The Role of Zinc-bound Water in Liver Alcohol Dehydrogenase Catalysis*

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To elucidate the role of zinc-bound water in liver alcohol dehydrogenase catalysis, chelation by 1,10-phenanthroline and 2,2-bipyridine was studied. The rate constants for association of both chelating agents to the active center zinc were pH-dependent with a pKₐ of 9.2 and preferential binding to a protonated form. The binary complex dissociation rate constants were pH-independent for both chelating agents. In the presence of saturating NAD⁺, the pKₐ for the equilibrium binding of 2,2-bipyridine was perturbed to 7.6, similar to the functional group previously shown to be involved in NAD⁺ binding. The presence of saturating imidazole resulted in pH-independent 2,2-bipyridine binding. Studies of these provide compelling evidence that the ionizing group involved in coenzyme binding, proton liberation, and conformational states is zinc-bound water. The limiting rate of chelation by 2,2-bipyridine was pH-independent, and no limiting rate was observed in the presence of saturating imidazole. These results indicate that the limiting rate of chelation is due to the rate of dissociation of zinc-bound water. The implications of this regarding the role of zinc in catalytic turnover of liver alcohol dehydrogenase are discussed.

Previous studies (1, 2) demonstrated the existence of a liver alcohol dehydrogenase functional group with a pKₐ > 9 in free enzyme, perturbed to a pKₐ of 7.6 in binary enzyme-NAD⁺ complex. This perturbation resulted in partial proton liberation, with subsequent alcohol binding resulting in net proton release stoichiometry of one upon ternary complex formation. Subsequent studies indicated that the same ionizing group controlled the conformational state of the enzyme (3) and the binding of NADH (4, 5) and ADP-ribose (6).

The abolition of the pH dependence of NAD⁺ binding in the presence of imidazole (7), and crystallographic evidence that the enzyme active center zinc ion contained a tightly bound water molecule replaceable by imidazole (8), lent strong support to the postulate that zinc-bound water was the ionizing functional group involved in coenzyme binding (9). However, no direct evidence for this existed, and a recent report that the pKₐ for 1,10-phenanthroline binding was 8.1 resulted in the interpretation that zinc-bound water played no role in the pH dependence of coenzyme association (4).

Considering the potential significance of the zinc-bound water molecule, which has also been reported to be a component of a proton relay system (10), we re-investigated the pH dependence of chelation by both 1,10-phenanthroline and 2,2-bipyridine, which also chelates the active center zinc ion (11, 12). In addition, the pH dependence of the previously reported limiting chelation rate (13, 14) was determined and the effect of imidazole on both the pH dependence of chelation and the limiting rate was studied. These investigations provide compelling evidence for zinc-bound water as the ionizing group involved in coenzyme binding, proton release, and conformational states in the liver alcohol dehydrogenase reaction mechanism.

MATERIALS AND METHODS

Alcohol dehydrogenase (EC 1.1.1.1) was prepared from frozen horse livers by the method of Theorell et al. (15). Enzyme concentration was determined by absorbance at 280 nm using ε₉₅₀ of 4.55 and by activity measurements (16). NAD⁺, grade III, rabbit muscle lactate dehydrogenase, sodium pyruvate, and imidazole were purchased from Sigma Chemical Co. NAD⁺, Chromato Pure-ethanol-free, was obtained from P-L Biochemicals and 2,2-bipyridine and 1,10-phenanthroline from Eastman Organic Chemicals. For pH dependence experiments, phosphate buffer was used at pH 7 to 8, pyrophosphate at pH 8.5 to 9.5, and carbonate at pH 9.5 to 11.5. EDTA (2 mM) was used in all studies to chelate trace metal ions and has been shown not to interfere with enzyme-bipyridine or enzyme-phenanthroline complexes (11, 12).

Stopped flow studies were performed using a Durrum-Gibson spectrophotometer and a stopped flow fluorimeter built by Dr. David Ballou of the University of Michigan. Both instruments are interfaced to a Data General Nova 2/10 computer by a system purchased from On-Line Instrument Co., enabling signal averaging and facilitated calculation of pseudo-first order rate constants. Equilibrium binding studies were performed using a Zeiss PMQ II spectrophotometer. All measurements were made at room temperature, 23°C.

Stopped flow techniques were used to measure the rate of 1,10-phenanthroline association by observing the increase in absorbance associated with binary complex formation (Δε = 8.0 × 10⁴ M⁻¹ cm⁻¹) at 297 nm (17). The rate of 1,10-phenanthroline binding was measured at 305 nm under pseudo-first order conditions by mixing liver alcohol dehydrogenase (10 to 15 μM after mixing) with varying concentrations of 1,10-phenanthroline (0.10 to 2.0 μM after mixing). This wavelength was used to minimize the background absorbance of 1,10-phenanthroline and the difference extinction coefficient of the binary complex is 4.0 × 10⁴ M⁻¹ cm⁻¹ at 305 nm (11). Association rate constants were calculated from a plot of observed pseudo-first order rate constants versus 1,10-phenanthroline concentration.

The rate of 2,2-bipyridine association was measured by mixing liver alcohol dehydrogenase (10 μM after mixing) with varying concentrations of 2,2-bipyridine (0.1 to 6.0 μM after mixing) and observing the increase in absorbance at 308 nm with Δε = 1.1 × 10⁴ M⁻¹ cm⁻¹ (11). Association rate constants were determined from a double reciprocal plot of 1/kₐ₉₋₀ versus 1/[bipyridine], with kₐ₀ equal to kₐ₋₀ - k₋₀ at each ligand concentration, using k₋₀, the dissociation rate constant, determined directly. The slope of the plot is the reciprocal of the association rate constant while the y intercept provides the limiting rate of binding.

The rate constants for 1,10-phenanthroline and 2,2-bipyridine dissociation from enzyme-ligand binary complexes, k₋₀, were determined by displacement of the ligand with NADH (50 to 150 μM) and isobutyramide (10 μM). The increase in NADH fluorescence upon
ternary complex formation was monitored with excitation through a Corning 7-60 filter and emission through a PBL-1-42 filter. The NADH concentration sufficient for rate-limiting ligand dissociation was calculated using the equation of Noble et al. (18) and experimentally verified by increasing the NADH concentration until a maximum rate was observed. At pH values greater than 9.5, a pH-jump was used to measure both association and dissociation rates due to instability of the enzyme at pH values greater than 10.0. The enzyme or binary complex in 5 mM sodium phosphate buffer, pH 7.0, was mixed with appropriate reagents in 100 mM carbonate buffer at higher pH values. The pH of the mixture was measured upon completion of the reaction.

Equilibrium binding constants were determined spectrophotometrically by monitoring the increase in absorbance resulting from enzyme-2,2-bipyridine or enzyme-NAD⁺-2,2-bipyridine complex formation. Bipyridine was titrated into a cuvette containing liver alcohol dehydrogenase (25 μM) in the presence and absence of either 10 mM imidazole or saturating NAD⁺ (15 mM ≤ pH 7; 19 mM ≥ pH 7). For experiments in the presence of NAD⁺, 1 × 10⁻⁵ M sodium pyruvate and catalytic amounts of rabbit muscle lactate dehydrogenase were present to ensure that the coenzyme remained oxidized. The increase in absorbance at 308 nm minus the absorbance of a blank containing an equivalent bipyridine concentration was monitored. Statistical nonlinear least squares analysis of the data was performed using a Nova 2/10 computer.

RESULTS

The rate of 1,10-phenanthroline and 2,2-bipyridine binding to liver alcohol dehydrogenase was studied from pH 7.0 to 10.5, and Fig. 1 shows a typical reaction trace of enzyme-2,2-bipyridine binary complex formation. The rate constants for association of both ligands were pH-dependent with slower association of both ligands were pH-dependent with slower

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FIG. 2. The rate of dissociation of 2,2-bipyridine from enzyme-2,2-bipyridine binary complex. Syringe 1, 10 μM liver alcohol dehydrogenase 7.0 mM 2,2-bipyridine. Syringe 2, 20 mM isobutyramide, 300 μM NADH, 50 mM pyrophosphate buffer, pH 9.0. Inset, first order plot of the kinetic data, x axis 0 to 100 ms.

At high concentrations of bipyridine, the pseudo-first rate constants for association approach a limiting rate (13, 14). It has been proposed that this limiting rate results from the displacement of zinc-bound water. As a result, we investigated the effect of pH on the limiting rate of bipyridine binding from pH 7.0 to 10.3 using a double reciprocal plot. These results demonstrate a pH-independent limiting rate of 185 to 225 s⁻¹ for bipyridine binding (Fig. 6). The rate of bipyridine dissociation is pH-dependent and the pH dependence of \(K_d\)

FIG. 1. The rate of binding of 2,2-bipyridine to liver alcohol dehydrogenase. 10 μM liver alcohol dehydrogenase, 0.29 mM 2,2-bipyridine, 50 mM pyrophosphate buffer, pH 9.0. Inset, first order plot of the kinetic data, x axis 0 to 50 ms.
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Therefore results from a pH-dependent association rate constant ranging from $2.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.0 to $1.6 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ at pH 10.3. The $pK_a$ of the rate of bipyridine association is 9.2, the same $pK_a$ as determined from $K_D$ values ranging from 0.26 mM at pH 7.0 to 3.13 mM at pH 10.3.

To assess the involvement of zinc-bound water in the limiting rate of 2,2-bipyridine binding, pseudo-first order rate constants for the association as a function of ligand concentration were obtained in the presence of saturating imidazole. As shown in Fig. 7, no evidence of a limiting rate was observed with values up to 210 s$^{-1}$. The association rate constant in the presence of imidazole was $1.3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ contrasted to $2.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ for bipyridine binding to native enzyme, while the rate of bipyridine dissociation was 60 s$^{-1}$ in both the presence and absence of imidazole. The $K_D$ for bipyridine binding in the presence of imidazole from this kinetic data is 4.3 mM, in agreement with the values of 4 to 6 mM determined by equilibrium binding studies (Fig. 5).

1,10-Phenanthroline is a bidentate ligand which binds to the active site zinc ion of liver alcohol dehydrogenase, displacing zinc-bound water, and changing the coordination number of the zinc ion to five (8). A previous study of 1,10-phenanthroline binding reported that the association rate constant was pH-dependent with a $pK_a$ of 8.1, while dissociation was pH-independent (4). Since the $pK_a$ of 8.1 was much lower than the value for the coenzyme binding rate constant, it was concluded that zinc-bound water was not the group regulating coenzyme association. These results were in conflict with the proposal that zinc water is the proton-donating group on the enzyme with a $pK_a > 9.0$ in the native enzyme that is perturbed to 7.6 with the release of a proton when NAD$^+$ binds (2) and with the pH independence of NAD$^+$ binding in the presence of imidazole (7). Due to the importance of this question, we chose to reinvestigate the effect of pH on the binding of 1,10-phenanthroline to liver alcohol dehydrogenase.
using the spectrophotometric signal resulting from the chelation of enzyme-bound zinc.

The pKa of 1,10-phenanthroline binding (Fig. 3) was 9.2 in contrast to the previously reported value of 8.1 (4). To reaffirm this value we also investigated the pH dependence of the binding 2,2-bipyridine, another bidentate ligand known to bind the active site zinc ion of liver alcohol dehydrogenase. The association rate constant for 2,2-bipyridine chelation showed the same pH dependence observed for 1,10-phenanthroline binding. The pK, of bipyridine binding was thus also 9.2 (Fig. 3), indicating that this value is a property of the enzyme-bound zinc ion and not the chelating agent.

A possible explanation for the discrepancy in pKa values for 1,10-phenanthroline binding results from the different methods used to determine the rate of association. DeTraglia et al. (4) measured the rate by monitoring protein fluorescence quenching of the enzyme due to chelation. In the present study, the rate was determined by observing the increase in absorbance at 365 nm associated with the formation of a zinc-phenanthroline complex. Use of the characteristic absorption signal is more specific for binding at the zinc ion than the fluorescence-quenching signal, which is complicated by possible binding at other hydrophobic regions of the protein. In addition, 1,10-phenanthroline is fluorescent when excited at 280 nm with an emission maximum at 360 nm which overlaps the protein emission peak (330 nm). As the concentration of 1,10-phenanthroline is increased, the fluorescence emission of the ligand, in addition to inner filter effects, makes observation of the protein fluorescence difficult because of decreased relative signal amplitude. Consequently, the range of 1,10-phenanthroline concentrations where the fluorescence signal can observe the binding of ligand to protein is very limited.

Because of the lower extinction of 2,2-bipyridine and the larger spectral change upon complex formation, contrasted to 1,10-phenanthroline, the remaining experiments were limited to a study of the binding of 2,2-bipyridine to enzyme. Although there is no crystallographic evidence that the binding of 2,2-bipyridine displaces zinc-bound water, binding studies have established that both 1,10-phenanthroline and 2,2-bipyridine bind to liver alcohol dehydrogenase in the same manner (11). This suggests that the two nitrogen atoms of each chelating agent coordinate to the zinc ion at the active site by displacement of the zinc-bound water molecule. In addition, the present study demonstrates that the binding of both ligands is regulated by the ionization of the same group, most reasonably attributed to zinc-bound water.

Another important reason for studying bipyridine binding is the existence of an E-NAD*-bipyridine ternary complex, demonstrated by kinetic and ligand-binding studies (11). The formation of a ternary complex enabled determination of the pKa of zinc chelation in the E-NAD* binary complex. The presence of the coenzyme weakens the binding of bipyridine by a factor of 3 (Fig. 4) but does not displace the chelating agent. The oxidized coenzyme thus remains bound in the presence of bipyridine. It is possible that the NAD* is bound in an unproductive manner in the ternary complex, similar to the 3-isodopropidine analog (19). However, even if this is the case the rate constant for binding of bipyridine could be expected to exhibit the pKa of the zinc-bound water in the enzyme-NAD* complex. The pathlength of the stopped flow cuvette and the high absorbance of NAD* precluded kinetic determination of the binding rate constants for ternary complex formation over the pH range desired, and Kd values were therefore determined by equilibrium binding studies. The pKa of 2,2-bipyridine binding to the E-NAD* binary complex was 7.6 (Fig. 4) indicating that the pKa of the group on the enzyme controlling bipyridine binding was perturbed to 7.6 in the E-NAD* binary complex from 9.2 in the native enzyme. These pKa values correlate with the functional group isolated by proton liberation due to NAD* binding (2) and confirm the identity of zinc-bound water as the group involved with the binding of NAD*.

To substantiate further that zinc-bound water is the group responsible for the pH dependence of chelation, we investigated 2,2-bipyridine binding in the presence of saturating imidazole. Imidazole abolishes the pH dependence of NAD* binding (7), and crystallographic studies have shown that it displaces the zinc-bound water (8). When bipyridine binds in the presence of saturating imidazole, it will displace the imidazole from the zinc since water is no longer present. This should result in pH-independent binding of bipyridine, similar to the effect of imidazole on E-NAD* binding (7). Imidazole was found to abolish the pH dependence of bipyridine binding (Fig. 5), as it had for NAD* binding. The absence of a pH effect further indicates that zinc-bound water is the group displaced when 2,2-bipyridine binds and consequently, that the ionization of zinc-bound water with a pKa of 9.2 is the group controlling the association of coenzymes and chelating agents. In the presence of NAD*, the pKa of zinc-bound water is shifted to 7.6.

Frolich et al. (14) corroborated the earlier observation made in this laboratory (13), that the rate of 2,2-bipyridine binding was limited by a rate of approximately 200 s⁻¹ and observed similar limiting rates for the binding of 1,10-phenanthroline and 5-chloro-1,10-phenanthroline. The mechanism proposed, consistent with their results, was rapid formation of an outer sphere complex followed by dissociation of an inner sphere water molecule. In the present study we have investigated the rate of 2,2-bipyridine binding over the pH range 7 to 10.2 (Fig. 6). A pH-independent limiting rate of 185 to 225 s⁻¹ was observed, suggesting pH-independent dissociation of the water molecule. The association of 2,2-bipyridine reflects the ionization of zinc-bound water but the absence of a pH effect on the limiting rate indicates water dissociation as the neutral molecule. This mechanism is shown in Scheme I.

In the presence of saturating NAD*, the 2,2-bipyridine binds in the following manner, with the pKa, perturbed to 7.6.

To reaffirm the relationship between water dissociation and the limiting rate of bipyridine binding, we investigated the rate of 2,2-bipyridine association in the presence of saturating imidazole. In the presence of imidazole, a different limiting rate of chelation might be expected due to the absence of zinc-bound water. As seen in Fig. 7, no evidence of a limiting rate

\[ \text{Bipyridine} \xrightarrow{\text{E-NAD}^*} \text{E-NAD}^* \text{bipyridine} \xrightarrow{\text{imidazole}} \text{E-NAD}^* \text{imidazole} \]

\[ \text{SCHEME I} \]

\[ \text{Bipyridine} \xrightarrow{\text{E-NAD}^*} \text{E-NAD}^* \text{bipyridine} \]

\[ \text{SCHEME II} \]

To reaffirm the relationship between water dissociation and the limiting rate of bipyridine binding, we investigated the rate of 2,2-bipyridine association in the presence of saturating imidazole. In the presence of imidazole, a different limiting rate of chelation might be expected due to the absence of zinc-bound water. As seen in Fig. 7, no evidence of a limiting rate
was observed up to rates of 210 s$^{-1}$. The association rate constant was 1.4 s$^{-1}$, an order of magnitude slower than binding to the native enzyme. This results from the competition between imidazole and 2,2-bipyridine for the enzyme-bound zinc ion. Considering crystallographic evidence indicating that imidazole replaces the zinc-bound water molecule, the absence of a limiting rate in the presence of imidazole confirms that the limiting rate of chelation is related to water dissociation. It is probable that the faster rate of dissociation of imidazole compared with water results from the weaker interactions between the imidazole and zinc or adjacent amino acid residues. The absence of an observable limiting chelation rate in the presence of imidazole confirms the structural studies which concluded that imidazole replaces zinc-bound water (8).

The limiting rate of chelation is at least 2 orders of magnitude slower than values generally reported for water dissociation from simple zinc complexes (20, 21). This can most readily be explained by the unique properties of the enzyme-bound zinc compared with the hydrated ion used for water exchange rate studies. As previously pointed out (14), the zinc of liver alcohol dehydrogenase possesses a distorted tetrahedral geometry with no formal charge due to the two cysteine ligands, while hydrated zinc has octahedral geometry. The enzyme-bound zinc is buried in a hydrophobic milieu, and its bound water molecule has been reported to be hydrogen-bonded to Ser 48. An additional possibility would be that the 200 s$^{-1}$ limiting rate is that of a conformational change due to chelation, too subtle to be observed in the 4.5 Å map (8) of the liver alcohol dehydrogenase-1,10-phenanthroline complex. However, the finding that the binding of structurally different bidentate ligands yields the same limiting rate (14), which is abolished by replacement of the water with imidazole, indicates that the conformational change is due to displacement of the water rather than the result of inner sphere zinc chelation. Although it is not presently possible absolutely to rule out an enzyme conformational change linked to displacement of zinc-bound water, the abolition of the limiting rate in the enzyme-imidazole indicates that rate of the conformational change must be limited by the rate of water dissociation.

The significance of our investigation may be considered in relation to the role of zinc in liver alcohol dehydrogenase catalysis. The present study provides strong evidence that the enzymatic function of the enzyme with a pK$_a$ greater than 9 and free enzyme, perturbed to 7.6 in the binary enzyme-NAD$^+$ complex, is zinc-bound water. Furthermore, the zinc-bound water is one of the ionizing groups controlling the rate of binding of oxidized and reduced coenzyme. The linkage between ionization state of the zinc-bound water and coenzyme binding, however, is not entirely clear since ADP-ribose, which does not bind at the active site pocket and causes no observable conformational change at 2.8 Å resolution (22), also binds to the protonated form of the enzyme with a pK$_a$ of 9 (6). It is possible that an as yet undefined interaction exists between the ADP-ribose binding site and zinc-bound water, or that another functional group is involved. An alternative explanation which we previously proposed (3), is that ionization of zinc-bound water results in an enzyme conformation unable to bind coenzyme or its fragments. The identical enzyme structure for the apoenzyme and the binary complex with ADP-ribose can readily be explained if the least soluble enzyme conformation, which crystallizes readily, is the protonated form with water rather than hydroxyi bound to the zinc ion. This interpretation, which seems most reasonable, will require experimental verification.

The precise mechanistic role of zinc in the catalytic step of the liver alcohol dehydrogenase reaction has been the focus of considerable discussion in recent years. It was originally suggested by Theorell and co-workers that alcohol substrate was bound to the zinc of the enzyme-NAD$^+$ complex as an alcololate, with the zinc acting as a Lewis acid catalyst (9). The pH dependence of alcohol binding (2), substituent effects on alcohol oxidation (23, 24) and NMR studies (25) indicated the possibility that zinc-bound water could be acting in general acid-base catalysis. More recently, however, the spectral shift of a chromophoric substrate (26) and crystallographic studies of ternary complexes (27) have provided evidence that the oxygen atoms of alcohol and aldehyde substrates are bound directly to zinc rather than to a zinc-bound water molecule or hydroxyl ion. In an attempt to reconcile the plethora of data on this subject, it has been proposed (28) that the zinc is pentacoordinate in the active ternary complex, with both the substrate and water molecules present.

If the 200 s$^{-1}$ limiting rate constant for chelation is measuring the dissociation of zinc-bound water, this rate constant should appear at some step in the turnover of the enzyme. Thus far, no limiting rate has been reported for alcohol binding, and aldehyde binding and substrate reduction occurs at rates considerably faster than 200 s$^{-1}$. It is possible that the conformational changes resulting from coenzyme binding in some way facilitate water dissociation, so that it does not limit the rate of substrate association. Alternatively, the water molecule may still be present, with pentacoordinate zinc able to also accommodate the substrate oxygen, as has been proposed (28). This aspect of the mechanism is currently being further investigated.

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