Neutral metal-bound water is the base catalyst in liver alcohol dehydrogenase

(electron paramagnetic resonance/oxygen-17/pH dependence of kinetic parameters/spin-lattice relaxation/zero-field splitting)

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ABSTRACT The catalytic role of the active site metal–water complex in horse liver alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) is investigated on the basis of a comparative analysis of the pH dependence of steady-state kinetic parameters of the native and active-site-specific Co²⁺-reconstituted enzyme and on the basis of assignment of the coordination environment of the Co²⁺ by electron paramagnetic resonance methods. The pH dependence of the kinetic parameters for the oxidation of benzyl alcohol reveals two ionizations ($pK_1 \approx 6.7$; $pK_2 \approx 10.6$) that govern k_{cat} and belong to the ternary enzyme–NAD⁺–alcohol complex and two ionizations ($pK_1' \approx 7.5$; $pK_2' \approx 8.9$) that govern k_{cat}/K_m and belong to the binary enzyme–NAD⁺ complex. The ionizations pK_2 and pK_2' decrease by 0.5–1 pK_a unit upon replacement of the active site Zn^{2+} by Co^{2+} . A similar metal ion dependence of pK_2 and pK_2' is observed for the oxidation of 2-propanol. We attribute these ionizations to a metal-bound water molecule. The zero-field splitting energy of the Co^{2+} in the binary enzyme–NADH complex and the ternary enzyme–NADH– CF_3CH_2OH complex is $\approx 22\text{ cm}^{-1}$, indicative of a pentacoordinate species. Binding of a water molecule to the metal ion as the fifth ligand in the ternary enzyme–NADH– CF_3CH_2OH complex is confirmed on the basis of magnetic interactions of $H_2^{17}O$ with Co^{2+} . The results indicate that the active site metal ion in catalytically competent ternary enzyme–coenzyme–substrate complexes is pentacoordinate and is ligated by a neutral water molecule in the physiological pH range. We suggest that the neutral metal-bound water molecule serves as the base catalyst for proton abstraction in alcohol oxidation.

The catalytically essential active site Zn^{2+} in liver alcohol dehydrogenase (LADH; alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) is tetracoordinate, with a water molecule as the fourth ligand. On the basis of difference Fourier syntheses, it is thought that the binding of inhibitor molecules to binary enzyme–coenzyme complexes results in displacement of the metal-bound water (1, 2), as in the free enzyme (3). For this reason it is generally assumed that the metal-bound water molecule has no catalytic role in the oxidation of alcohols. However, an ionization with $pK_a \approx 7$ in the ternary enzyme–NAD⁺–alcohol complex that controls the temporal separation of abstraction of the proton of the alcoholic hydroxyl group from the chemical step of hydride transfer has been attributed (i) to the metal-bound substrate in a tetracoordinate complex (4, 5), (ii) to the metal-bound water in a pentacoordinate complex (6, 7), or (iii) to histidine-51 (8). Because the assignment of the residue governing this pK_a is not clear and the functional role of the active site metal–water complex is not established, we have carried out an investigation of the native and active-site-specific Co^{2+} -reconstituted enzyme (CoLADH; ref. 9) with the use of steady-state kinetic and elec-

tron paramagnetic resonance (EPR) methods. In this communication, we report the results of our studies from which we conclude that the active site metal ion in the catalytically competent ternary enzyme–NAD⁺–alcohol complex is pentacoordinate with a neutral water molecule as the fifth ligand in the physiological pH range. We suggest that the metal-bound water molecule serves as the base catalyst in proton abstraction, and we also discuss the electronic interactions through which the water molecule may modulate the Lewis acid activity of the metal ion.

EXPERIMENTAL METHODS

Crystalline horse LADH packed for transit at 0°C was purchased from Boehringer Mannheim. CoLADH was prepared and characterized as described by Maret *et al.* (9). *p*-*N,N*-dimethylaminocinnamaldehyde (DACA; Aldrich) was recrystallized to uniformity of color and to constant melting point before use. The inhibitor 2,2,2-trifluoroethanol (CF_3CH_2OH) was obtained from Sigma and used without further purification. Tris (ultra pure) was obtained from Schwarz/Mann. The zwitterionic buffers 1,4-piperazinediethanesulfonic acid (Pipes), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches), and 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) were obtained from Sigma and were used as their potassium salts. All other chemicals were of the highest purity available. Deionized, distilled water was used throughout. ^{17}O -enriched water (52.79% ^{17}O , 41.79% ^{18}O) was obtained from Monsanto Research (Miamisburg, OH).

Initial velocity data corresponding to the reduction of NAD⁺ were collected with a Perkin–Elmer MPF-44A spectrofluorimeter by following the change in fluorescence emission intensity at 460 nm with excitation at 340 nm. The fluorescence emission was calibrated with solutions of NADH, the concentrations of which were determined by enzymatic assay (10). The reaction was initiated by addition of enzyme, and the reported values of kinetic parameters are derived on the basis of the mean of at least two initial velocity measurements at each substrate concentration. More detailed description of kinetic studies will be provided elsewhere.

Complexes of CoLADH were prepared for EPR studies as described (11). The continuous wave (CW) microwave power saturation technique developed in this laboratory (12) was employed to estimate the zero-field splitting (ZFS) of Co^{2+} and to determine the influence of ^{17}O -enriched H_2O on the electronic spin-lattice relaxation time of the metal ion.

Abbreviations: Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CW, continuous wave; DACA, *p*-*N,N*-dimethylaminocinnamaldehyde; LADH, liver alcohol dehydrogenase; CoLADH, active-site-specific Co^{2+} -reconstituted LADH; Pipes, 1,4-piperazinediethanesulfonic acid; ZFS, zero-field splitting.

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RESULTS AND DISCUSSION

We have previously shown by application of EPR methods developed in this laboratory that the tetracoordinate active site metal ion of CoLADH becomes pentacoordinate in binary and ternary complexes formed with NAD⁺ (11). In the present investigation we have extended this analysis to complexes formed with NADH. The EPR spectrum of the ternary CoLADH-NADH-CF₃CH₂OH complex is shown in Fig. 1. There are several overlapping resonance features in the spectrum. A detailed comparison of the power saturation dependence of this spectrum to the dependences of independently prepared CoLADH-NADH and CoLADH-CF₃CH₂OH complexes shows that the resonance at $g \approx 3.4$ is cleanly separated from possible contributions of the binary complexes. The *Inset* in Fig. 1 illustrates the differential power saturation behavior of the $g \approx 3.4$ resonance line for the ternary complex in the presence of natural-abundance and ¹⁷O-enriched H₂O. The saturation data are plotted in a form (14, 15) that yields a graphical estimate of the parameter $\log P_{1/2}$ as the intersection of the asymptotes. The parameter $P_{1/2}$ is the value of the microwave power at which the saturation factor $(1 + \gamma^2 H_1^2 T_1 T_2)^{-1}$ is equal to 0.5. As shown by the data, there is no change in the slope although there is a change in the value of $P_{1/2}$ induced by the presence of ¹⁷O-

FIG. 1. First-derivative EPR absorption spectrum of the ternary CoLADH-NADH-CF₃CH₂OH complex. The complex was prepared in solutions buffered to pH 8.0 with 0.05 M Tris-HCl containing enzyme at 0.6 mM in active sites, 10 mM NADH, and 50 mM CF₃CH₂OH. The spectrum was recorded at 20.0-mW incident microwave power with 20-G (1 G = 10⁻⁴ T) modulation amplitude with the sample at 4.7 K. The arrows indicate the resonance lines at g values of ≈ 6.1 , 3.4, and 2.1 that belong to the CoLADH-NADH-CF₃CH₂OH complex. In separate experiments we have determined that the binary CoLADH-NADH complex exhibits resonances with g values of ≈ 6.5 , 2.5, and 1.7, whereas the CoLADH-CF₃CH₂OH complex exhibits resonances at g values of ≈ 6.3 , 2.4, and 1.7. Thus, the $g_1 \approx 6.1$ and $g_3 \approx 2.1$ components of the ternary CoLADH-NADH-CF₃CH₂OH complex overlap substantially with the corresponding high- and low-field components of these two binary complexes. By selecting the $g \approx 3.4$ resonance of the ternary complex for power saturation studies, we are able to eliminate contributions from other species. (*Inset*) CW power saturation curve of the $g \approx 3.4$ component for the complex in natural abundance water (●) and 26 mol % ¹⁷O-enriched water (Δ). Estimates of the $\log P_{1/2}$ values are 0.97 and 1.16, respectively. The standard error in the estimation of $\log P_{1/2}$ is $\pm 10\%$. For all of the complexes described here, we have been unable to detect broadening due to the ligand hyperfine interaction of metal-bound H₂¹⁷O, probably because of the intrinsically very broad resonance absorption lines of the spectrum. Similar behavior has been noted for Co²⁺-substituted carboxypeptidase A (13). For all of the complexes of CoLADH described here, there is no change in the linewidth at high levels of incident microwave power and no change in the slope of the saturation curve in the 4–12 K range.

enriched water. We have shown in our earlier studies using Co²⁺-substituted carboxypeptidase A and model compounds of high-spin Co²⁺ that the shift in the value of $P_{1/2}$, with no alteration in the linewidth of the resonance band or in the slope of the saturation curve at high values of P , reflects a change in the spin-lattice relaxation time (T_1) of the high-spin Co²⁺ that is induced by a metal-coordinated ¹⁷O-enriched oxygen-donor ligand (13). Therefore, the data in Fig. 1, indicating that the spin-lattice relaxation probability of the Co²⁺ is sensitive to ¹⁷O-enriched H₂O, reveal the presence of a water molecule coordinated to the active site metal ion.

In Fig. 2 is illustrated the EPR spectrum of the ternary complex of CoLADH formed with NADH and DACA stabilized under *nonturnover* conditions at high pH (16). The spectrum exhibits a rhombic pattern with broad resonance features at $g_1 \approx 7.23$, $g_2 \approx 2.77$, and $g_3 \approx 2.10$ and hyperfine structure due to the ($I = 7/2$) ⁵⁹Co nucleus. The *Inset* compares the differential power saturation behavior of the complex in the presence of natural-abundance and ¹⁷O-enriched water. Although there is a decrease in the signal-to-noise ratio in the spectrum of this complex, resulting in some increased scatter of the saturation data, there is no apparent difference in the saturation behavior of this complex prepared in the presence of ¹⁷O-enriched or natural-abundance water. This result stands in contrast to that of the ternary complex formed with CF₃CH₂OH illustrated in Fig. 1.

In Table 1 we have summarized the ZFS constants of the active site metal ion of several binary and ternary complexes of CoLADH formed with NADH for comparison to the results of our earlier studies of NAD⁺ complexes (11). The ZFS of the CoLADH-NADH-DACA complex falls into the range observed for structurally defined tetracoordinate complexes. This interpretation is confirmed by the absence of the influence of H₂¹⁷O on the spin-lattice relaxation probability of Co²⁺, as shown in Fig. 2. Also, the assignment of a tetracoordinate Co²⁺ in this complex is consistent with a recent difference Fourier analysis (18) of the ternary complex formed with DACA and the NADH inhibitor analog 1,4,5,6-tetrahydro-NADH (19). For this inhibitor complex, it has been shown on the basis of difference Fourier syntheses calculated with phase refinement that the active site metal ion is tetracoordinate and is directly coordinated by the carbonyl oxygen of the aldehyde. Only distant water molecules (≥ 5 Å) are localized in the active site region. On the other hand, the ZFS of the binary CoLADH-NADH complex and the ternary complex formed with CF₃CH₂OH indicate a pentacoordinate environment of the active site metal ion. We have previously outlined reasons why nearby amino acid side chains are too distant to account for the formation of a pentacoordinate environment (11). Therefore, the fifth ligand in both complexes must be a water molecule. This assessment is confirmed for the ternary CoLADH-NADH-CF₃CH₂OH complex by the results of Fig. 1.

Since we have not succeeded hitherto in determining conditions for stabilizing kinetically competent reaction intermediates of LADH for spectroscopic characterization, as in the case of Co²⁺-reconstituted carboxypeptidase A (13, 20), we have tested for the presence of a metal-coordinated water molecule in the ternary enzyme-NAD⁺-alcohol complex by kinetic methods. To this end, the pH dependences of the kinetic parameters k_{cat} and k_{cat}/K_m governing the oxidation of benzyl alcohol are compared in Fig. 3 for both native LADH and the Co²⁺-substituted enzyme. In the high pH range, the limiting values of k_{cat} and k_{cat}/K_m are slightly lower for CoLADH than for the native enzyme, but the general shape of the profile is similar for both enzymes. Comparable results are observed for the pH dependence of the kinetic parameters governing the oxidation of 2-

FIG. 2. First-derivative EPR absorption spectrum of the ternary CoLADH-NADH-DACA complex. The complex was prepared in solutions buffered to pH 9.1 with 0.1 M Ches containing enzyme at 0.52 mM in active sites, 20 mM NADH, and 2 mM DACA (introduced in 0.01 ml of purified diglyme). The spectrum was recorded at 2.5-G modulation amplitude with the sample at 3.5 K. The average hyperfine coupling at g_1 due to the ($I = 7/2$) ^{59}Co nucleus is 0.017 cm^{-1} . (Inset) CW power saturation curve of the signal intensity of the lowest field hyperfine component of the complex prepared in natural-abundance ($\bullet$) and 26 mol % ^{17}O -enriched (Δ) water. Other conditions as in Fig. 1. The weak signal at about 3,200 G arises from impurities in the quartz sample tube. The nonturnover conditions employed here to stabilize this ternary complex are identical to those employed in optical studies (16).

propanol. Estimates of the ionization constants from the pH profiles for the oxidation of benzyl alcohol and 2-propanol are summarized in Table 2.

In these kinetic studies, we have employed the substrates benzyl alcohol and 2-propanol, for it is well established that NADH dissociation limits k_{cat} at most pH values in the oxidation of benzyl alcohol (4, 5), whereas hydride transfer is at least partially rate limiting in the oxidation of 2-propanol (28). According to the theory of the steady-state treatment of a one-substrate enzyme-catalyzed reaction, the pH profile of the kinetic parameter k_{cat}/K_m reflects ionizations in the free enzyme or free substrate, and the pH profile of k_{cat} reflects ionizations of the enzyme-substrate complex that occur prior to or in the rate-limiting step (cf. ref. 21). Therefore, since the pH profiles governing the kinetic parameters for both substrates are similar, they must reflect ionizations of the same catalytic groups that occur prior to hydride transfer and NADH dissociation in

both reactions. On this basis, application of the theory of a one-substrate enzyme-catalyzed reaction to the compulsory-order ternary complex mechanism (24–26) of LADH under conditions of saturating coenzyme requires that the ionizations detected in the pH profiles of k_{cat} and k_{cat}/K_m belong, respectively, to the ternary enzyme-NAD⁺-alcohol complex and to the binary enzyme-NAD⁺ complex.

As seen in Table 2, the values of pK_1 and pK_1' are insensitive to substitution of the active site metal ion in the oxidation of either benzyl alcohol or 2-propanol. However, for both substrates the values of pK_2 controlling k_{cat} and of pK_2' controlling k_{cat}/K_m at high pH are influenced significantly by metal substitution. The decrease in both ionization constants by 0.5–1 pK_a unit is compatible with ionization of a metal-coordinated ligand under the conditions of replacement of closed-shell Zn^{2+} in the native enzyme by open-shell Co^{2+} . These observations, therefore, mean that only these two ionizations governing the kinetic parameters at high pH are attributable to the active site metal-bound water molecule and are consistent with our earlier suggestion (11) that the active site metal ion is pentacoordinate in the catalytically competent ternary enzyme-NAD⁺-alcohol complex.

This assignment of pK_2 and pK_2' specifies discrete ionizations of the metal-bound water molecule that occur separately at the binary and ternary complex level in the course of the enzyme-catalyzed reaction. This interpretation differs substantially from those made by others, whereby only the ionization corresponding to pK_1 in the oxidation of benzyl alcohol (6) or the ionization with $\text{pK}_a \approx 7.6$ regulating the binding of NAD⁺ to the enzyme (29) has been attributed to metal-bound water. The latter ionization corresponds to pK_1' . As seen in Table 2, both pK_1 and pK_1' are insensitive to metal ion substitution and, therefore, are not compatible with ionization of a metal-bound water molecule. While the interpretation of Dworschack and Plapp (6) implies a pentacoordinate active site metal ion complex, a metal hydroxide species is invoked in both studies as the catalytically active form. However, the results in Fig. 3 demand a different interpretation of the mechanism of action of LADH.

Table 1. Comparison of ZFS constants of complexes of CoLADH

Enzyme complex	$2 D $,* cm^{-1}	Coordination number of active site metal ion [†]
CoLADH	$\leq 9.3^\ddagger$	4
CoLADH- $\text{CF}_3\text{CH}_2\text{OH}$	≤ 3.1	4
CoLADH-NADH-DACA	13	4
CoLADH-NADH	21	5
CoLADH-NADH- $\text{CF}_3\text{CH}_2\text{OH}$	22	5

*The values of the ZFS constants are determined on the basis of the temperature dependence of the parameter $P_{1/2}$ by the CW microwave power saturation technique developed in this laboratory (12).

[†]The coordination number is assigned on the basis of the magnitude of the ZFS. The ZFS constants of the high-spin Co^{2+} are observed to fall in three ranges according to coordination number (11, 17). They are 0–13 cm^{-1} for tetracoordinate complexes, 20–60 cm^{-1} for pentacoordinate complexes, and 90–310 cm^{-1} for hexacoordinate complexes.

[‡]Ref. 11.

FIG. 3. Dependence of the kinetic parameters k_{cat} and k_{cat}/K_m on pH for the oxidation of benzyl alcohol catalyzed by LADH. Initial velocity data were collected at 21°C under conditions corresponding to the steady-state approximation. The buffers were Pipes (pH 6.1–7), Hepes (pH 7.5–8), Ches (pH 8.6–9.7), and Caps (pH 10.3–11) used at 0.1 M. The initial NAD^+ concentrations, 5.75 mM for solutions of pH < 6 and 1.15 mM for solutions of pH > 6, were confirmed by experiment to provide saturating levels for both metalloenzymes. Depending upon the pH, the initial substrate concentration ranged from $0.1 \times K_m$ to $(1-10) \times K_m$. No substrate inhibition was observed in the concentration range used to estimate kinetic parameters. The kinetic parameters k_{cat} and k_{cat}/K_m were estimated by use of the algorithm ENZKIN provided by J. L. Westley of the Department of Biochemistry at The University of Chicago, as described in earlier studies (20). The pK_a values indicated by arrows [↓ for native LADH (●) and ↑ for CoLADH (▲)] are compared in Table 2 to those observed for the oxidation of 2-propanol. The pH profile of $\log k_{\text{cat}}$ at high pH shows only a small flattening indicative of an ionization; this effect can be seen more clearly in the pH profile of k_{cat} , however. A similar flattening at high pH is also observed in the pH profile of $\log k_{\text{cat}}$ for the oxidation of cyclohexanol (8). According to the steady-state treatment of the analysis of the influence of hydrogen ions on kinetic parameters (21), the presence of an ionization indicated only by a flattening in the pH profile of k_{cat} at high pH is confirmed by the approach to a plateau in the pH profile of k_{cat}/K_m . Therefore, the reported values of pK_2 are those that provide the best fit to the pH profile of $-\log K_m$ (unpublished data) because the ionization constant pK_2 is not accurately estimated on the basis of k_{cat} data alone. The values for pK_2 are, therefore, schematically illustrated as broken arrows in the pH profile of $\log k_{\text{cat}}/K_m$.

With assignment of pK_2 to ionization of a metal-bound water molecule in a pentacoordinate reaction intermediate, the location of both the plateau in the pH profile of k_{cat} and the maximum in the pH profile of k_{cat}/K_m in Fig. 3 requires that the active site metal ion is ligated by a neutral metal-bound water molecule. This complex is the physiologically active species, and the demonstration of a neutral metal-bound water molecule in the immediate proximity of the alcoholic OH group in the catalytically active ternary complex suggests that it plays an integral part in controlling substrate oxidation.

On the basis of these investigations and the observation (30)

Table 2. Comparison of the values of ionization constants governing the oxidation of benzyl alcohol and 2-propanol by native LADH and CoLADH

Substrate	Enzyme	pK_a^*			
		k_{cat}		k_{cat}/K_m	
		pK_1	pK_2	pK_1'	pK_2'
Benzyl alcohol	ZnLADH	6.7	10.6	7.5	8.9
	CoLADH	6.3	10.0	7.6	7.9
2-Propanol†	ZnLADH	7.0	>11.5‡	8.1	>11.5§
	CoLADH	6.7	11.0	8.3	10.2§

* The pK_a values are estimated to an accuracy of ± 0.2 unit and are obtained by fitting the experimentally determined kinetic parameters to calculated theoretical curves with use of the algorithm BELL (22). Only values for the native enzyme (ZnLADH) and CoLADH differing by ≥ 0.5 unit are considered as indicating ionizations of a metal-ion-coordinated ligand.

† Initial velocity data were collected in the presence of 0.1 M potassium chloride with buffers of 0.1 M potassium phosphate (pH 5–7), sodium pyrophosphate (pH 7.5–9), and potassium glycinate (pH 9.5–11).

‡ No decrease from the plateau level could be detected at pH 11; therefore, the ionization must occur at higher pH. The observed values of pK_2 for this substrate are kinetically shifted from those observed for benzyl alcohol.

§ Dalziel and Dickinson (23) have shown that the oxidation of 2-propanol is significantly more random than the compulsory-order ternary complex mechanism for primary alcohols (24–26). This observation, together with the results of Wolfe *et al.* (27) showing that NAD^+ binds kinetically faster to the enzyme at low pH, suggests that both the binary enzyme- NAD^+ and enzyme-2-propanol complexes contribute to this pK_a in the pH profile of k_{cat}/K_m . Nonetheless, the influence of metal ion substitution parallels that seen in the oxidation of benzyl alcohol, which does proceed according to a compulsory-order ternary complex mechanism (24, 25).

that proton abstraction precedes the hydride transfer step, we envision the catalytic events at the metal ion to occur as schematically illustrated in Fig. 4. In the catalytically active, ternary enzyme- NAD^+ -alcohol complex, both the alcoholic OH group and the water molecule are tightly coordinated to the active site metal ion. With this ligand configuration, it is probable that the substrate and water molecule are hydrogen bonded to each other. It has been demonstrated that covalent metal-oxygen interactions polarize OH bonds and strengthen hydrogen bridging between the metal-bound ligands in multihydrate metal ion systems (32). These interactions, applied to the substrate OH group and the metal-bound water in the ternary enzyme- NAD^+ -alcohol complex, may facilitate ionization of the proton from the metal-bound OH group of the substrate to form a transient species best described as an alcoholate-metal- OH_3^+ complex, probably as a result of the activated processes that lead to substrate oxidation. The proton produced by ionization of the alcoholic OH group is then rapidly transferred from the metal-bound water to bulk solvent, probably via the hydrogen-bonded proton-relay system of serine-48 and histidine-51 (31). In this manner, a neutral water molecule remains coordinated to the metal ion, and the chemical step of hydride transfer then completes the reaction. In this scheme, the neutral metal-bound water molecule serves as the base catalyst for abstraction of the proton from the hydroxyl group of the alcohol.

In addition to its function as the base catalyst, the water molecule may regulate the role of the metal ion as a Lewis acid. In aldehyde reduction, the function of the metal ion is to polarize the carbonyl bond of the substrate. This action in turn removes electron density from the carbon atom and thereby facilitates the approach of the incoming hydride anion to the substrate. Indeed, the strength of the metal ion as a Lewis acid, as shown through metal ion substitution studies, is directly cor-

FIG. 4. Catalytic role of the active site metal-bound water molecule of LADH during the conversion of the ternary enzyme-NAD⁺-alcohol complex to the ternary enzyme-NADH-aldehyde complex. The Zn²⁺ is represented by a circle, and the donor ligand atoms are those of cysteine-46, cysteine-174, and histidine-67 (31). The pathway begins with the metal-bound alcohol forming a hydrogen bridge to the metal-bound water molecule and proceeding to transfer of the proton to form a hydronium ion that is fleetingly coordinated to the metal ion. With release of the proton to bulk solvent, abstraction of the hydride ion by the nearby pyridinium group of NAD⁺ can occur. The process of hydride ion abstraction at this point presumably constitutes the primary origin of the activation barrier. It is not known whether the species drawn, beginning with proton abstraction, correspond to stable intermediates—i.e., troughs in the reaction coordinate scheme—or intrinsically unstable species. Reduction of the aldehyde to alcohol can proceed directly through the same species by the reverse reaction. See text for discussion.

related with the rate of hydride transfer in aldehyde reduction (33, 34). On the other hand, a metal ion with strong electron withdrawing ability would hinder alcohol oxidation and removal of the hydride anion by decreasing the electron density on the methylene carbon atom adjacent to the metal-bound OH group. In support of this notion, we point out the results of Shore and Santiago (35) showing that the rate of hydride transfer in alcohol oxidation is slower for the Co²⁺-substituted enzyme than for native LADH. In aldehyde reduction, the covalent interactions between the metal ion and carbonyl oxygen lead to effective polarization of the carbonyl bond because of the intrinsically delocalized π -electronic structure of a carbonyl group. On the other hand, the presence of the metal-bound water molecule in the enzyme-NAD⁺-alcohol complex can increase the electron density around the metal ion, reducing its capacity to function as a Lewis acid. This reduces the removal of electron density from the methylene carbon atom, and consequently abstraction of the hydride ion is favored. In this manner, the presence of the water molecule may modulate the electronic structure of the metal ion and its catalytic role. Structural alterations that occur in the protein upon binding of NAD⁺ and NADH must be responsible for the change in the coordination number of the active site metal ion and stabilization of the metal-bound water molecule. Their identification remains a challenging problem in the study of the structural basis of LADH action.

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