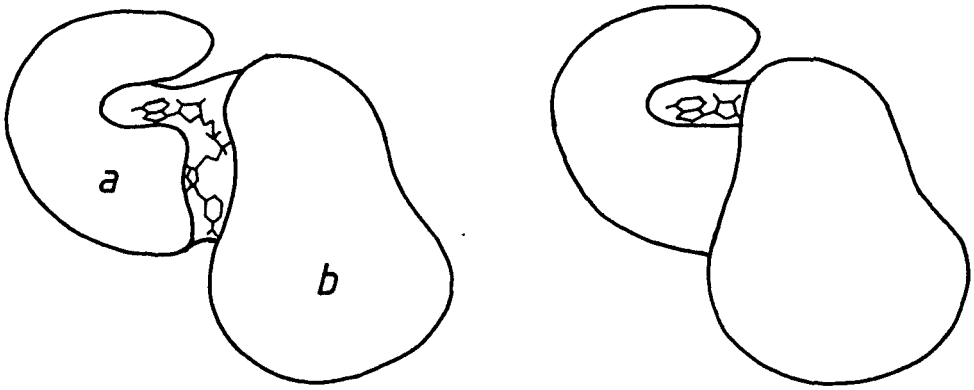


FIGURE 1. Highly schematic representation of the liver alcohol dehydrogenase subunit, illustrating the coenzyme-induced closure of the cleft which separates the coenzyme-binding (a) and catalytic (b) domains.[28]

The general fold is largely identical in the closed and open forms. The enzyme subunit is divided into two domains by a cleft with a deep pocket (Figure 1). This pocket accomodates the substrate and the nicotinamide part of the coenzyme. The smaller one of the domains is comprised of residues 178 to 318 and contains most of the enzymic groups participating in coenzyme binding. The larger domain provides ligands to the protein-bound zinc ions and contains most of the residues controlling substrate binding and catalytic activity. The coenzyme-binding domains are joined to form a central core in the enzyme dimer. The conformational transition from the open to the closed form involves a rigid 10° body rotation of each catalytic domain relative to the central core (Figure 2) with almost complete preservation of the internal structures of the subunit domains.

B. Metal Sites

One of the two zinc ions in the enzyme subunit is catalytically essential.[2] This active-site zinc ion is positioned at the bottom of the domain-separating cleft about 20 Å from the protein surface. It is bound in a distorted tetrahedral coordination sphere by

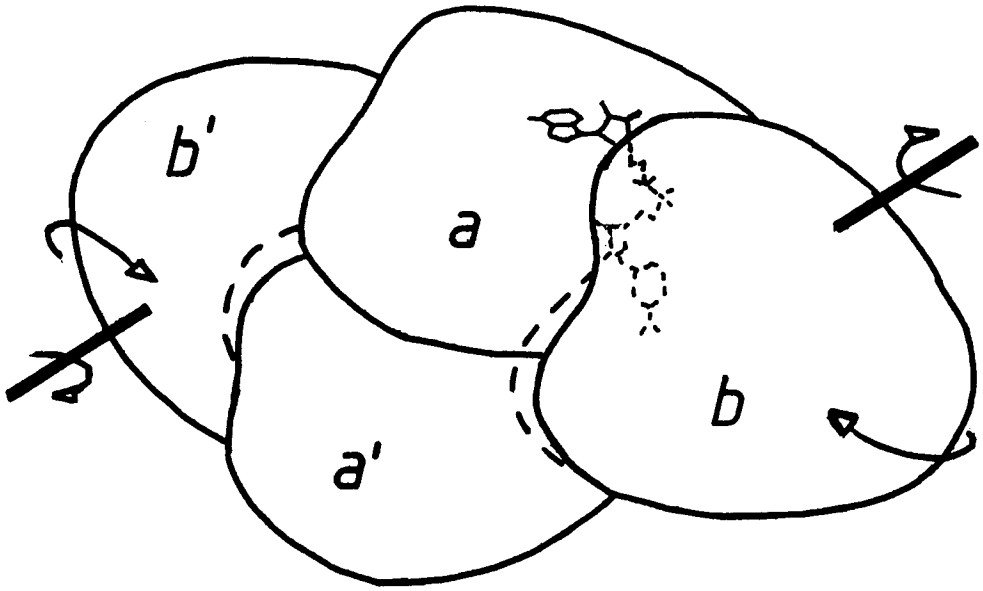


FIGURE 2. The liver alcohol dehydrogenase dimer and the rigid body rotations associated with the open to closed conformational transition.[22]

three protein ligands (Cys-46, His-67, and Cys-174), with the fourth coordination position being accessible to water and other ligands from solution. The peptide parts of the three protein ligands are firmly integrated into secondary structural elements, and the side chain of His-67 is positioned through a hydrogen bond from the imidazole N-3 to the carboxyl group of Asp-49.[29]

The second zinc ion is tetrahedrally coordinated by sulfur atoms from cysteine residues 97, 100, 103, and 111. It is located about 20 Å from the catalytic metal site in a polypeptide lobe that projects out of the catalytic domain. The function of this zinc remains unknown, although recent results have been taken to support a structural role.[30]

C. Coenzyme Binding Site

Each subunit binds one coenzyme molecule in extended conformation to an approximately 30-A-long crevice constituted by the deep domain-separating cleft and a more shallow cleft at the surface of the coenzyme-binding domain. The latter cleft binds the adenosine part of the coenzyme. The adenine moiety is accomodated in a hydrophobic cavity which may interact with hydrophobic groups in general.[2,4] The adenosine ribose moiety forms hydrogen bonds to the side chains of Asp-223 and Lys-228. The former interaction involves both of the ribose hydroxyl groups, which explains why the enzyme cannot utilize NADP as a coenzyme.[2] The detailed coenzyme-protein hydrogen bonding pattern indicated by high-resolution studies of species in the closed conformational state is shown in Figure 3.

The coenzyme pyrophosphate group is bound close to the rim of the domain-separating cleft and makes a bend over the edge of the pleated-sheet structure of the coenzyme-binding domain. This conformation of the group appears to be stabilized by hydrogen bonds from the phosphate oxygen atoms to main-chain nitrogen atoms, as well as by charge interactions with the side chains of Arg-47, Arg-369, and Lys-228. The dipole moment created by adjacent protein helices may add to the cationic character of the pyrophosphate-binding subsite,[22] which acts as a binding site for the heavy-metal marker $Pt(CN)_4^{2-}$ and inorganic anions in general.[2]

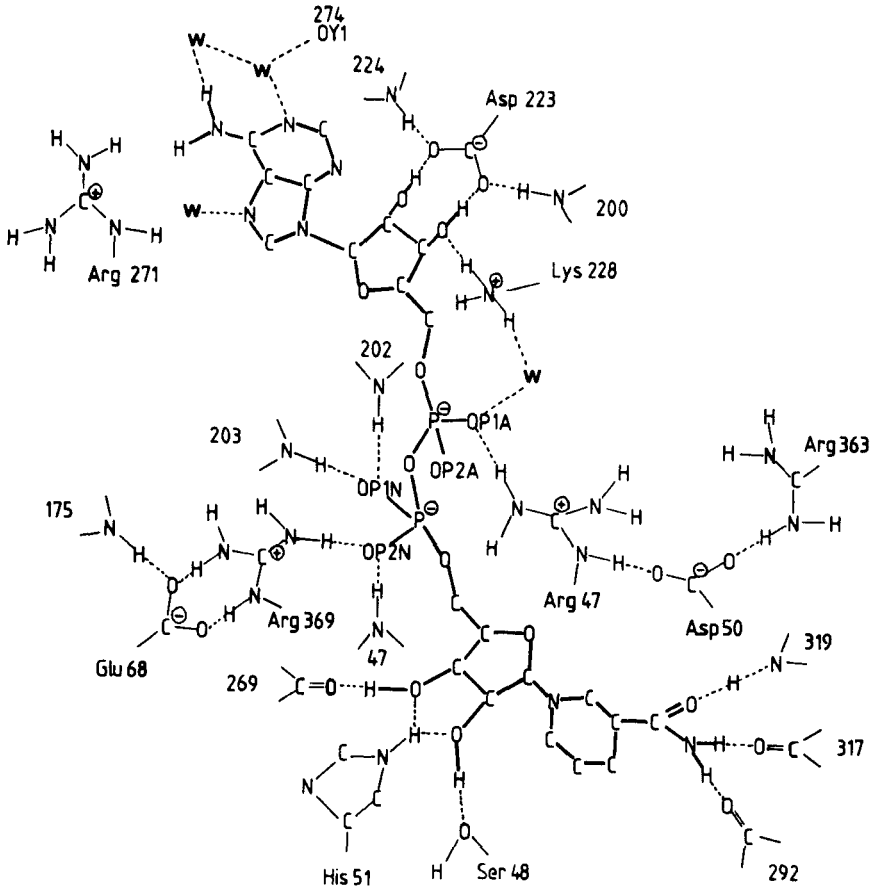


FIGURE 3. Schematic drawing of the hydrogen bond interactions between coenzyme and liver alcohol dehydrogenase. (From Eklund, H., Samama, J. P., and Jones, T. A., *Biochemistry*, 23, 5982, 1984. With permission.)

The nicotinamide ribose of the coenzyme is tightly bound in the narrow cleft between the two domains of the subunit. One of the ribose hydroxyl groups forms hydrogen bonds to the side chains of Ser-48 and His-51 (Figure 3). This system of hydrogen bonds has been proposed to act as a relay for proton transfer from the active site to solution.[27]

The coenzyme nicotinamide ring is positioned in a slot near the bottom of the domain-separating cleft. One side of the slot is constituted by hydrophobic residues from the coenzyme-binding domain. The other side is highly polar, being constituted by the catalytic zinc ion and its ligands. The orientation of the nicotinamide ring appears to be determined mainly by hydrogen bonds from its carboxyamide group to main-chain protein atoms at the bottom of the slot. The latter interactions lead to a stereospecific binding of the nicotinamide ring with its A-side directed towards the catalytic zinc ion, and a B-specific binding of the ring is sterically hindered in the closed conformation.[22]

Bonding interactions described above lock the coenzyme in a fixed conformation that is almost identical in all examined complexes having a closed structure, including complexes formed with enzyme depleted of catalytic zinc[31] or containing Cd(II) at the catalytic metal site.[32]

D. Substrate Binding Site

Almost all crystal structures reported by Brändén and co-workers refer to enzymic

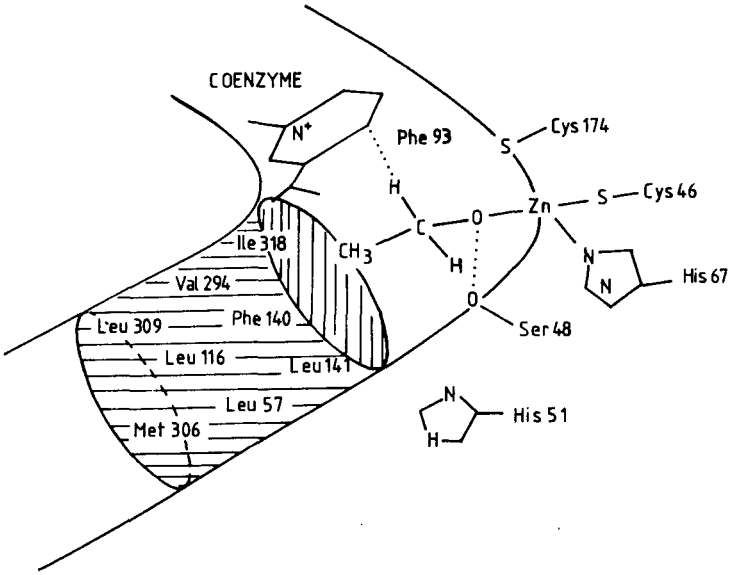


FIGURE 4. Schematic representation of the active site region in liver alcohol dehydrogenase, showing catalytic zinc with its ligands, the nicotinamide-binding site, and the hydrophobic substrate-binding barrel. (From Eklund, H., and Brändén, C.-I., *Biological Macromolecules and Assemblies*, Vol. 3, Jurnak, F. and McPherson, A., Eds., John Wiley & Sons, New York, 1987. With permission.)

complexes containing either a substrate or a substrate-competitive inhibitor. These ligands are bound directly to or in the vicinity of catalytic zinc at a 5- to 10-Å wide and about 20-Å long pocket that leads from solution to the active site metal ion, and which represents a continuation of the domain-separating cleft (Figure 4). The inner part of the pocket can be described as a hydrophobic barrel formed by residues from the catalytic domain. Closer to the protein surface, the substrate-binding pocket of one subunit is lined also with residues contributed by the coenzyme-binding domain of the second subunit. The fluorescent dye auramine O interacts specifically with the substrate-binding pocket and has been used as a reporter ligand in studies of complex formation at this site.[2]

The bonding interactions between substrate and protein have been studied to 2.9-Å resolution with the ternary enzyme-H_2NADH-dimethylaminocinnamaldehyde and enzyme-NAD$^+$-bromobenzylalcohol complexes, which crystallize in the closed conformation.[23,26] The oxygen atom of these substrates is directly bound to catalytic zinc and, in the case of bromobenzyl alcohol, positioned within hydrogen-bonding distance to the hydroxyl group of Ser-48. The crystallographic data are neither compatible with outer-sphere coordination of the substrate, nor with the presence of a penta-coordinated zinc-bound water molecule. The substrate molecules in the averaged X-ray structures are not optimally oriented for direct hydride ion exchange with bound coenzyme, but can be readily brought to such an orientation through rotation around the oxygen-metal bond. Figure 5 shows the productive mode of substrate binding as deduced from X-ray diffraction data for the complex formed with bromobenzyl alcohol.

Bignetti et al.[33] concluded from microspectrophotometric measurements on crystal complexes that X-ray data reported for ternary enzyme-NAD$^+$-alcohol complexes may actually refer to the abortive complexes containing NADH instead of NAD$^+$, a criticism which appears justified in the light of results recently presented for Cd(II) substi-

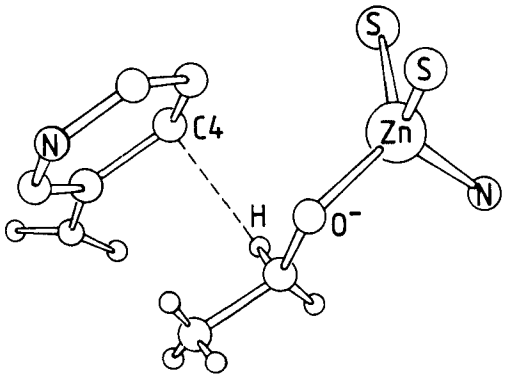


FIGURE 5. Schematic drawing of the geometry for hydride transfer between ethanol and NAD^+. (From Eklund, H. and Branden, C.-I., *Biological Macromolecules and Assemblies*, Vol. 3., Jurnak, F. and McPherson, A., Eds., John Wiley & Sons, New York, 1987. With permission.)

tuted enzyme.[32] They also found that substrate diffusion into crystals of the enzyme-NADH complex leads to reversible oxidation of the bound coenzyme without disruption of the crystal lattic.[33] The latter observation, the principle of microscopic reversibility, and structural data obtained with enzyme-NAD^+-pyrazole and enzyme-H_2NADH-aldehyde complexes[21,23] indicate that the coenzyme bonding pattern observed with enzyme-NADH-alcohol complexes is also largely representative for the productive mode of NAD^+ binding.

E. Crystal Water Sites

Refinement of high-resolution data for different enzymic complexes has provided some detailed information on the hydration state of the crystal structures.[22,23] About 300 well-ordered water molecules have been located at the surface of each subunit in the absence of coenzyme, and some 40 of these are in the domain-separating cleft and the substrate-binding pocket. The majority of the latter water molecules are displaced on the binding of coenzyme and substrate, but approximately 10 water molecules remain positioned in the active site region of the enzyme-H_2NADH-methylpentanediol complex which crystallizes in the open enzyme conformation. The transition from the open to the closed conformation narrows the domain-separating cleft and brings the catalytic zinc ion about 1 Å closer to the coenzyme binding region. This results in complete dehydration of the active site region.

The latter observation indicates that hydrophobic stabilization provides a major driving force for the open to closed conformational transition, i.e., this transition is likely to be dependent on the net energetic outcome of a multitude of solvent-ligand-site interactions. Occupation of the nicotinamide-binding part of the coenzyme binding site appears to be necessary to stabilize the closed form.[3] The sensitivity of the conformational transition to ligand structure is illustrated by the observations of an open conformation for the enzyme-H_2NADH-methylpentanediol complex, but closed conformations for the enzyme-NADH-methylpentanediol and enzyme-H_2NADH-dimethylaminocinnamaldehyde complexes.[23,26] Results recently obtained with carboxymethylated enzyme indicate that, in solution, there may be an equilibrium distribution of species in open, closed, and partially closed states.[30]

III. CHEMICALLY MODIFIED ENZYME

A. Metal Substitution

Liver alcohol dehydrogenase can be fully depleted of protein-bound zinc by dialysis at low pH against chelating reagents.[2] Zinc-free enzyme is catalytically inactive. It looses SH-groups on prolonged exposure to air, but has been reconstituted by treatment with zinc ions in the presence of reducing reagents.[34]

Dialysis of native enzyme against metal salts may lead to partial or complete metal substitution at the zinc site, with noncatalytic zinc exchanging more rapidly than catalytic zinc. Equilibrium dialytic methods are now available for complete substitution of enzyme-bound zinc by Co(II) and Cd(II), as well as for the preparation of hybrides containing either of these two metals at the noncatalytic site and zinc at the catalytic site.[35-37] Selective metal substitution at the noncatalytic site does not appear to affect the catalytic activity of the enzyme.

Zeppezauer and co-workers in 1979 reported that alcohol dehydrogenase can be selectively depleted of catalytic zinc by treatment of crystalline suspensions of the enzyme with chelating reagents under anaerobic conditions.[38] Recent X-ray diffraction studies establish that the inactive enzyme derivative thus obtained lacks catalytic zinc, but otherwise retains the crystal structure of native enzyme except for minor changes in position of the freed protein ligands at the metal site.[39] The selectively zinc depleted enzyme can be fully reactivated by treatment with zinc ions[38] and has been analogously reconstituted by insertion of Co(II), Cd(II), Cu(II), Ni(II), Fe(II), Pb(II), or Hg(II) into the catalytic metal site.[38,40-43] Crystal structures of the Co(II) and Cd(II) reconstituted enzymes have been determined and found to be essentially identical with those of native enzyme.[31,32,39]

Ultraviolet-visible absorption spectra of Co(II), Ni(II), Cd(II), and Cu(II) substituted enzyme show transitions attributable to metal-sulfur charge transfer and are consistent with a tetrahedral coordination geometry at the catalytic as well as the noncatalytic metal site.[35,38,40-42] Tetrahedral metal coordination at the catalytic site in free enzyme is also supported by circular dichroic, resonance Raman,[44] and EPR[45] spectral evidence. X-band EPR spectra of enzyme containing Cu(II) at the catalytic site show superhyperfine splitting typical of inner-sphere nitrogen coordination to the metal,[46] and evidence for inner-sphere coordination of a water molecule comes from NMR studies of this enzyme derivative and the corresponding Co(II) substituted enzyme.[47] Particular attention has been drawn to the optical and EPR spectral properties of the selectively Cu(II) substituted derivative,[40,46] which are similar to those of Type 1 Cu^{2+} in "blue" copper proteins.

B. Modification of Amino Acid Residues

While chemical modification experiments have failed to provide evidence for a functional role of tyrosine residues in liver alcohol dehydrogenase catalysis, pronounced effects on enzyme activity were observed with a variety of reagents that react more or less specifically with histidine, lysine, arginine, and cysteine residues.[2] Histidine modification by diethyl pyrocarbonate leads to inactivation of the enzyme, but the affected reaction steps and residues involved remain unidentified.[48] The activity increase following alkylation of lysine residues has been firmly established to derive from an increased rate of coenzyme dissociation caused specifically by modification of Lys-228.[2] Plapp et al.[49] determined the X-ray structure for enzyme in which 23 of the 30 lysine residues per subunit were substituted for isonicotinamidine groups. The overall conformation of the modified enzyme was closely similar to that of native enzyme, which explains why catalytic activity is retained despite the extensive chemical modification. Alkyla-

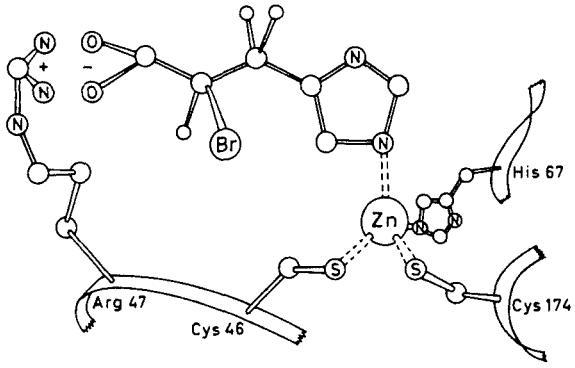


FIGURE 6. Proposed mode of reversible binding of α-bromo-β(5-imidazolyl)-propionate prior to the irreversible alkylation of Cys-46. (From Dahl, K. H. and McKinley-McKee, J. S., *Eur. J. Biochem.*, 118, 507, 1981. With permission.)

tion of Lys-228 appears to interfere with the interactions that normally exist between the unmodified amino group and the AMP portion of bound coenzyme, but the early observation that NADH and AMP (or ADP-ribose) binding are differently affected by the modification indicates that additional factors may contribute to the impaired binding of the coenzyme.[50] Enhancement of the coenzyme dissociation rate is likely to account also for the increase in enzyme activity recently reported to result from specific glycosylation of Lys-315.[51]

Treatment of the enzyme with iodoacetate leads to selective modification of the zinc ligand Cys-46 by a mechanism involving prior reversible binding of the anionic reagent to the cationic pyrophosphate-binding subsite.[2] The carboxymethylated enzyme is catalytically active, and crystallographic studies of the modified enzyme and its complex with NADH indicate that the structural features of native enzyme are largely preserved.[30,52] The reaction of Cys-46 with iodoacetate is promoted by imidazole binding to catalytic zinc, an effect which can be attributed to metal-mediated electron delocalization from the imidazole ligand to the cysteinyl ligand.[53] McKinley-McKee and coworkers have demonstrated that a particularly rapid and specific modification of Cys-46 is achieved with α-bromo-β-(5-imidazolyl)propionic acid,[54,55] a reagent which may interact simultaneously with catalytic zinc and Arg-47 at the pyrophosphate-binding subsite (Figure 6). Evidence that this reagent does combine to the catalytic metal ion was provided in subsequent spectral and kinetic studies of its interaction with Co(II) and Cd(II) substituted enzyme.[56]

Kinetic data for enzyme inactivation by a series of halogenated carboxylic acids were reported by Dahl et al.[57] and discussed with the assumption that observed effects derive from alkylation of Cys-46. Chadka and Plapp[58] found that the enzyme inactivation caused by 3-bromopropionate derives from modification of Cys-174 and suggested that alkylation of the latter ligand of catalytic zinc is facilitated by salt bridge formation between 3-bromopropionate and Arg-369 at the pyrophosphate-binding subsite. However that may be, *the particular reactivity of Cys-46 and Cys-174 appears to be attributable partly to metal coordination of the residues, but mainly to the availability of adjacent sites for prior binding of the alkylating reagents.*[30,57] Selective modification of either Cys-46 or Cys-174 has been accomplished also with halogenated coenzyme analogues.[59-61]

Butanedione or phenylglyoxal treatment of liver alcohol dehydrogenase results in modification of two arginine residues per subunit with concomitant loss of enzyme

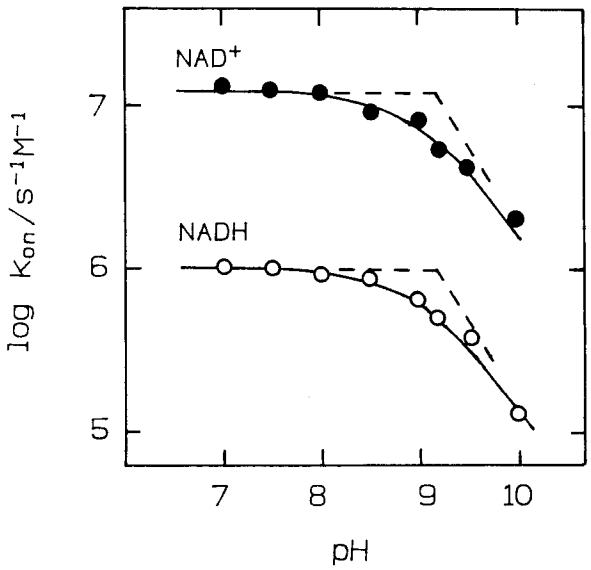


FIGURE 7. pH dependence of association rate constants for coenzyme binding to liver alcohol dehydrogenase.

activity due to abolition of coenzyme binding.[62] One of these residues has been identified as Arg-84,[63] and several lines of evidence indicate that the second modified residue is one of the arginyl constituents of the pyrophosphate-binding subsite. The importance of Arg-47 for tight coenzyme binding has been established by studies of isozymes containing a histidine residue at position 47,[64] but no arginine modification experiments with such isozymes have been reported.

IV. BINDING PROPERTIES

The binding properties of liver alcohol dehydrogenase have been probed with a large variety of coenzyme or substrate analogues and coenzyme or substrate competitive inhibitors.[2] The results are generally consistent with the structural information obtained from crystallographic data, and more or less detailed explanations for the specificity patterns and inhibitory effects observed in early solution studies have been provided in relation to X-ray diffraction studies of different enzymic complexes.[2-4] More recent binding studies, therefore, have centred on kinetically or mechanistically related questions to which crystallographic results do not give an immediate answer, and particular attention has been paid to the pH dependence of complex formation at the substrate and coenzyme binding sites.

A. Coenzyme Binding to Native Enzyme

Early investigations showed that the affinity of the enzyme for NAD^+ increases drastically when the pH is raised from 6 to 10, whereas the affinity for NADH remains essentially constant up to pH 9 and then decreases.[2] The pH dependence of NADH binding has been established by stopped-flow kinetic methods to derive mainly from the coenzyme association step (Figure 7), which requires protonation of an enzymic group with a pK_a within the range 9.0 to 9.5;[65-68] a value of 9.2 appears to be consistent with all NADH binding data so far reported and is supported also by measurements of the pH dependent proton uptake occurring on formation of the enzyme-NADH complex.[69]

The pH dependence of NAD^+ binding derives from combined effects of pH on the coenzyme association and dissociation rates and was earlier interpreted in terms of a ligand-induced perturbation of the pK_a of an enzymic group from 8.8 to 9.6 in free enzyme to 6.9 to 8.0 in the enzyme-NAD^+ complex.[2] Off-velocity constant determinations by stopped-flow techniques have confirmed that coenzyme dissociation from the enzyme-NAD^+ complex requires protonation of an enzymic group with a pK_a of 7.6,[66-68] as concluded by Shore et al. from studies of the proton release associated with formation of the binary complex.[70] On-velocity constant determinations have given slightly varying estimates of the pK_a that controls NAD^+ association to free enzyme (Figure 7), with values of 9.1 to 9.2 being indicated by the most recent reports.[67,68] Results of a critically designed displacement experiment corroborate the observation of DeTraglia et al.[66] that NADH and NAD^+ association rates are controlled by the same pK_a.[67]

Theorell earlier attributed the pH dependence of NAD^+ binding to electrostatic interactions of the coenzyme nicotinamide ring with an ionizing zinc-bound water molecule at the active site.[71] Crystallographic results lend crucial support to that idea by showing that catalytic zinc does coordinate a water molecule, that the nicotinamide ring of NAD^+ is bound in close proximity to this zinc-bound water, and that NADH and NAD^+ are bound at one and the same site through largely identical bonding interactions.[2-4] The latter observation confirms the supposition of Theorell that any pronounced difference in the affinity of the enzyme for oxidized and reduced coenzyme must be related to the positive nicotinamide ring charge of NAD^+, consistent with the conclusion drawn from solution studies of phenyl-AD and pyridine-AD^+ binding to the enzyme.[72] Early information on the differential effect of pH on NADH and NAD^+ binding has been extended by equilibrium constant determinations over the pH range 10 to 12.[73] The latter results, combined with previously available binding data,[2] indicate that the stability of the enzyme-NAD^+ complex relative to that of the enzyme-NADH complex increases by a factor of about 4000 as the pH is raised from 6 to 12 (Figure 8). Andersson et al.[74] pointed out that the pH profile for the differential effect of pH on NADH and NAD^+ binding must include the accumulated stabilizing contributions from deprotonation of *all* ionizing groups that interact with the NAD^+ ring charge. Hence they concluded that (1) such interactions are of detectable strength only in one instance; (2) the interacting group is the one that accounts for the pK_a-7.6-dependence of NAD^+ dissociation; (3) this group has its pK_a perturbed to 11.2 in the enzyme-NADH complex; and (4) the exceptional strength of the interaction (ΔpK = 3.6, i.e., stronger than the electrostatic interaction of the two carboxylic groups in oxalic acid[75]) implies that the group must be located in the immediate vicinity of the nicotinamide ring of bound NAD^+.

The only polar groups in close proximity to the nicotinamide binding site are the two cysteinyl ligands of catalytic zinc, zinc-bound water, and the side chains of Ser-48 and Thr-178.[4] The latter two groups would be expected to ionize with pK_a values well above 13 in free enzyme and, therefore, have been disregarded in discussions of the pH dependence of coenzyme binding. Anderson and Dahlquist speculated recently that Cys-46 or Cys-174 could be the group that has its pK_a perturbed from a value above 9 to 7.6 on NAD^+ binding.[76] That idea cannot be reconciled with the available information on the effect of metal coordination on ligand pK_a,[77] according to which the two cysteinyl zinc ligands would be expected to ionize with pK_a values below 6 already in free enzyme. The possibility that the active site zinc ions in liver alcohol dehydrogenase might exhibit atypical properties in this respect is unlikely *a priori* and inconsistent with evidence showing that HCN undergoes a drastic pK_a perturbation on coordination to catalytic zinc.[74] Cys-46 and Cys-174 have been generally assumed to be fully ionized

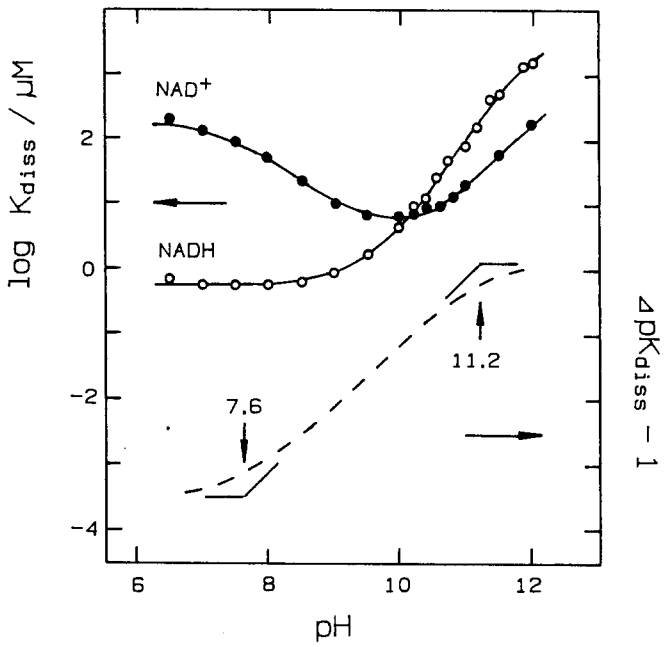


FIGURE 8. pH dependence of equilibrium constants for coenzyme binding to liver alcohol dehydrogenase. The dashed curve indicates the differential effect of pH on NADH and NAD^+ binding.

above pH 6,[1,2] and direct evidence in support of that view comes from the observation that metal reinsertion into enzyme depleted of catalytic zinc leads to the release of two protons per enzyme subunit.[78]

Cook and Cleland proposed from steady-state kinetic measurements that the pK_a-7.6-dependence of NAD^+ binding may derive from electrostatic perturbation of His-51.[79] Observed pKs of 9.7 to 10.4 for kinetic parameters reflecting substrate or inhibitor binding to the enzyme-NADH and enzyme-NAD^+ complexes were tentatively assigned to zinc-bound water, which would imply that the latter group is less strongly perturbed by the positive ring charge of NAD^+ than is His-51. Such an interpretation of the experimental results would seem to be incompatible with the available crystallographic information in two fundamental respects. First, no interactions have been detected which could force His-51 to ionize with a pK_a of about 9 in free enzyme;[23] the imidazole group of this surface residue appears to be extensively solvated in the open enzyme conformation and therefore would be expected to show a pK_a below 7 already in the absence of bound NAD^+. Second, His-51 is considerably more distant and dielectrically shielded from the nicotinamide binding site than is zinc-bound water, which precludes that the NAD^+ induced pK_a perturbation may be more pronounced for the former group than the latter.

Objections of the latter kind can be raised against any proposal that the drastic differential effect of pH on NADH and NAD^+ binding may derive from an ionizing group farther away than zinc-bound water from the nicotinamide binding site; Lys-228 and Tyr-286 have been mentioned as possible candidates.[1,2] Such a proposal would not only make it extremely difficult to explain the magnitude of the pH effect which is observed, but would also seem to require unreasonable assumptions about the ionization properties of zinc-bound water to explain the absence of an even greater effect of deprotonation of the latter group.[74] *If the NAD^+ ring charge interacts detectably*

strongly with a single ionizing enzymic group, then elementary electrostatic theory indicates that this group should be the nearest one with adequate ionization properties, and crystallographic evidence identifies the latter group as zinc-bound water. The possibility that zinc-bound water may show a pK_a as low as 7.6 in the enzyme-NAD^+ complex has never been strongly questioned, and arguments presented by Andersson et al.[73] indicate that a pK_a of 11.2 for zinc-bound water in the enzyme-NADH complex is compatible with the structure of the catalytic metal site in liver alcohol dehydrogenase as judged from reported data for ionization of metal-bound water in other zinc enzymes and in model zinc complexes.

Subramanian and Ross confirmed by calorimetric methods that NAD^+ binding is controlled by a perturbed enzymic group with a pK_a of 7.6 in the binary complex.[80] The enthalpy for ionization of this group was estimated at 8.8 to 9.8 kcal/mol, consistent with values reported for deprotonation of zinc-bound water. The only amino acids whose heats of deprotonation are close to the measured value are α- and ε-amino groups. The N terminus of the liver alcohol dehydrogenase subunit is acetylated,[18] and no lysine residues are located in the immediate vicinity of the nicotinamide binding site.

Shore et al.[65] attempted to rationalize pH dependence data for coenzyme binding and the quenching of LADH fluorescence in terms of a proton-dependent conformational change affecting NAD^+ binding as well as protein fluorescence. Subsequent studies showed that the fluorescence of free enzyme is quenched at alkaline pH with a pK_a of 9.8,[81] unaffected by imidazole binding to catalytic zinc and by carboxymethylation of the zinc ligand Cys-46.[82] The original interpretation of these observations was that the pH dependence of NAD^+ binding cannot be attributed to ionization of zinc-bound water, but it now seems more reasonable to conclude that the pK_a-9.2 group controlling coenzyme and imidazole binding is distinct from the pK_a-9.8 group which accounts for the quenching of protein fluorescence at alkaline pH. Laws and Shore presented spectral evidence indicating that the latter group may be a tyrosine residue and proposed resonance energy transfer from Trp-314 to Tyr-286 as a common mechanism for the decrease in protein fluorescence occurring at alkaline pH or on ternary complex formation with NAD^+ and trifluoroethanol.[83,84] Subramanian et al. in a thorough study of absorption and fluorescence spectra of coenzyme-containing complexes found no evidence for a ligand-induced ionization of tyrosine residues.[85] On the other hand, there are several reports confirming that coenzyme and substrate analogue binding may lead to a selective quenching of Trp-314,[86-88] which is buried inside the protein close to the subunit interface region. The mechanism for such quenching is still under debate and has not yet been convincingly related to any specific conformational change or ionizing enzymic group.[88-91] As pointed out by Abdallah et al.,[86] fluorescence quenching could reflect subtle environmental changes that may not be revealed by the available crystallographic data.

The early report of Theorell and co-workers that binding constants for NADH and ADP-ribose are affected analogously by pH over the range 6 to 10 has been corroborated by more recent measurements.[65] Gunnarsson and Petterson[92] found that this analogy extends also to the coenzyme fragment AMP, while there is no corresponding effect of pH on the binding of adenosine. Hence they proposed that the pH dependence of NADH binding derives from a proton-dependent modulation of the anion-binding capacity of the pyrophosphate binding subsite. Kinetic inhibition and Cys-46 modification studies have confirmed that anion binding at this subsite requires protonation of an enzymic group with a pK_a (8.8 to 9.2) differing insignificantly from that controlling NADH, ADP-ribose, and AMP binding.[92-95] Additional evidence for a common origin of the observed effects of pH on NADH and anion binding comes from proton-

release measurements indicating that complex formation between enzyme and NADH, ADP-ribose, AMP, or $Pt(CN)_4^{2-}$ perturbs the pK_a of an ionizing enzymic group from 9.2 to a value above 10.[67,69] Attribution of the pK_a-9.2 dependence of NADH association to effects on binding of the coenzyme pyrophosphate group is also consistent with the observation that NAD^+ association is affected analogously by pH (Figure 7).

Solution studies have shown that the interaction of anions with the pyrophosphate binding subsite conforms to the lyotropic series of affinity order,[93] which indicates that such complex formation is stabilized by charge interactions in a hydrophobic environment. This confirms the conclusions drawn from crystallographic results,[2,22,52] according to which Arg-47, possibly also Arg-369 and Lys-228, may provide positive charges at the anion binding subsite. The two arginyl residues interact with carboxylate groups of neighboring acid residues in the structure determined for free enzyme and can be anticipated to ionize with pK_a values well above 12. The crystallographic evidence for direct bonding interactions between the coenzyme adenine ribosyl group and Lys-228 (Figure 3) suggests that deprotonation of the latter group should affect not only coenzyme binding, but also the binding of adenosine; this argues against the possibility that Lys-228 may account for the pK_a-9.2 dependence of coenzyme binding. Andersson et al.[73] reported that the binding of NADH and NAD^+ is destabilized by deprotonation of an ionizing group with a pK_a of 10.4 in free enzyme (Figure 8). It seems reasonable to believe that the latter effect may reflect deprotonation of Lys-228; crystallographic results indicate that Lys-228 is extensively solvated in free enzyme,[49] suggesting that its pK_a should be close to that (10.2) of an unperturbed ϵ-amino group. Evidence relating the pK_a-9.2 dependence of anion and coenzyme binding to ionization of zinc-bound water will be considered in the following section.

Complex formation between enzyme and coenzyme has usually been described in terms of a single-step binding mechanism. Applying pressure-jump relaxation techniques, however, Coates et al.[96] found that the binding of NAD^+ can be resolved into a rapid association step followed by a slower isomerization of the binary complex:

$$E + NAD^+ \overset{\text{rapid}}{\rightleftharpoons} E - NAD^+ \overset{\text{slow}}{\rightleftharpoons} E^* - NAD^+$$

The slow step was shown to account for the pK_a-7.6 dependence of NAD^+ dissociation and was later proposed by Hardman to reflect a rate-limiting change in protein conformation.[97] A more obvious inference provided by the experimental results is that the pK_a perturbation of the ionizing group controlling NAD^+ binding must be associated with the isomerization step. This indicates that the latter step reflects accomodation of the coenzyme nicotinamide ring in proximity to zinc-bound water, supporting the early proposal of Shore that binding of the nicotinamide ring occurs subsequent to binding of the adenine ring.[98] Such an interpretation of the relaxation data would also be consistent with crystallographic results showing that coenzyme analogues may be bound in a nonproductive mode with the nicotinamide ring positioned at the surface of the protein.[99,100] Evidence for a multistep binding of NADH comes from protein fluorescence measurements performed at subzero temperatures in cryosolvents.[101,102]

B. Inhibitor Binding to Catalytic Zinc

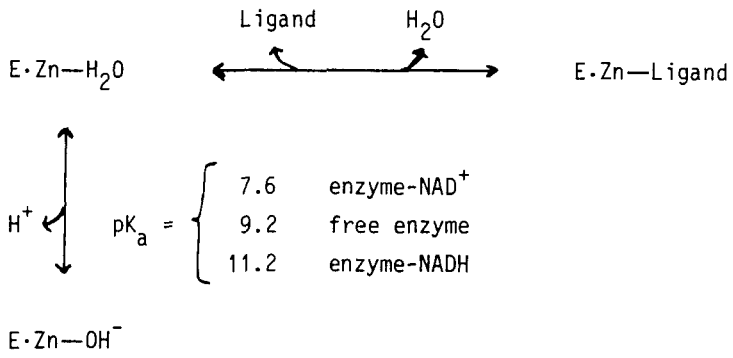
The first indications that zinc may have an essential mechanistic function came from inhibition studies involving heterocyclic metal binding bases such as imidazole, pyra-

zole, 1,10-phenanthroline, and 2,2′-bipyridine.[2] Spectroscopic results obtained with metal-substituted enzyme leave no doubt that these inhibitors are bound as inner-sphere ligands of catalytic zinc,[42] but have led to partly conflicting inferences with regard to the coordination number in pyrazole-containing species. Thus, resonance Raman spectra of Cu(II) enzyme indicate that the tetrahedral structure of the catalytic metal site is maintained after pyrazole binding,[44] whereas perturbed angular correlation spectroscopy on Cd(II) enzyme led to the conclusion that a five-coordinate complex is formed.[103] Solvent magnetic relaxation data for Cu(II) enzyme suggest that pyrazole adds as a fifth ligand in the absence of coenzyme and forms a four-coordinate complex in the presence of bound NAD^+.[47] The converse inference was drawn from EPR spectral parameters determined with Co(II) enzyme, which were taken to be indicative of four-coordination in the enzyme-pyrazole complex and five-coordination in the enzyme-NAD^+-pyrazole complex.[45] *The disparity of the above spectral evidence might reflect methodological difficulties in establishing the metal coordination number, but could also be due in part to a differential coordinative behavior of the different metal substituted enzyme derivatives.*

Crystallographic results, therefore, presently appear to provide the most reliable information on the detailed mode of binding of heterocyclic bases to native enzyme. Difference Fourier maps for the binary complex formed with 1,10-phenanthroline were determined at a resolution of 4.5 Å and indicate that the inhibitor is directly bound to catalytic zinc, probably as a bidentate ligand.[20] X-ray diffraction studies of binary and ternary complexes formed with imidazole and pyrazole have been performed at higher resolution and demonstrate that the latter inhibitors are bound as monodentate zinc ligands with retention of four-coordination at the metal site in the absence as well as the presence of bound coenzyme.[21,24]

Pyrazole, 1,10-phenanthroline, and bipyridine have been extensively used as reporter ligands in spectroscopic studies of the interaction of the enzyme with nonchromophoric ligands. Such studies have shown that catalytic zinc may function as a binding site for a variety of compounds with inhibitory action, including anionic ligands such as CN^- and higher homologues of fatty acids.[2,73] While acetate is bound at least 15 times more tightly to the pyrophosphate-binding subsite than to catalytic zinc, propionate and higher homologues appear to combine preferentially to the metal site;[73] this could explain why alkylations with iodoacetate and bromopropionate lead to modification of different cysteine residues.[58] Spectroscopic results obtained with Ni(II), Cu(II), and Co(II) substituted enzyme would seem to establish that anions such as CN^-, SH^-, and N_3^- are directly bound to catalytic zinc and suggest that a tetrahedral coordination is preserved in the complexes formed.[41,46,104]

DeTraglia et al.[66] pointed out that ligand binding to catalytic zinc should be strongly affected by ionization of the zinc-bound water molecule. They estimated the pK_a for this group to 8.1 from pH-dependence studies of 1,10-phenanthroline binding to free enzyme, and hence originally arrived at the conclusion that another group must account for the pH dependence of the NADH and NAD^+ association rates (Figure 7). Subsequent stopped-flow kinetic measurements demonstrated that 1,10-phenanthroline, bipyridine, and pyrazole association to free enzyme requires protonation of an enzymic group with an actual pK_a of 9.2,[69,105,106] consistent with Scheme 1 and the pK_a value assigned to zinc-bound water on the basis of Theorell's proposal and data in Figure 7.



Scheme 1. Displacement mechanism for the binding of nonionizing ligands to catalytic zinc in liver alcohol dehydrogenase.

An analogous dependence on the protonated form of the pK_a-9.2 group has been observed for the equilibrium binding of imidazole,[69] the alkylating imidazole derivative in Figure 6,[95] decanoate,[107] and cyanide.[74] Evans and Shore found that saturating NAD^+ shifts the effect of pH on bipyridine binding to a pK_a of 7.6,[105] and the same pK_a shift has been documented for pyrazole and decanoate binding to the enzyme-NAD^+ complex.[106,107] These results strongly support attribution of the observed effects of pH to ionization of zinc-bound water and provide compelling evidence that such ionization accounts for the pH dependence of NAD^+ binding.

Saturating NADH shifts the pK_a controlling imidazole, pyrazole, or decanoate binding from 9.2 to a value above 10,[69,106,107] consistent with the assignment of a pK_a of 11.2 to zinc-bound water in the enzyme-NADH complex. This pK_a shift for inhibitor binding to catalytic zinc is induced also by anionic coenzyme-competitive ligands such as AMP and $Pt(CN)_4^{2-}$.[69] Conversely, the pK_a-9.2 dependence of NADH, AMP, and $Pt(CN)_4^{2-}$ binding is abolished by imidazole, bipyridine, or 1,10-phenanthroline binding to catalytic zinc.[2,69,105] These observations, which provide clear evidence for a common origin of the observed effects of pH on ligand binding to catalytic zinc and complex formation at the pyrophosphate-binding subsite, led Andersson et al.[69] to propose that ionization of zinc-bound water destabilizes the binding of anionic ligands at the latter subsite through repulsive electrostatic interactions, mediated possibly by dislocation of the main cationic constitutent (Arg-47) of the subsite. According to that view, NADH binding perturbs the pK_a of zinc-bound water from 9.2 to 11.2 due to the electrostatic effect of the negatively charged pyrophosphate group of the coenzyme. NAD^+ binding gives rise to similar repulsive interactions, but these are counteracted by the stronger electrostatic effect of the positive nicotinamide ring charge leading to a net perturbation of the pK_a of zinc-bound water from 9.2 to 7.6.

Using crystallographic data and reported semiempirical relationships for the effective dielectric permittivity in water-accessible regions of proteins,[108] Pettersson and Eklund recently applied a point charge model for estimation of the electrostatic effects of bound coenzyme on the acidity of ionizing groups in the liver alcohol dehydrogenase subunit.[109] The results confirm that zinc-bound water would be expected to undergo substantial and opposite electrostatic pK_a shifts on the binding of oxidized and reduced coenzyme (Table 2). The calculated strength of interaction of groups such as His-51, His-67, and Lys-228 with the NAD^+ nicotinamide ring charge was at least 400 times too low to account for the drastic differential effect of pH on NADH and NAD^+ binding indicated by data in Figure 8, and none of these groups would be expected to have its pK_a shifted to a lower value following NAD^+ binding.

Table 2
pK$_a$ SHIFTS ATTRIBUTED TO AND CALCULATED FOR IONIZATION OF ZINC-BOUND WATER[73,109]

		Δ pK	
Species	pK$_a$	Observed	Calculated
Enzyme	9.2	—	—
Enzyme-NAD$^+$	7.6	−1.6	−1.5
Enzyme-NADH	11.2	+2.0	+1.5

Table 3
EFFECT OF COENZYMES ON ANION BINDING TO CATALYTIC ZINC[74,107]

	Cyanide		Decanoate	
Species	K_{diss} (μM)	Δ pK	K_{diss} (μM)	Δ pK
Enzyme	12	—	50	—
Enzyme-NAD$^+$	0.23	+1.7	2	+1.4
Enzyme-NADH	600	−1.7	2400	−1.7

Experimental information on the actual strength of coenzyme interactions with zinc-bound anions comes from equilibrium binding studies showing that the affinity of cyanide and carboxylates for catalytic zinc is weakened about 50-fold by NADH binding and strengthened to approximately the same extent by NAD$^+$ binding (Table 3).[74,107] This corresponds to coenzyme-induced opposite pK shifts of about 1.7, which can be anticipated to be representative also for hydroxide ion binding to catalytic zinc and hence for the thermodynamically equivalent process of ionization of zinc-bound water. The theoretically calculated and experimentally established effects of NADH and NAD$^+$ on anion binding to catalytic zinc are thus in the direction and of the magnitude required to account for the postulated pK$_a$ perturbation of zinc-bound water. The demonstrated existence and estimated strength of these effects provides the additional inference that coenzyme binding *minimally* must show a most pronounced pH dependence attributable to ionization of zinc-bound water. Such ionization must be assumed to add a contribution to the differential effect of pH on NADH and NAD$^+$ binding just as drastic as the single contribution observed experimentally (Figure 8), and the possibility that the pH effect contributed by zinc-bound water might fall entirely below pH 6 or above pH 12 cannot be reconciled with the available information on the coordination chemistry of catalytic zinc.[74] *Data in Tables 2 and 3, therefore, would seem to definitely establish that the observed differential effect of pH on NADH and NAD$^+$ binding derives from ionization of zinc-bound water.*

The NADH induced raise in pK$_a$ of zinc-bound water was recently attributed to the close proximity and low polarity of the reduced nicotinamide ring,[110] but it is far from obvious that a decrease in polarity of the environment of catalytic zinc should tend to decrease the acidity of zinc-bound water. AMP and $Pt(CN)_4^{2-}$ have no significant effect on 1,10-phenanthroline binding to catalytic zinc,[69,111] but their destabilizing effect on decanoate binding to zinc is similar to that of NADH and indicative of a predominantly electrostatic origin of the observed negative heterotropic cooperativity effects.[107] Syvertsen and McKinley-McKee[95] determined cooperativity factors for ligand binding to catalytic zinc and iodoacetate binding at the Arg-47 subsite from Cys-46

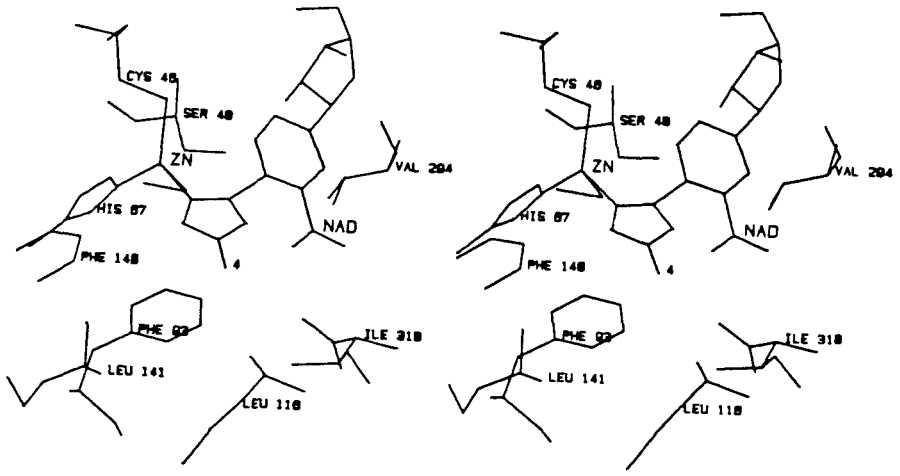


FIGURE 9. Stereo diagram of 4-iodopyrazole bound at the active site in liver alcohol dehydrogenase. (From Eklund, H., Samama, J.-P., Wallén, L., *Biochemistry*, 21, 4858, 1982. With permission.)

modification experiments. While no pronounced synergistic effects were observed with a variety of neutral metal ligands tested, sulfhydryl ion binding to catalytic zinc was found to be destabilized by a factor of 60 in the presence of bound iodoacetate. The latter results confirm that repulsive charge interactions between anions bound at catalytic zinc and at the Arg-47 subsite are of general significance and of sufficient strength to support the proposal that NADH induced effects on anion binding to the metal site (Table 3) and on the acidity of zinc-bound water (Table 2) derive mainly from electrostatic effects of the coenzyme pyrophosphate group.

The stabilizing effect of NAD^+ on anion binding to catalytic zinc has been demonstrated also with ligands that combine preferentially to the Arg-47 subsite in their interaction with free enzyme. Stopped-flow kinetic measurements of anion effects on NAD^+ binding led to the conclusion that acetate and thiocyanate interact more strongly with catalytic zinc in the enzyme-NAD^+ complex than with the Arg-47 subsite in free enzyme,[74] which indicates that the inhibitory effect of these anions derives primarily from the interaction with the metal site. Spectroscopic studies of Co(II) enzyme have demonstrated inner-sphere coordination of acetate and chloride ions to the metal site in the presence of NAD^+, and the equilibrium constant for anion dissociation from the enzyme-NAD^+-chloride complex was estimated to 0.1 *M*.[113,114] Assuming a cooperativity factor of 50 for the stabilizing effect of NAD^+ (Table 3), the latter datum would correspond to an equilibrium constant of 5 *M* for chloride dissociation from the metal site in the absence of coenzyme, consistent with ^{35}Cl NMR spectral evidence showing that 0.5 *M* chloride does not interact detectably with catalytic zinc in free enzyme.[111]

The stabilizing effect of NAD^+ on pyrazole binding (and vice versa) is well documented and has been extensively used for various methodological purposes.[1,2] Kinetic measurements have established that the exceptional stability of the enzyme-NAD^+-pyrazole complex relates to a reaction leading to proton release and indicates that pyrazole (analogously with water) undergoes a drastic pK_a shift on binding to catalytic zinc in the presence of NAD^+.[106] Crystallographic data for the ternary complex show that pyrazole is bound with one of its nitrogen atoms coordinated to zinc and the other positioned at a distance of 2 Å from carbon C-4 of the coenzyme nicotinamide ring (cf Figure 9). These results lend support to early proposals that pyrazole forms a covalent adduct with NAD^+,[2] and additional evidence in that direction comes from chemical

shifts observed for the nicotinamide and pyrazole nitrogens in ^{15}N NMR studies of the enzyme-NAD^+-pyrazole complex.[115] The strong inhibitory action of aldoxime derivatives has been similarly attributed to covalent adduct formation with bound NAD^+.[116,117]

Imidazole, bipyridine, and 1,10-phenathroline binding to catalytic zinc decreases the affinity of the enzyme for NADH and NAD^+.[2] X-ray diffraction studies of the enzyme-NADH-imidazole complex indicate that this synergistic destabilization can be related to steric factors; the inhibitor interferes with NADH binding and prevents the bonding interactions contributing to stabilization of the closed enzyme conformation from being established.[24]

C. Coenzyme Binding to Modified Enzyme

Enzyme selectively depleted of catalytic zinc by the anaerobic method of Zeppezauer and co-workers shows a slightly weakened affinity for NAD^+ and a drastically enhanced affinity for NADH. The latter effect derives from a decreased rate of coenzyme dissociation and has been attributed to the decreased polarity of the active site after zinc removal.[118] Dietrich et al.[119] found that effects of pH on coenzyme binding to the selectively zinc-depleted enzyme are similar to those reported for native enzyme, with the pK_a for coenzyme association being shifted from 9.2 to about 8.8 on removal of catalytic zinc.[119] This led them to question the role proposed by Andersson et al.[69] for zinc-bound water in the binding process and to attribute the observed effects of pH to an unspecified complex set of events considered likely to include the ionizations of Lys-228 and His-51.

The observed similarity in pH dependence of coenzyme binding to native and zinc-depleted enzyme is precisely what one would expect according to the proposal of Andersson et al., when zinc-bound water is replaced by a group (one of the freed cysteine ligands of catalytic zinc) with similar location and acidity. Crystallographic studies of the zinc-depleted enzyme provide clear evidence that the freed cysteine ligands remain unoxidized and located in positions where they should be subjected to coenzyme dependent electrostatic field effects of a strength similar to that established for zinc-bound anions (Table 3).[31] This argues strongly against the idea that another group or system of groups may account for the observed effects of pH on coenzyme binding to zinc-depleted enzyme; the effects caused by ionization of one of the freed cysteine groups must minimally be assumed to be at hand and are sufficient to account for the pH dependencies observed. Results presented by Dietrich et al.[119] would thus seem to support, rather than bring into question, the proposal of Andersson et al.[69]

^{113}Cd NMR studies of fully Cd(II) substituted enzyme failed to provide evidence for any spectral shifts attributable to ionization of metal-bound water between pH 7 and 10,[120] but a small shift detected at pH 10.3 was interpreted as the onset of water deprotonation with an estimated pK_a of 11.3. While the latter assignment and pK_a estimate may require more convincing experimental support, perturbed angular correlation spectroscopy on ^{111}Cd inserted selectively into the catalytic site has confirmed that cadmium-bound water probably ionizes with a pK_a above 9.5 whether NAD^+ is present or not.[103] The latter results were related to unpublished evidence indicating a pH dependence between pH 7 and 8 of K_m for the kinetic interaction of NAD^+ with Cd(II) enzyme and hence originally led to the conclusion that zinc-bound water is unlikely to account for the pK_a-7.6 dependence of NAD^+ dissociation from native enzyme. Subsequent stopped-flow kinetic measurements have shown that the rate of NAD^+ dissociation from Cd(II) enzyme actually remains unaffected by pH changes in the range 6 to 8,[68] consistent with the assignment of a pK_a above 9.5 to cadmium-bound water in the enzyme-NAD^+ complex and supporting Theorell's proposal that the pH depen-

Table 4
KINETIC PARAMETERS FOR COENZYME BINDING TO NATIVE AND Co(II) SUBSTITUTED ENZYME

Parameter	Zn(II)-E	Co(II)-E
NADH		
k_{on}, $\mu M^{-1}sec^{-1}$	9.8	8.7
pK_{aon}	9.35	9.15
k_{off}, sec^{-1}	4	6
NAD^+		
K_{on}, $\mu M^{-1}sec^{-1}$	38	23
pK_{aon}	9.1	8.8
k_{off}, sec^{-1}	65	61
pK_{aoff}	7.7	8.1

From Sartorius, C., Diplomarbeit, Universität des Saarlandes, Saarbrücken, 1984. With permission.

dence of NAD^+ binding reflects the ionization state of metal-bound water.[71] Effects of pH on coenzyme binding to native and Cd(II) substituted enzyme appear to be qualitatively dissimilar in other respects as well,[68] and may possibly derive in part from nonanalogous ionization processes. *Until that point has been clarified, pH dependence data obtained with Cd(II) enzyme should be interpreted with great caution.*

Effects of pH on coenzyme binding to native and selectively Co(II) substituted enzyme, however, are closely similar (Table 4) and can be anticipated to derive from analogous ionizations. Bertini et al.[121] examined proton NMR spectra of the Co(II) enzyme and assigned an isotropically shifted resonance appearing in aqueous solution to the imidazole δ-NH proton of the metal ligand His-67. This signal was shown to undergo a pH-dependent intensity decrease with a pK_a of 9.0 in the absence of coenzyme and a chemical shift change with a pK_a of 8.3 in the presence of NAD^+. While the latter effect was considered likely to reflect ionization of metal-bound water, the intensity decrease was tentatively attributed to deprotonation of the His-67 δ-NH group and led to the proposal that such deprotonation may account for the pK_a-9.2 dependence of coenzyme and inhibitor binding to native enzyme. The pK_a of the δ-NH group of free imidazole is 14.2 (i.e., lower than that (15.6) of free water), and the authors argued that this acidity order might be maintained after metal coordination of the ligands such that ionization of His-67 will occur prior to and suppress the ionization of metal-bound water.

Similar ideas have been advanced in discussions of the zinc site in carbonic anhydrase, but were rejected by Demoulin et al. on the basis of *ab initio* molecular orbital calculations indicating that the acidity order of imidazole and water is reversed on coordination to zinc.[122] In the case of liver alcohol dehydrogenase, the possibility that zinc-bound water might be less acidic than the δ-NH group of His-67 would seem to be definitely excluded by crystallographic evidence showing that the latter group is hydrogen bonded to the negatively charged carboxylate group of Asp-49;[29] this structural arrangement must cause a drastic increase in pK_a of the His-67 δ-NH group. Ionization of zinc-bound water, on the other hand, might be facilitated by electrostatic interactions with the positively charged side chain of Arg-47.[69] As pointed out by Bertini et al.,[121] the pK_a-9.0 dependence of the NMR signal assigned to His-67 can also be explained in terms of secondary effects of ionization of cobalt-bound water. The latter

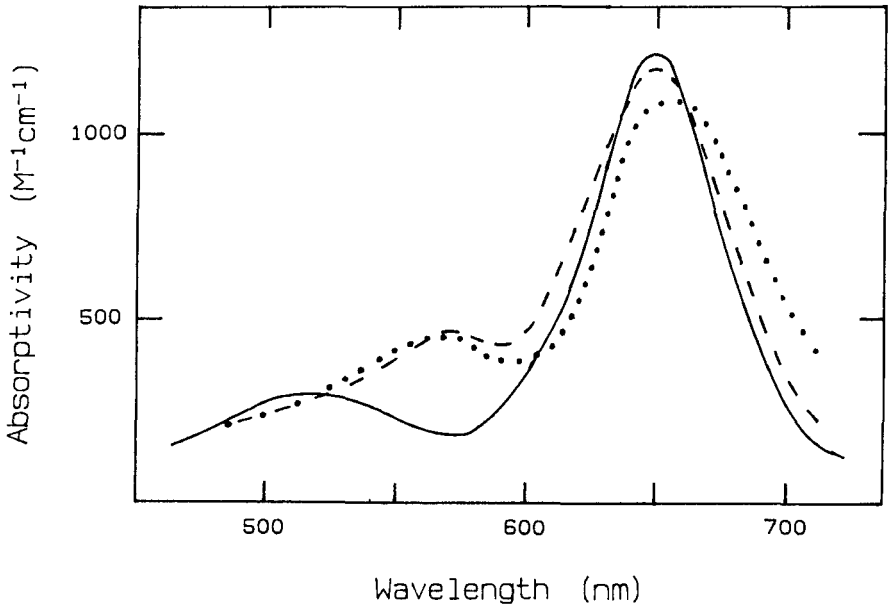


FIGURE 10. Optical absorption spectra of liver alcohol dehydrogenase containing cobalt(II) at the catalytic metal site, recorded at pH 5.3 (full curve) and pH 9.6 (dashed curve).[104] The dotted curve illustrates the spectral shift induced by azide binding at acid pH.[38]

interpretation seems more reasonable and is supported by their observation that NADH binding renders the signal pH independent in the range 7.3 to 9.3.

Zeppezauer and co-workers have examined the effect of ligands and pH on optical spectra of selectively Co(II) substituted enzyme.[42,104] The position of the metal-dependent band at 525 nm (Figure 10) remains essentially unaffected by pyrazole and other neutral ligands that are likely to combine to the active site metal ion. The binding to catalytic cobalt of anions such as Cn^-, N_3^-, and SH^- causes a characteristic redshift of the band to about 575 nm, indicating that this shift should also occur on hydroxide ion binding and hence may serve as an indicator of ionization of cobalt-bound water. Indeed, a raise in pH of solutions of free Co(II) enzyme was found to induce the characteristic shift to 575 nm of the 525-nm band with a pK_a close to 9.2.[104] The corresponding shift occurred with a pK_a of about 7.9 in the presence of saturating NAD^+, but could not be detected below pH 9.5 in the presence of saturating NADH. *These results corroborate the kinetic evidence for a coenzyme-dependent pK_a perturbation of an ionizing enzymic group (Table 2) and lend exceptionally strong support to the proposals identifying this group as metal-bound water by showing that the spectral effects of its ionization are indistinguishable from those caused by substitution of metal-bound water for an anion.*

Most kinetic and spectral studies of selectively metal substituted enzyme have been performed in solutions containing chloride ion, and quantitative data thus obtained may be affected by anion binding to the Arg-47 subsite or to the catalytic metal site.[74,93] Using chloride free solutions of Co(II) enzyme, Maret and Zeppezauer recently found a pK_a of 6.9 for the ionization shifting the 525-nm band to 575 nm in the presence of NAD^+,[114] consistent with the expectation that cobalt-bound water should be more acid than zinc-bound water; extension of these studies to include free enzyme and the enzyme-NADH complex would seem highly desirable. The ionization of the enzyme-NAD^+ complex, as well as chloride binding to the complex, was shown to affect also the main absorption band at 650 nm, causing a redshift of the band similar to that induced by NADH binding. This redshift was attributed to the transition from the

Table 5
DEPENDENCE ON THE CATALYTIC METAL ION OF KINETIC AND SPECTRAL PARAMETERS FOR DIMETHYLAMINOCINNAMELDEHYDE BINDING TO THE ENZYME-NADH COMPLEX

Parameter	Co(II)-E	Ni(II)-E	Zn(II)-E	Cd(II)-E
k_{on}, $\mu M^{-1}sec^{-1}$	20	16	40	25
k_{off}, sec^{-1}	80	110	180	380
k_{cat}, sec^{-1}	10.7	7.1	7.2	0.17
$\Delta\lambda_{max}$, nm	80	77	66	59

From Dunn, M. F., Dietrich, H., MacGibbon, A. K. H., Koerber, S. C., and Zeppezauer, M., *Biochemistry*, 21, 354, 1982. With permission.

open to the closed enzyme conformation and led the authors to propose that the presence of a negatively charged ligand at the active site metal ion is required to stabilize a closed conformational state for the enzyme-NAD^+ complex.

D. Substrate Binding

Early kinetic and equilibrium binding studies indicated the presence in liver alcohol dehydrogenase of a hydrophobic region for complex formation with substrate molecules and established that such complex formation may be competitively affected by ligands that combine to the active site zinc ion.[2] Mildvan and co-workers proposed from NMR studies of fully substituted cobalt enzyme that substrates are bound via first-sphere water to the second coordination sphere of catalytic zinc; metal-proton distances calculated from assumed paramagnetic effects of cobalt on relaxation rates of the protons of enzyme-bound ethanol, isobutyramide, and imidazole were found to be incompatible with inner-sphere coordination of the ligands.[1,2] More recently, Drysdale and Hollis obtained similar results for ligands used in X-ray diffraction studies of the enzyme,[123] which led them to conclude that the inner-sphere coordination of substrate analogues observed in crystal structures of ternary complexes may not be representative for the structures in solution. In a subsequent NMR study, however, Zeppezauer and associates could detect no paramagnetic contribution from the active site cobalt ions to the magnetic relaxation rate of solvent water and methanol protons, despite attempts to enhance the detection of such effects.[47] They concluded that previous high-field cobalt NMR data require reinterpretation since any paramagnetic effects that might be present are small and not easily separable from a variety of diamagnetic effects.

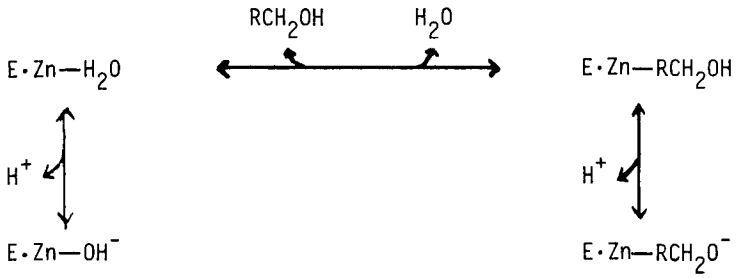
Spectral data indicative of direct ligation of a substrate to catalytic zinc were first presented by Dunn and co-workers, who found that the catalytically competent complex formed between enzyme, NADH, and the chromophoric 4-*trans*-(*N,N*-dimethylamino)cinnamaldehyde (DACA) exhibits a pronounced ligand redshift similar to that observed in model metal complexes of the aldehyde.[1] Analogous studies of metal-substituted enzyme have provided unambiguous evidence for inner-sphere coordination of DACA by demonstrating that the redshift, as well as the rate of aldehyde dissociation from the complex, show the expected correlation with Lewis acid strength of the active-site metal ion (Table 5).[124] Dimethylaminobenzaldehyde forms a similar redshifted complex with enzyme and NADH. Jagodzinski et al.[125] reported that resonance Raman spectra of that complex lacks a carbonyl group vibration deriving from the aldehyde and concluded from studies of model zinc complexes that this spectral feature can be reconciled only with strong zinc-oxygen bond formation.

Using Co(II) enzyme, Makinen et al. demonstrated the absence of a solvent isotope

effect on EPR spectra of the enzyme-NADH-DACA complex.[126] This corroborates the crystallographic evidence showing that DACA is bound to zinc with displacement of the inner-sphere water molecule and leads to the expectation (cf. Scheme 1) that formation of the redshifted complex should exhibit a pH dependence reflecting the ionization state of zinc-bound water.[1] Spectrophotometric equilibrium measurements have confirmed that expectation and support the attribution of a pK_a of 11.2 to zinc-bound water in the enzyme-NADH complex.[73]

No redshifted intermediate is formed on the interaction of DACA with free enzyme, the enzyme-NAD^+ complex at high pH, or with enzyme depleted of catalytic zinc.[1,127] These observations have been taken to indicate that the aldehyde does not bind to the enzyme in the absence of the metal or reduced coenzyme.[127,128] Alternatively, the aldehyde might be bound in the substrate-binding pocket without combining directly to catalytic zinc. Using auramine O and bipyridine as reporter ligands, Andersson et al.[129] found that the latter case applies for DACA and benzaldehyde binding to free native enzyme, and similar methods were recently used by Kovař et al.[130] to demonstrate complex formation between aldehydes and zinc-depleted enzyme. Auramine O displacement experiments also provided evidence for the formation of an enzyme-NAD^+-DACA complex, the stability of which was shown to remain essentially unaffected by pH changes over the range 6 to 10.[129] Dahl and Dunn later observed that the chromophoric properties of this complex are strongly pH dependent,[128] with a redshift characteristic of inner-sphere bonding of the aldehyde appearing below a pK_a of 7.6 attributable to ionization of zinc-bound water in the presence of NAD^+. The available evidence, therefore, indicates that aldehydes may be bound in the inner as well as the outer coordination sphere of catalytic zinc, with inner-sphere coordination predominating in coenzyme-containing complexes at pH values where zinc-bound water is predominantly unionized.

Evidence that alcohol binding to the enzyme-NAD^+ complex is dependent on the ionization state of the pK_a-7.6-group was first presented by Shore et al.[70] and the tighter binding of trifluoroethanol observed at alkaline pH was originally taken to indicate that alcohols interact with the unprotonated form of the ionizing group. Kvassman and Pettersson[131] later found from stopped-flow kinetic measurements that trifluoroethanol association to the complex requires protonation of the pK_a-7.6 group, as observed for the association of decanoate and other ligands which combine to catalytic zinc (cf. Scheme 1). The tight equilibrium binding of the alcohol at high pH was shown to derive from an ionization of the enzyme-NAD^+-trifluoroethanol complex, characterized by a pK_a of 4.3 and an at least 100,000-fold lower rate of alcohol dissociation from the unprotonated form. The consistency of the observed pH-dependence pattern with that expected for metal coordination of an ionizing ligand led the authors to propose that alcohols combine to catalytic zinc in a displacement reaction controlled by analogous ionizations of zinc-bound water and the zinc-bound alcohol (Scheme 2).[131,132]



Scheme 2. Displacement mechanism proposed to account for the pH dependence of alcohol binding to the enzyme.[132] Table 6 lists the pK_a values applying for different species in the scheme.

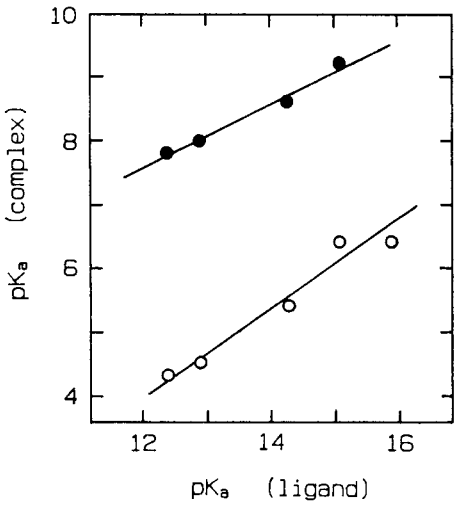


FIGURE 11. Brönsted plot of the pK_a controlling alcohol dissociation from enzyme-alochol (•—•) and enzyme-NAD^+-alcohol (O—O) complexes vs. the pK_a of the free alcohol ligand.[132-134]

Attribution of the pK_a controlling alcohol dissociation to the alcohol ligand, as opposed to an enzyme functional group such as His-51,[2,27,79] is supported not only by the drastic kinetic effect of the ionization,[134] but also by the absence of a corresponding effect of pH on the rate of dissociation of decanoate and other nonionizing zinc-bound ligands.[69,105,131] The possibility that zinc-bound water might have its pK_a perturbed from 7.6 to 4.3 through trifluoroethanol binding as a fifth ligand has been considered,[1] but would neither seem to be compatible with the higher intrisic acidity of the latter ligand, nor with the expectation that formation of a pentacoordinate complex should decrease rather than increase the acidity of zinc-bound water. Compelling evidence that the pK_a controlling alcohol dissociation reflects formation of a bound alkoxide ion comes from kinetic data demonstrating that this pK_a is linearly dependent on the intrisic pK_a of the alcohol ligand (Figure 11) with a Bronsted α-value as high as 0.6.[132,133]

Unlike aldehydes,[129] primary alcohols displace zinc-bound reporter ligands in the absence of coenzyme, and alcohol-binding constants calculated from such displacement reactions agree with those obtained using auramine O as reporter ligand.[134] This indicates that alcohols are bound predominantly as inner-sphere zinc ligands already at the binary-complex level. Additional evidence in that direction comes from the observation that alcohol binding to free enzyme shows a pH dependence analogous to that observed in the presence of NAD^+, but shifted towards higher pH.[134] As would be expected from Scheme 2, the ligand-dependent ionization occurred with the pK_a of 9.2 assigned to ionization of zinc-bound water in the absence of coenzyme, and the ligand-dependent ionization exhibited a linear Bronsted correlation ($\alpha = 0.5$) with the intrisic acidity of the alcohols examined (Figure 11). Kamlay and Shore found no pK_a-9.2 dependence for ethanol binding to free enzyme.[135] This may suggest that there are alcohols that do not combine to catalytic zinc at the binary-complex level, although data in Figure 11 would seem presently to favor the alternative possibility that zinc-bound ethanol (analogously with benzylalcohol) exhibits a pK_a experimentally indistinguishable from that of zinc-bound water in the absence of coenzyme. Alcohol binding to the enzyme-NADH complex has been found to be pH independent up to pH 10,[134,135] consistent with the evidence indicating a pK_a of 11.2 for zinc-bound water in

Table 6
pK$_a$ VALUES ASSIGNED TO ZINC-BOUND ALCOHOLS IN DIFFERENT ENZYMIC SPECIES[131-134]

Ligand (L)	pK$_a$			
	L	E-L	E-NAD$^+$-L	E-NADH-L
Water	15.6	9.2	7.6	11.2
Ethanol	15.9	—	6.4	—
Benzylalcohol	15.1	9.2	6.4	>10
2-Chloroethanol	14.3	8.6	5.4	>10
2,2-Dichloroethanol	12.9	8.0	4.5	>10
2,2,2-Trifluoroethanol	12.4	7.8	4.3	>10

the presence of reduced coenzyme.[73] The pK$_a$ values assigned to zinc-bound alcohols from kinetic and equilibrium binding studies are listed in Table 6.

The kinetic and crystallographic evidence for inner-sphere zinc coordination of primary alcohols is supported by spectroscopic results obtained with metal-substituted enzyme. Proton magnetic relaxation studies of Cu(II) enzyme showed that methanol displaces metal-bound water in its interaction with free enzyme and the enzyme-NAD$^+$ complex.[47] Makinen et al. concluded from EPR data for Co(II) enzyme that trifluoroethanol displaces metal-bound water in the absence of coenzyme and adds as a fifth ligand in the presence of NADH.[126] Bobsein and Myers found in NMR studies of Cd(II) enzyme that the catalytic ^{113}Cd resonance is deshielded by 75 ppm on pyrazole and trifluoroethanol binding to the enzyme-NAD$^+$ complex; the similarity of these shifts was related to the sensitivity of ^{113}Cd NMR to nitrogen vs. oxygen ligation and hence led to the proposal that neither ligand combines directly to the metal.[120] The magnitude of the shifts would seem to be more compatible with inner-sphere coordination of both ligands, however;[120,136] however, the large effect of trifluoroethanol (unexpected for the substitution of one neutral oxygen ligand by another) suggests that the alcohol is bound as an alkoxide ion. Perturbed angular correlation spectra of Cd(II) enzyme provides clear evidence for first-sphere metal binding of trifluoroethanol in the presence of NAD$^+$ and indicates that a five-coordinate complex is formed.[103]

Anderson and Dahlquist concluded from ^{19}F NMR studies of the enzyme-NAD$^+$-trifluoroethanol complex that the alcohol does not change ionization state in the pH range 6 to 9, but could not decide whether the ligand is ionized or not.[76] Using Co(II) enzyme, Dietrich and Zeppezauer[104] demonstrated that optical absorption spectra of the complex exhibit the 575-nm band characteristic for the presence of metal-bound anions; the band remained unaffected by pH in the range 4.5 to 10 and showed the onset of an intensity decrease below pH 4.5. Spectra of the enzyme-NADH-ethanol complex were found to be pH independent in the range 4.5 to 9.5 and to exhibit the 525-nm band characterizing the presence of neutral zinc-bound ligands, while spectra of the enzyme-NAD$^+$-ethanol complex displayed a pH-dependent shift of the 525-nm band to 575 nm with a pK$_a$ of 6.3. These observations strongly support the binding mechanism in Scheme 2 and the pK$_a$ values assigned to zinc-bound alcohols in Table 6.

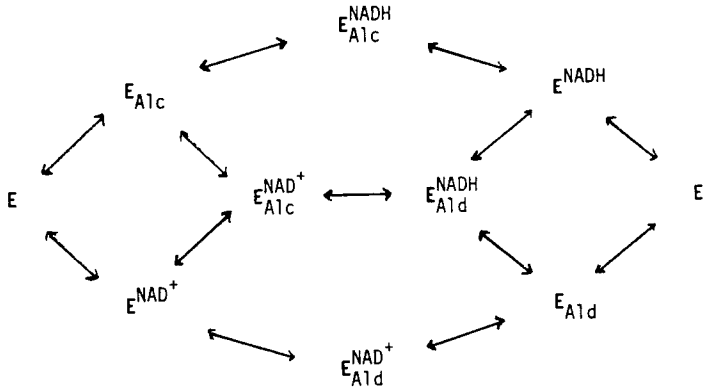
As earlier reported for fatty acid amides,[2] the binding of aldehydes and alcohols appears to be generally stabilized by a factor of 5 to 20 in the presence of saturating NADH.[129,134] This synergistic stabilization of the ternary complexes is dependent on the coenzyme nicotinamide group, as well as on the presence of the active site metal ion,[130] and has been attributed to hydrophobic interactions associated with dehydra-

tion of the active site region on simultaneous occupation of the adjacent nicotinamide and substrate binding sites.[129] NAD^+ has a similar stabilizing effect on the binding of amides and aldehydes,[88,129] and may drastically increase the affinity of the enzyme for alcohols due also to effects on the ionizing groups controlling substrate binding.[76,134] The synergism between substrate and coenzyme binding could be of physiological importance, since it reduces the K_m values for catalytic turnover of alcohol and aldehyde substrates.

V. KINETIC PROPERTIES

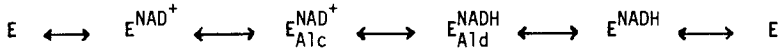
A. Random-Order Mechanism

Liver alcohol dehydrogenase operates by a ternary-complex mechanism. The equilibrium binding studies showing that substrates and coenzymes form binary as well as abortive ternary complexes indicate that the catalytic reaction must be basically assumed to proceed by the generalized random-order mechanism in Scheme 3.



Scheme 3. Random kinetic scheme for the catalytic interconversion of alcohols (Alc) and aldehydes (Ald).

Evidence that the reaction is not compulsory ordered came from early isotope exchange measurements, and the kinetic significance of species such as the abortive enzyme-NADH-alcohol complex has been demonstrated by early and more recent analyses of the substrate inhibition or activation patterns observed at high alcohol concentrations.[2,135] Over concentration ranges where Michaelis-Menten kinetics are obeyed, however, the compulsory-order mechanism in Scheme 4 has been firmly established to account for the steady-state rate behavior of the enzyme with most alcohol and aldehyde substrates tested.



Scheme 4. Ordered kinetic scheme usually applied for interpretation of the rate behavior of the enzyme.

The view has been widely held that the enzyme under such conditions operates by an effectively ordered mechanism with preferential utilization of the pathway involving prior binding of the coenzyme,[1,2] and several attempts have been made to explain why this binding order is preferred.[2,4,27,77]

Andersson et al.[137] recently arrived at an unexpected answer to that question. Using

DACA as the substrate, they found that NADH and aldehyde association rates in the alternative pathway involving prior substrate binding agree with those in the alledgedly preferred pathway for formation of the productive enzyme-NADH-aldehyde complex; this establishes that both pathways are fully catalytically competent. Analysis of the kinetic data indicated that, depending on substrate and coenzyme concentrations, either pathway may provide a major contribution to net reaction flux, whereas *the apparent steady-state rate equation will be of the Michaelis-Menten type corresponding to Scheme 4 irrespective of what pathway is actually preferred.* Consequently, the observation that this type of rate equation obtains cannot be taken as evidence that the actual reaction is correspondingly (preferentially) ordered. The catalytic reaction appears to be perfectly random with regard to the order of NADH and aldehyde binding, and Scheme 4 may give a completely misleading picture of the dominating pathways and accumulating reaction intermediates. Similar considerations are likely to apply for catalysis in the direction of alcohol oxidation,[134] at least with substrates (e.g., ethanol[135]) which do not affect the rate of NAD^+ association to the enzyme. On the other hand, there is no reason to doubt that product desorption is an effectively ordered process with coenzyme as the last-leaving product.[134,137] Furthermore, parameter values obtained with standard substrates in standard kinetic experiments will provide information only on rate constants for reaction steps in Scheme 4.[137] This may justify the standard approach (applied below) of discussing the rate behavior of the enzyme primarily in terms of the latter kinetic scheme.

B. Subunit Equivalence

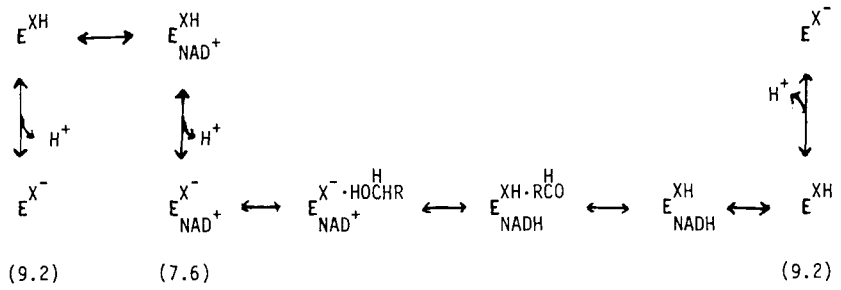
Binding studies and steady-state kinetic measurements have failed to provide evidence for any nonequivalence of the two subunits in the liver alcohol dehydrogenase molecule.[2] Bernhard et al. in 1970 reported that the transient-state reduction of aromatic aldehydes occurs in two distinct phases with consumption of exactly one half of the limiting reactant in each phase; this led them to propose a "half of the sites" reactivity for the enzyme, later attributed to effects of the ligation state at one subunit on the catalytic activity of the other.[1,2] That proposal initiated extensive studies of the biphasic aldehyde reduction process, but was ultimately strongly disfavored by results showing that the amplitude of the rapid one of the two transients approaches a saturation value corresponding more closely to 100 than 50% of the total concentration of active sites.[1] Theoretical analyses based on the assumption of equivalent and noninteracting sites indicated that the transient-state rate behavior of the enzyme is fully compatible with the ordered mechanism in Scheme 4,[138] with the biphasic time course for the reduction of tightly bound aldehydes being attributable to transient accumulation of the enzyme-NAD^+-alcohol complex.[139]

More recently, Dunn et al.[140] in a rebuttal of the criticism raised against the "half of the sites" reactivity hypothesis reported amplitude data for the transient-state reduction of a series of *para*-substituted benzaldehydes by NADH and NAD^2H. Isotope and substituent effects indicated by these data were claimed to be in substantial disagreement with Scheme 4 and to support a more complicated mechanism considered likely to involve subunit cooperativity. The detailed predictions of Scheme 4 with regard to transient amplitudes were later analyzed by Andersson and Pettersson[141] and the extended kinetic theory was found to account with remarkable accuracy for reported transient-state kinetic results, including those of Dunn et al.[140] By ^{19}F NMR studies of ternary complexes containing *p*-trifluoromethyl derivatives of benzylalcohol and benzaldehyde, Anderson and Dahlquist confirmed that the equilibrium for catalytic hydride transfer favors accumulation of enzyme-NAD^+-alcohol complexes.[142] It was further concluded from such experiments that a single lifetime describes the exchange of ligands from both subunits of the protein.[143] The absence of a ligand-induced subunit

cooperativity is indicated also by kinetic results obtained with enzyme in which 50% of the active sites were prelabeled with NAD^{3}H,[144] inhibited by pyrazole or trifluoroethanol binding,[145] or inactivated by carboxymethylation of Cys-46.[146] No sound experimental evidence is presently available for a kinetic nonequivalence of the two subunits of the enzyme.

C. Catalytic Proton Release

The steady-state kinetic evidence locating catalytic hydride transfer between substrate and coenzyme to the ternary-complex interconversion step in Scheme 4 was corroborated by early transient-state kinetic results showing that this step is subjected to a primary isotope effect in reactions performed with deuteriated alcohols or NADH.[1] Subsequent kinetic studies have focused on the pH dependence of the individual reaction steps in Scheme 4 and on the mechanism for the net release of protons that must be associated with alcohol oxidation by NAD^+. Shore et al.[70] found that such proton release occurs prior to and uncoupled from the hydride transfer step at neutral and alkaline pH, being triggered by NAD^+ binding and the concomitant pK_a perturbation of the ionizing group that controls the binding of the coenzyme. The observation that the ionization state of this group affects also the binding of substrate analogues led them to propose the mechanism in Scheme 5, according to which the ionizing group has a dual function as a binding site for substrates and as a mediator of proton transfer from bound alcohols to solution.

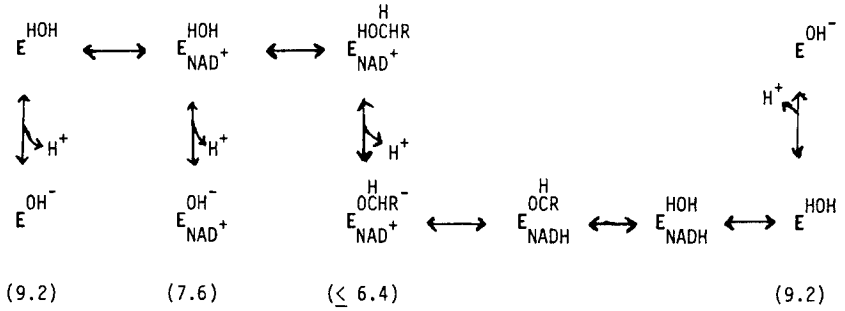


Scheme 5. Reaction mechanism proposed by Shore et al.[70] The catalytic step of proton transfer is located to the binary enzyme-NAD^+ complex. pK_a values for the ionization steps are indicated within brackets.

Modifications of Scheme 5 in the latter respect were considered by Branden et al.[2] and Dworschack et al.[147] who identified the ionizing group as zinc-bound water and suggested that the zinc-bound hydroxide ion acts as a base catalyst for proton abstraction from the alcohol in the substrate binding step and the ternary-complex interconversion step, respectively.

The need for a kinetically more fundamental modification of Scheme 5 was indicated by the early observation of Brooks et al. that hydride transfer from ethanol to NAD^+ is dependent on the basic form of an ionizing group with a pK_a of 6.4 in the productive ternary complex.[148] Hydride transfer from benzylalcohol to NAD^+ shows a similar pK_a-6.4 dependence, but there is no corresponding effect of pH on hydride transfer from NADH to benzaldehyde.[149] Kvassman and Pettersson concluded from the latter observations that deprotonation of the enzyme-NAD^+-alcohol complex represents an obligatory catalytic step preceding hydride transfer to NAD^+, and in subsequent kinetic studies showed that alcohol desorption takes place exclusively from the protonated form of the complex.[131,132] Hence they found that all main effects of pH (in the range 6 to 10) on the catalytic reaction can be accounted for in terms of Scheme 6, according

to which catalytic proton exchange with solution occurs at the ternary-complex level rather than being linked to the binding or desorption of NAD^+.



Scheme 6. Reaction mechanism proposed by Kvassman and Pettersson.[132] The catalytic step of proton transfer is located to the ternary enzyme-NAD^+-alcohol complex and attributed to ionization of the bound alcohol.

Consistent with Scheme 6, proton release/uptake associated with formation of the enzyme-NAD^+-alcohol complex during alcohol oxidation or aldehyde reduction could be experimentally detected when reactions were performed at adequate pH below 6.4.[131,132]

Kvassman and Pettersson attributed the pH-dependent reactivity of the enzyme-NAD^+-alcohol complex to ionization of the enzyme-bound alcohol, which was assumed to be directly coordinated to catalytic zinc.[132,149] Crucial evidence in favor of that proposal came from the subsequent observation that the corresponding pK_a is dependent on the intrisic acidity of the alcohol ligand (Figure 11); this was demonstrated for the hydride transfer step as well as for the alcohol desorption step.[133] The idea that hydride transfer to aldehydes results in formation of a zinc-coordinated alkoxide ion prior to product desorption was also favored by Morris et al., who found that catalytic breakdown of the enzyme-NADH-DACA complex is rate limited by hydride transfer at low pH and by a pH-dependent alcohol dissociation at high pH.[150] The pK_a value accounting for the latter pH dependence could not be established, but must be lower than the apparent pK of 6.0 shown to characterize the shift in the rate-limiting step; this provides additional evidence for a ligand dependence of the pK_a controlling the reactivity of the enzyme-NAD^+-alcohol complex.

Early kinetic evidence suggesting that an isomerization of binary enzyme-coenzyme complexes may contribute to limitation of the rate of coenzyme dissociation was reinterpreted by Kamlay and Shore and shown to reflect synergistic effects of substrate and chloride on the coenzyme desorption rates.[2,135] Andersson et al. concluded from DACA association studies that neither the open to closed conformational change, nor ligation of the substrate to catalytic zinc, provides a rate-limiting first-order contribution detectable by stopped-flow techniques.[137] Zinc-chelating reagents such as bipyridine and 1,10-phenathroline, however, exhibit a limiting rate of about 250 sec^{-1} in their chromophoric association to the enzyme; this has been proposed to reflect rapid outer-sphere complex formation followed by inner-sphere metal coordination of the chelator in a slower step rate limited by dissociation of zinc-bound water.[105,151,152] The absence of a corresponding limitation of the rate of association of monodentate inhibitors was demonstrated by Frolich et al.[152] and led them to suggest that such ligands (and substrates, by analogy) combine to catalytic zinc without displacing zinc-bound water. Hence they favored the proposal of Dworschack and Plapp[147] that a pentacoordinate water molecule may play an essential role in the catalytic process of proton transfer

from substrate to solution. Andersson et al.[69] argued that a limiting rate of 250 sec^{-1} is far too low to represent the dissociation rate of zinc-bound water and more likely reflects the structural rearrangements associated with chelate formation due to the increase in metal coordination number from 4 to 5. This would indicate that monodentate ligands are bound without any change in metal coordination number, an interpretation which is favored by crystallographic results and by kinetic evidence showing that the pK_a-9.2 dependence of bipyridine binding derives from the initial rapid association step.[69,105] Bertini et al. pointed out that the absence of pronounced intensity changes in optical absorption spectra of Co(II) enzyme argues against a shift in metal coordination number following coenzyme or substrate binding.[121] The observation that binding of the bidentate ligant bipyridine to Co(II) enzyme causes a substantial decrease in intensity of the 650-nm absorption band adds weight to that argument.[153]

D. Kinetic Studies of Modified Enzyme

Early observations that alkylation of Cys-46 by iodoacetate affects rates and pH dependencies of reaction steps in Scheme 4 were related primarily to the increase in effective charge of catalytic zinc resulting from carboxymethylation of the negatively charged thiolate ligand.[1] Recent crystallographic results indicate that distortion of the metal site and steric or electrostatic field effects of the introduced carboxymethyl group are likely additional factors of mechanistic significance.[30] Evidence that such factors are of greater importance than the modulation of metal Lewis-acid strength comes from kinetic data showing that carboxymethylated enzyme exhibits a drastically reduced rate of hydride transfer from NADH to aldehydes,[154] and a significantly increased pK_a for the ionization controlling hydride transfer from ethanol to NAD^+.[155] Regression analysis of the latter data suggested that the protonated enzyme-NAD^+-alcohol complex may have about one seventh of the activity of the unprotonated form, but the statistical level of significance for rejection of the alternative possibility of no detectable activity of the protonated form was low. The evidence relating the pH-dependent reactivity of the complex to alcohol ionization provides the inference that it might be extremely difficult to demonstrate an activity of the protonated complex; insertion of a chloro substituent at C-2 of ethanol decreases the rate of catalytic hydride transfer from C-1 by a factor exceeding 1000,[133] and the conversion of a neutral alcohol into an alkoxide ion would be expected to have an even more drastic electronic effect on the hydride transfer step.

Shore and Santiago[156] earlier reported that the kinetic (and hence mechanistic) properties of fully Co(II) substituted enzyme are closely similar to those of native enzyme. An analogous similarity was noted by Dunn et al.[124] for the kinetics of DACA reduction by native and selectively Co(II), Ni(II), or Cd(II) substituted enzyme; their observation that rate constants for hydride transfer from NADH to the aldehyde exhibit the expected positive correlation with Lewis-acid strength of the active site metal ion (Table 5) provides clear evidence that the spectroscopically detected substrate-metal coordination is of catalytic significance. Koerber et al.[157] in a subsequent study of *p*-nitrobenzaldehyde reduction concluded that the mechanism of enzyme action is substantially altered by the substitution of Zn(II) for Co(II). The reported differences in kinetic behavior of native and Co(II) enzyme with this substrate would rather seem to reflect effects on magnitudes of individual rate constants than a fundamental change in catalytic mechanism; however, the indirect evidence presented for a kinetically significant isomerization of the enzyme-NAD^+-aldehyde complex is not convincing.

Compelling evidence supporting the mechanistic proposal in Scheme 6 was recently presented by Zeppezauer and associates.[110] Applying rapid-scanning stopped-flow techniques to Co(II) enzyme, they confirmed that the biphasic time course for single-

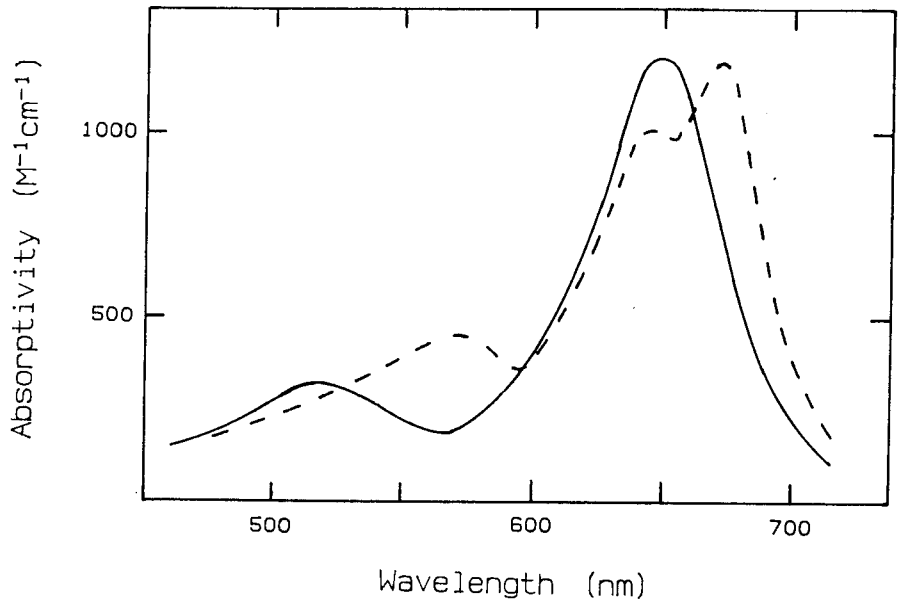


FIGURE 12. Optical absorption spectra of liver alcohol dehydrogenase containing cobalt(II) at the catalytic metal site (full curve) compared with the spectrum of the enzymic complex formed with NAD^+ and alcohols (dashed curve) at neutral pH.[110]

turnover benzaldehyde reduction reflects transient accumulation of an intermediate formed subsequent to hydride transfer and prior to product release, i.e., representing the enzyme-NAD^+-alcohol complex; the properties of this complex were examined also in benzylalcohol oxidation experiments. Spectra of the kinetically detected intermediate exhibited a pH-dependent contribution from the 575-nm absorption band characterizing the presence of cobalt-bound anions and indicated that the substrate is predominantly bound as a metal coordinated alkoxide ion at pH values above 6 (Figure 12). Similar results were obtained with other substrates. The approximate pK_a values for alkoxide formation estimated from the disappearance of the 575-nm band were 7.5 for methanol, 5.5 for ethanol, benzylalcohol and anisylalcohol, and <4.5 for trifluoroethanol, consistent with the pK_a values assigned to zinc-bound alcohols in the presence of NAD^+ (Table 6). As noted from static spectra of the enzyme-NAD^+-trifluoroethanol complex, ionization of the kinetically detected ternary complexes was associated also with the 650-nm band redshift suggested to characterize the open to closed conformational change. While this indicates that alkoxide formation may be an important factor triggering the conformational transition, no evidence was obtained for a temporal separation of the 575- and 650-nm spectral changes and there presently seems to be no strong reason to include the open/closed conformational transition as a separate reaction step in kinetic schemes for liver alcohol dehydrogenase catalysis.

Catalytically active enzyme containing covalently bound coenzyme has been prepared and the bound coenzyme could be recycled with other dehydrogenases.[158,159] Bernhard and co-workers suggested from recent studies of native enzyme that coenzyme recycling or exchange without prior release of the dinucleotide to solution may be a general characteristic of the metabolic operation and interplay of NAD^+-dependent dehydrogenases.[160,161]

E. Aldehyde Dehydrogenation

^{19}F NMR studies of the interaction of liver alcohol dehydrogenase with *p*-trifluoromethylbenzaldehyde have confirmed that the enzyme exhibits an aldehyde dehydrogen-

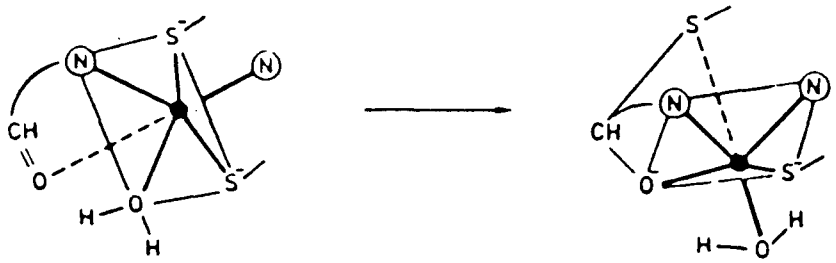


FIGURE 13. Thiohemiacetal formation step in the mechanism proposed for liver alcohol dehydrogenase catalysis of histidinal oxidation by NAD^+. (From Dutler, H. and Ambar, A., *The Coordination Chemistry of Metalloenzymes*, Bertini, I., Drago, S., and Luchinat, C., Eds., Reidel, London, 1983, 135. With permission.)

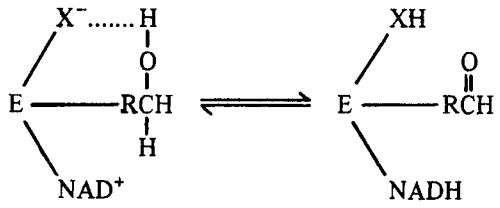
ase (aldehyde dismutase) activity.[162] The oxidation of aldehydes by NAD^+ may involve the hydrated form of the substrate and proceed by the same mechanism as alcohol oxidation, but there is no good evidence to show that such is actually the case. Dutler and Ambar have demonstrated that the enzyme catalyzes a NAD^+-dependent oxidation of histidinol to histidine with intermediate formation of histidinal.[163] This oxidation process was proposed to involve bidentate binding of the substrate to catalytic zinc and to proceed via thiohemiacetal formation between histidinal and Cys-174 (Figure 13) in a series of reaction steps associated with distal-to-proximal positional changes in a coordination sphere containing one distal and five proximal ligands. Although that proposal presently lacks sufficiently convincing experimental support, it draws attention to the fact that results obtained with monodentate primary or secondary alcohols are not necessarily representative of the biological action of the enzyme. The natural substrate for liver alcohol dehydrogenase remains unknown, and the enzymic oxidation of histidinol represents a reaction of potential physiological interest that certainly merits further investigation.

VI. MECHANISTIC CONCLUSIONS

A. Proton Transfer

It would now seem firmly established that the drastic differential effect of pH on NAD^+ and NADH binding to liver alcohol dehydrogenase derives from ionization of zinc-bound water, and this group has been convincingly shown to account also for the pH dependence of anion binding to the Arg-47 subsite and of ligand binding to catalytic zinc. The observed effects of deprotonation of the group on the kinetics of alcohol and aldehyde binding are consistent with the spectroscopic and crystallographic evidence for inner-sphere zinc coordination of substrates in the productive ternary complexes, but also preclude that ionization of zinc-bound water at the binary-complex level may be on the main catalytic pathway; kinetic results, including reported solvent isotope effects,[1,164] provide clear evidence that catalytic proton release to solution during alcohol oxidation occurs at the ternary-complex level in a distinct reaction step preceding hydride transfer to NAD^+. The alcohol substrate must be the ultimate source of the catalytically produced proton, but the crystallographic evidence for a complete dehydration of the active site in productive ternary complexes indicates that the proton cannot be directly released from substrate to solution. All recent mechanistic proposals, therefore, have attributed a proton-relaying function to the system of hydrogen bonds which appears to link the hydroxyl group of zinc-bound alcohols to the solvent-accessible imidazole group of His-67 (Figures 3 and 4). There have been differences in

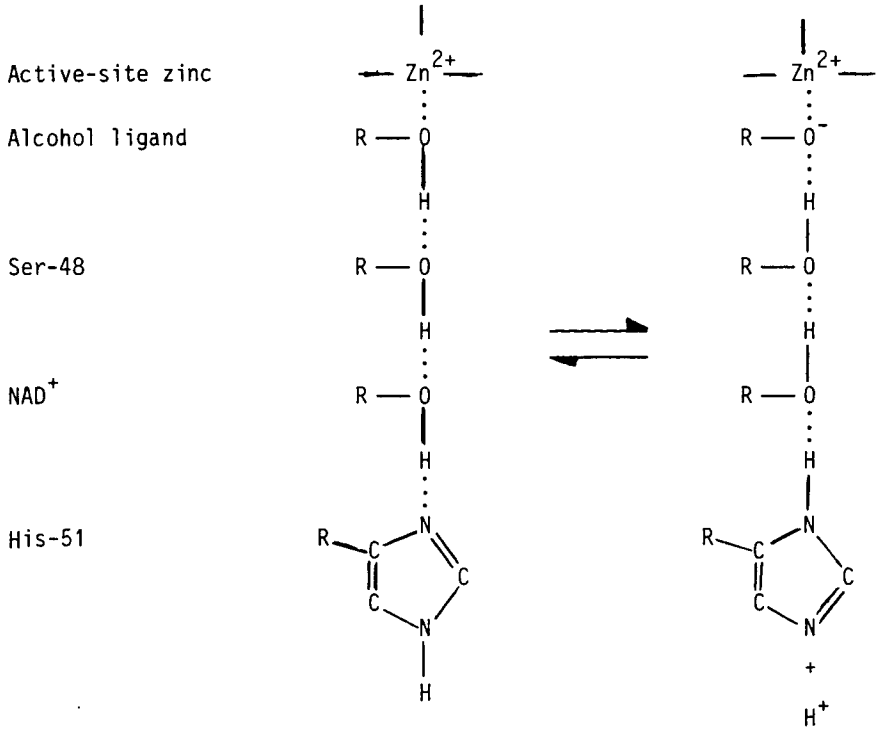
opinion only as to what group in the hydrogen bonded structure is the ionizing one which accounts for the actual proton release, i.e., which is associated with the pK_a controlling the reactivity of enzyme-NAD^+-alcohol complexes with regard to the hydride transfer and alcohol dissociation rates. His-51,[2,27,79] a pentacoordinated zinc-bound water molecule,[126,147,164] and the zinc-bound alcohol itself have been considered as possible candidates. In the latter case,[149] ionization of the alcohol must be assumed to take place in the proton release step (Scheme 6) and the system of hydrogen bonds merely serves the purpose of ensuring rapid transfer of the proton from substrate to solution. In the former two cases, proton abstraction can be envisaged to be temporally concerted with the hydride transfer step and to be subjected to direct or hydrogen-bond-mediated base catalysis by the deprotonated form of the ionizing group:



The proposal of Makinen et al.[126] that a *neutral* pentacoordinated water molecule might act as the base catalyst would assign a pK_a of 6.4 (with typical substrates) to a zinc-bound hydronium ion and must be rejected; the latter group would be expected to show a pK_a well below zero. Their data are compatible with catalytic participation of a neutral pentacoordinated water molecule as a constituent of the proton-relaying system of hydrogen bonds, however.

The possibility that ionization of His-67 may account for the pH-dependent reactivity of the productive ternary-complex would not seem to be supported by the available kinetic and spectroscopic data;[104,134] as noted by Eklund et al.[27] there may even be reason to question that His-67 necessarily forms part of the crystallographically indicated proton-relay system. Reported spectroscopic evidence for pentacoordination of a metal-bound water molecule may well be reliable, but refers to observations made with Co(II) enzyme in frozen solutions at 4 K,[45,126] or with substituted enzyme containing metal ions (Cu^{2+} or Cd^{2+}) that normally show a low tendency to form tetrahedral complexes.[47,103] The physiological relevance of the spectral results obtained at 4 K seems most uncertain, particularly since disagreeing structural inferences have been drawn from spectral data obtained with Co(II) enzyme at room temperature.[121] The absence of a detectable catalytic activity of Cu(II) enzyme,[40] and the presence of pronounced differences between native and Cd(II) substituted enzyme with regard to kinetic properties that relate to the structure of the catalytic metal site,[68,124] suggests that results obtained with Cu(II) and Cd(II) enzyme may not be entirely representative for the coordinative properties of native Zn(II) enzyme. The observation of a four-coordinate catalytic metal site in crystallographically examined species of Cd(II) enzyme indicates that the attainment of a higher coordination number (normally preferred by Cd^{2+}) is energetically disfavored by the enzyme polypeptide structure,[32] and the same conclusion has been drawn from structural model-building experiments.[3] This renders the possibility less likely that catalysis by native enzyme involves the formation of pentacoordinate species, except possibly as transient intermediates in the process of ligand exchange at catalytic zinc.[124] Zinc-chelating reagents are the only ligands that have been firmly established to form five-coordinate complexes with native enzyme, and the kinetic and spectroscopic characteristics of such complex formation appear to be qualitatively distinct from those of complex formation with monodentate ligands.

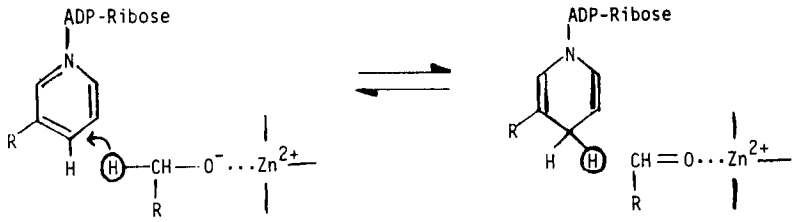
Results so far reported, therefore, lend no convincing support to the idea that productive ternary complexes formed with native enzyme contain a pentacoordinated water molecule. The weight of evidence strongly indicates that the pH-dependent reactivity of enzyme-NAD^+-alcohol complexes reflects formation of a four-coordinated zinc-bound alkoxide ion, supporting early and more recent proposals that alcohols are activated for hydride transfer through ionization.[2,149] Data in Table 6 indicate that such activation is accomplished by a pK_a perturbation (to a value of 6.4 or lower) that ensures that the substrate is bound predominantly as an alkoxide ion at physiological pH, and Scheme 7 depicts the ionizing structure and deprotonation step that seems likely to account for the catalytic proton transfer process.



Scheme 7. Probable mechanism for the catalytic proton-transfer step, involving ionization of the zinc-bound alcohol and proton release to solution via a system of hydrogen bonds.

B. Hydride Transfer

It was earlier recognized that ligation of the substrate to catalytic zinc may facilitate hydride ion transfer from NADH to aldehydes through Lewis-acid catalysis,[1,2] i.e., through electron withdrawal from C-1 of the substrate. It now seems apparent that metal coordination is also essential for the enzymic activation of alcohol substrates; the unfavorable direct Lewis-acid polarization of zinc-bound alcohols is counteracted by the metal and coenzyme induced alkoxide formation, which renders the substrate molecule negatively charged and hence lowers the energetic barrier or hydride ion abstraction from C-1. These electronic consequences of the metal-substrate interaction, combined with the crystallographic evidence for a juxtaposition of the reactive parts of substrate and coenzyme (Figure 5), leave little doubt that the catalytic redox step presently can be adequately described in terms of direct hydride exchange between the reactants (Scheme 8).



Scheme 8. Probable mechanism for the catalytic hydride-transfer step, involving direct hydride ion exchange between substrate and coenzyme.

The detailed mechanism for the hydride transfer step has been discussed on the basis of kinetically observed reactant and solvent isotope effects;[165-167] the latter data may be of minor informative value in that respect, considering the evidence indicating that hydride transfer takes place from an alkoxide ion in a completely solvent-free environment. Eklund and Branden pointed out that the absence of solvent might be required to avoid side reactions between water and the hydride ion.[3] Dehydration of the active site may be of importance also to favor inner-sphere zinc coordination of the substrate and to enhance the electrostatic stabilization of zinc-bound alkoxide ions.[3,129] The latter effect could explain why the NAD^+-induced pK_a perturbation is greater with zinc-bound alcohols than with zinc-bound water (Table 6).

C. Catalytic Principles

Presently available crystallographic, kinetic, and spectroscopic information on the structure and function of liver alcohol dehydrogenase is remarkably coherent and points to a relatively uncomplicated mechanism of enzyme action. The two subunits of the enzyme operate independently of each other, and there is no reason to believe that formation of the productive ternary complexes requires a certain order of substrate and coenzyme addition. The ligation of alcohols and aldehydes to catalytic zinc brings the substrate in suitable position for rapid hydride ion exchange with the coenzyme and proton exchange with solution, and alcohol/aldehyde interconversion through these hydrogen exchange reactions appears to proceed by the simplest possible mechanism. Thus, alcohol binding to zinc in the presence of NAD^+ results in immediate deprotonation of the substrate, which facilitates subsequent direct hydride transfer to NAD^+. Hydride transfer from NADH is facilitated by the polarizing effect of zinc on the aldehyde substrate, and protonation of the resulting alkoxide ion triggers desorption of the alcohol product. The main catalytic principles which apply (and that are all dependent on metal coordination of the substrate) are those of approximation and orientation effects, Lewis-acid catalysis, and transition-state stabilization; considering that free alcohol substrates ionize with pK_a values around 15 and hence have no significant tendency to dissociate a proton at physiological pH, it seems reasonable to characterize the alkoxide ion as a transition-state intermediate.

The open-to-closed conformational transition appears to be of crucial importance for adequate positioning of the reactants and for the creation of a favorable anhydrous site environment; the evidence relating this transition to coenzyme binding makes it reasonable to include induced fit among the applying catalytic principles. *From an energetic point of view, however, the drastic pK_a perturbation of alcohols in the productive ternary complex (ΔpK = 8 to 9) would seem to represent the key step of the catalytic process.* This stabilization of the transition-state intermediate by a factor exceeding 10^8 appears to result primarily from electrostatic-field effects of the principal nature discussed by Theorell and co-workers to support their ideas about the ionization

properties of zinc-bound water.[71] Thus, data for binary enzyme-alcohol complexes in Table 6 indicate that ligation of the substrate to catalytic zinc provides a major contribution ($\Delta pK = 5$ to 6) to the net pK_a perturbation. The additional pK_a shift contributed by NAD^+ binding is less pronounced, but of particular mechanistic interest since it is greater with alcohols ($\Delta pK \approx 3$) than with water (Δ pK = 1.6). This ensures that zinc-bound water will remain predominantly unionized (and hence exchangeable) in all substrate-binding species at physiological pH. In other respects, the ionization properties of zinc-bound water and alcohols appear to be entirely analogous and analogously affected by coenzyme binding, as would be expected from the structural analogy between these ligands. The much discussed pH dependencies attributable to ionization of zinc-bound water are of no major mechanistic interest per se, but simply reflect that the enzyme is designed to facilitate a corresponding ionization of alcohol substrates.

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