The pH Variation of Steady-state Kinetic Parameters of Site-specific Co²⁺-reconstituted Liver Alcohol Dehydrogenase

A MECHANISTIC PROBE FOR THE ASSIGNMENT OF METAL-LINKED IONIZATIONS

(Received for publication, March 12, 1991)

Wolfgang Maret‡ and Marvin W. Makinen§

From the Department of Biochemistry and Molecular Biology, The University of Chicago, Cummings Life Science Center, Chicago, Illinois 60637

To identify ionizations of the active site metal-bound water in horse liver alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase; EC 1.1.1.1), the pH, solvent isotope, temperature, and anion dependences of the steady-state kinetic parameters $k_{cat}$ and $k_{cat}/K_M$ have been evaluated under initial velocity conditions for the native and the active site-specific Co²⁺-reconstituted enzyme. In the oxidation of benzyl alcohol, a bell-shaped pattern of four prototropic equilibria was observed under conditions of saturating concentrations of NAD⁺. It is shown that the ionizations governing $k_{cat}$ ($pK_1 = 6.7, pK_2 = 10.6$) belong to the ternary enzyme-NAD⁺-alcohol complex, whereas the ionizations governing $k_{cat}/K_M$ ($pK_1' = 7.5, pK_2' = 8.9$) belong to the binary enzyme-NAD⁺ complex. The ionizations $pK_1$ and $pK_2$ are not influenced by metal substitution and are ascribed to His-51 on the basis of experimental estimates of their associated enthalpies of ionization. On the other hand, $pK_1'$ and $pK_2'$ are significantly decreased ($\Delta pK_a = 1.0$) in the Co²⁺-enzyme and are attributed to the active site metal-bound water molecule. The shape of the pH profiles requires that the active site metal ion coordinates a neutral water molecule in the ternary enzyme-NAD⁺-alcohol complex under physiological conditions. The possible catalytic role of the water molecule within a pentacoordinate metal ion complex in the active site is discussed.

The mechanism of action of liver alcohol dehydrogenase (LADH, alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) centers on different ligand states of the active site Zn²⁺ ion during the catalytic cycle (2), conformational changes in the protein (3–5) that govern the affinity and kinetics of coenzyme binding (6,7), and temporal separation of proton abstraction from the chemical step of hydride transfer (7–11). These molecular events are controlled by ionizing groups for which assignment has remained conjectural despite numerous structural, chemical, and kinetic studies. The ionization with $pK_a \approx 7$ that controls turnover has been attributed to the Zn²⁺-bound alcohol in a tetracoordinate species (8–10) and to His-51 (11) or to a Zn²⁺-bound water molecule (5, 12) in a pentacoordinate species. Moreover, two ionizations with $pK_a \approx 9.2$ in the free enzyme and $pK_a \approx 7.6$ in the binary complex formed with NAD⁺ have been attributed to the metal-bound water molecule (10, 13–16). However, it has been demonstrated that the ionization with $pK_a \approx 7.6$ is unchanged by removal of the active site metal ion (17, 18), strongly indicating that an amino acid side chain is responsible for the observed pH effects. Accordingly, it has been suggested that Lys-228 is responsible for the ionization with $pK_a \approx 9.2$ in the free enzyme (19). There is, thus, no agreement on basic features of the mechanism of action of LADH, such as assignment of ionizations of the metal-bound water or the alcohol molecule, or of the coordination number of the active site metal ion.

A direct resolution of many of the ambiguities concerning the molecular origins of $pK_a$ values can be achieved by identifying the ionizations of metal-bound ligands. In particular, metal substitution provides an experimental test for metal-sensitive ionizations. For instance, substitution of the open-shell Co²⁺ for the closed-shell Zn²⁺ may be expected to result in a detectable decrease in the $pK_a$ of a metal-bound ligand. In this respect the active site-specific Co²⁺-reconstituted enzyme characterized by Zeppezauer and co-workers (1) provides a particularly incisive derivative of LADH because it has been demonstrated that the structure of the enzyme and especially of the active site metal ion region are essentially identical with the native enzyme (20). Ionizations of molecules that reversibly ligate the metal ion can be further probed by substituting deuterium oxide for natural abundance water or by varying the structure of alcoholic substrates since their ionization constants cover a range of $10^{-14}$–$10^{-19}$ M in magnitude (21).

In a preliminary study of the influence of the metal ion on ionizations affecting catalysis of LADH, we observed that an ionization with $pK_a \approx 10.6$ controlling $k_{cat}$ and an ionization with $pK_a \approx 8.9$ controlling $k_{cat}/K_M$ were decreased by substitution of Co²⁺ for the active site Zn²⁺ (22). To characterize these ionizations in more detail, we have carried out a comparative investigation of the pH, solvent isotope, substrate, and temperature dependences of the steady-state kinetic parameters that govern the oxidation of alcohols catalyzed by both native LADH and the active site-specific Co²⁺-reconstituted enzyme. Our analysis shows that these two metal-
independent ionizations are best ascribed to metal-bound water in the ternary enzyme-NAD\(^+\)-alcohol complex and the binary enzyme-NAD\(^+\)-alcohol complex, an assignment that differs from proposals made by others (10, 15, 19). In particular, McConnell and coworkers (15, 19) proposed that the active site metal ion is pentacoordinate. They indicate how the metal-bound water molecule may play a central role in the reaction mechanism in proton abstraction in light of structural relationships of a putative proton relay system in the active site cleft (15, 23).

**EXPERIMENTAL PROCEDURES**

**Materials**—NAD\(^+\) and NADH (grade 1) were obtained from Boehringer Mannheim. Coenzyme purity was found by enzymatic assay (28) to be 94 and 94%, respectively. 2-Propanol (spectroscopic grade) and TFE (Gold Label) were obtained from Alrich; Tes, Pip, Heps, Caps, and deuterium oxide (99.8% D\(_2\)O) from Sigma; Chex from Calbiochem; and Tris (Ultra-pure) from Schwan/Mann. Analytical reagent grade BzOH was distilled under reduced pressure. All other chemicals were of analytical reagent grade. Deionized, distilled water was used throughout.

Crystalline ZnLADH packed for transit at 0 °C was obtained from Boehringer-Mannheim and used without further purification. Amorphous and flocculent protein precipitate was discarded. The remaining crystalline enzyme was then dissolved, exhaustively dialyzed against 50 mM Tris-HCl at pH 8.1 and crystallized by stepwise addition of mutarotally distorted tert-butanol to the dialysate. The active site specific Co\(^{2+}\)-reconstituted form of LADH was prepared and characterized according to Maret et al. (1). All preparations of CoLADH corresponded to no less than 85% active site metal ion substitution (1.7 g-atoms of Co\(^{2+}/\)dimer). Stock solutions of CoLADH in 25 mM Tes at pH 7, prepared and kept under a nitrogen atmosphere, showed no change in activity for at least 1 week. Incubation of CoLADH for up to 15 min in the reaction mixtures used for kinetic studies in the pH range 7–11 resulted in no detectable loss of catalytic activity.

**Methods**—Initial velocity data were collected with a Perkin-Elmer MPF-44A spectrofluorometer by following NADH fluorescence at 460 nm with excitation at 340 nm. The increase in emission intensity was evaluated with use of the algorithm of liver alcohol dehydrogenase. Initial velocity data were evaluated with use of the algorithm

\[ \text{Initial velocity data} = \text{enzyme} \times \text{substrate} \times \text{coenzyme} \]

**RESULTS**

**Influence of Metal Ion on Ionizations Governing Substrate Oxidation**

**Oxidation of Benzyl Alcohol and 2-Propanol**—No detailed evaluation of the LADH-catalyzed oxidation of BzOH or of 2-propanol has been reported previously on the basis of steady-state kinetic methods. We have chosen BzOH and 2-propanol as substrates in this study because (i) BzOH has been used extensively in transient state kinetic studies (8–10), (ii) the rate-limiting step with 2-propanol is well established as hydride transfer (31), and (iii) the pK\(_i\) values of these alcohols vary between 17 and 20 (21). Moreover, the use of these substrates is not influenced by factors that complicate the interpretation of kinetic results. For instance, the oxidation of ethyl alcohol is sensitive to substrate inhibition (32, 33), and the oxidation of cyclohexanol is subject to substrate activation or substrate inhibition, depending upon the concentration of NAD\(^+\) (11, 34). On the other hand, the oxidation of 2-propanol near neutral pH is not subject to substrate inhibition within the range of substrate concentrations used (35), and in this investigation we demonstrated that saturating concentrations of BzOH can be reached without deviation of initial velocities from a hyperbolic relationship.

The influence of pH on the kinetic parameters \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_M\) in the oxidation of BzOH is compared in Fig. 1 for ZnLADH and CoLADH. The bell-shaped profiles define four prototropic equilibria. The ionizations pK\(_i\) and pK\(_{i}\)' are observed in the pH profile of kcat, whereas pK\(_i\)' and pK\(_{i}\)" are observed in the pH profile of kcat/KM. Although the profiles are similar for the two enzymes, the limiting values of kcat and kcat/KM are lower for CoLADH than for the native enzyme in the high pH range. The decrease in the limiting value of kcat/KM for CoLADH results from the lower plateau value of kcat.

The acidic portion of the pH profiles of log kcat shows a distinct ionization with pK\(_i\) = 6.2–6.5 for both metalloenzymes. On the other hand, the alkaline portion of the pH profile of log kcat for CoLADH shows a hollow accompanied by an approach to a plateau in the pH profile of log kcat/KM. These two observations specify an ionization in the alkaline pH range that governs kcat (36), i.e. the dashed arrows in the

\[ \text{pH} = \text{pK}_i \]

\[ \frac{d\text{pH}}{dt} \]

\[ \text{pK}_i \]
values for $pK_1$ and $pK'_1$ were obtained, no decrease from the limiting plateau value in the pH profile of $k_{cat}/K_M$ up to pH 11 was observed for the oxidation of 2-propanol catalyzed by ZnLADH. In contrast, for CoLADH the onset of the ionization of $pK_2'$ was observed yielding a value of ~10.2. A metal-dependent shift similar in magnitude to the one observed for BzOH places this ionization at $pK_2$ values $>11.5$ for ZnLADH and, hence, outside of the range of detection by steady-state methods. Similarly, we conclude that the ionization $pK_2$ governing $k_{cat}$ in the oxidation of 2-propanol must be shifted also to values $>11.5$ since the onset of $pK_2$ was observed for CoLADH near pH 11. Thus, in the oxidation of 2-propanol, the ionizing groups governing $k_{cat}$ and $k_{cat}/K_M$ at high pH are altered by metal substitution, whereas the values of $pK_1$ and $pK'_1$ are insensitive to the metal, corroborating the results with BzOH.

Estimates of the ionization constants governing the kinetic parameters for BzOH and 2-propanol oxidation are summarized in Table I for the various reaction conditions employed in this investigation and are compared with corresponding values obtained in studies with other substrates (9, 11, 13, 33, 34, 39). We have also included in this table the $pK_1$ values of ionizations governing the kinetic parameter $\delta_2$ as derived for ethanol oxidation (40) and applied for steady-state measurements of the oxidation of BzOH (9). The parameter $\delta_2$ corresponds to the reciprocal of $k_{cat}/K_M$ and the associated $pK_1$ values are in not unreasonable agreement with ours.

A variety of anion binding effects on kinetic parameters governing substrate oxidation have been previously described by others (33, 41). In Fig. 2 we have compared the pH dependence of the kinetic parameters $k_{cat}$ and $k_{cat}/K_M$ for the ZnLADH catalyzed oxidation of BzOH in the presence and absence of added chloride, as a characteristic anion influencing kinetic parameters. As seen in Fig. 2, the ionization constants governing $k_{cat}/K_M$ are shifted to lower values, whereas the ionizations governing $k_{cat}$ are not affected. Also, it is seen that the plateau value of $k_{cat}$ is elevated in the presence of chloride, an effect noted by Theorell et al. (32) and subsequently attributed (42) to an increase in rate-limiting dissociation of NADH through displacement of the coenzyme by the chloride anion. Comparable effects were observed in this study for CoLADH, and the results for both metalloenzymes are summarized in Table I.

We have similarly compared the pH and pD dependence of the kinetic parameters governing the oxidation of BzOH. The pD profiles of the kinetic parameters of ZnLADH are illustrated in Fig. 3, and the results are summarized for both metal enzymes in Table I. The $pK_1$ values governing $k_{cat}$ are uniformly increased in the presence of D$_2$O, and, consequently, the ionization designated by $pK_2$ is not observed for ZnLADH. Correspondingly, for both metal-enzymes, the value of $pK'_1$ is more influenced by the presence of D$_2$O than is the value of $pK'_2$. In the pH 7-10 region, there is a small but significant deuterium solvent isotope effect (1.8-1.1) on $k_{cat}$ governing the oxidation of BzOH catalyzed by both metalloenzymes. The effect increases at pH values below $pK_1$ but decreases at pH values above $pK_1$ according to the isotopically induced shift in each ionization constant. The solvent isotope effect on $k_{cat}/K_M$ is of the same magnitude and similarly increases below $pK'_1$ and decreases above $pK'_1$. The parallel nature of the solvent isotope effect for both $k_{cat}$ and $k_{cat}/K_M$ indicates that the pH-sensitive and the isotope-sensitive processes are the same and determine the parameter value over the range of the pH profile for both metalloenzymes.

Inhibition of Substrate Oxidation by Trifluoroethanol—The theory of the influence of hydrogen ions on kinetic parameters...
show that kinetically unperturbed ionization constants intrinsic to an enzyme are obtained from the pH dependence of the binding of a competitive inhibitor (36, 37). The pH dependence of the inhibitor constant of TFE for both metallo-enzymes has been determined for the oxidation of several alcoholic substrates, a comparison of which is made for the oxidation of BzOH in Fig. 4. The pH dependence of Kf reveals ionizations with approximate pK values of 7.5 and 8.1 for ZnLADH, designated as pKa and pKbh respectively. For CoLADH, the two ionizations are more strongly overlapping. Comparison of the shape of the pH profile of CoLADH to that of ZnLADH shows that the alkaline ionization has been shifted to a lower pKa value, and only an average value of ~7.5 can be estimated as the pK, of both ionizing groups. Identical results were obtained for the pH dependence of Kf in the oxidation of both 2-propanol and ethanol catalyzed by ZnLADH, confirming that these ionizations are independent of substrate. Such results have not been previously reported in the literature for LADH. 

Under “Experimental Procedures,” we have described in detail the conditions for investigating the inhibition of BzOH oxidation by TFE, including the order of addition of reagents. Above pH 8, incubation of the enzyme in the presence of NAD+ and TFE, even for the brief period of time required for subsequent substrate addition, led to formation of a weakly fluorescent complex with no detectable change in the absorbance of the reaction mixture at 340 nm. Under these conditions, there is an apparent inactivation of the enzyme since the subsequent steady-state velocities upon substrate addition were much lower than when the reaction was initiated by enzyme addition. As illustrated in Fig. 4, incubation of the enzyme with NAD+ and TFE under alkaline conditions leads to obscuring of the ionization pKb = 8.1 that governs the binding of TFE in the alkaline pH range. Since NAD+ is nonfluorescent and no weak fluorescence increase was observed when TFE was added to the enzyme-NAD+ complex at neutral pH, we conclude that the weak fluorescence reflects a protein conformational change within the enzyme-NAD+-TFE complex that is induced only in the alkaline pH range. Fluorine-19 nuclear magnetic resonance studies (43) have revealed that the lifetime of bound TFE in the enzyme-NAD+-TFE complex is ~400 ns at pH ~8.7. Such a long lived bound state is incompatible with behavior as a competitive inhibitor under steady-state conditions, and we, therefore, conclude that this long lived species is responsible for the apparent inactivation of the enzyme at alkaline pH.

pK, values for the ionizing groups controlling TFE inhibition in the oxidation of cyclohexanol and ethanol have been reported by others (11, 13). Our value of pK, = 7.5 agrees directly with their estimates of 7.2 (11) and 7.5 (13). However, Cook and Cleland (11) report a pK, of 10.1 for the ionization governing TFE inhibition of cyclohexanol oxidation in the alkaline range, whereas Shore et al. (13) report no ionization at high pH in the oxidation of ethanol. We note that in both instances kinetic data were collected by absorption measurements rather than by the more sensitive fluorescence method employed here, and no particular mention was made for the order of addition of reagents. We, therefore, conclude that the experimental conditions employed by others (11, 13) obscured the pK, of ~8.1 through formation of the altered, inactivated complex. Although an ionization controlling kcat/Km in the alkaline range was reported by Cook and Cleland (11) for oxidation of cyclohexanol, their estimates of pKp (10.1) or of pKp’ (10.3) do not agree well with the results reported by Taylor (39) for identical substrate and zwitterionic buffer conditions (cf. Table I). Moreover, the earlier steady-state

| Substrate | Enzyme     | kcat | pK,  | pK,b | kcat/Km |
|-----------|------------|------|------|------|---------|
| BzOH      | ZnLADH     | 6.5  | 6.9  | 10.7 | 8.1     |
|           | CoLADH     | 6.2  | 7.1  | 9.7  | 10.1    |
|           | ZnLADH     | 6.7  | 6.7  | 10.6 | 7.5     |
|           | CoLADH     | 6.3  | 6.3  | 10.9 | 7.6     |
| 2-Propanol| ZnLADH     | 7.0  | 7.0  | >10.5| 8.1     |
| Ethanol   | ZnLADH     | 6.3  | 6.3  | 10.0 | 7.5     |
| Cyclohexanol| ZnLADH | 6.2  | 6.2  | 9.0  | 7.1     |

* Measurements were carried out at 21 °C under the conditions of added potassium chloride, as described in the legend to Fig. 2; in general, an error of less than or equal to ±0.2 pK, units is estimated for the pK, values determined in this investigation.
* pK, values in parentheses refer to measurements in D2O under conditions of added potassium chloride.
* Measurements were carried out in the presence of zwitterionic buffers in the absence of added chloride.
* From Ref. 9. The pK, values of ionizations governing pK1' are given as pK1' and pK2' since pK2' corresponds to the reciprocal of kcat/Km.
* Calculated by fitting the data of Theorell et al. (32) to theoretical curves. Since these data have not been analyzed previously in this manner, a copy of the calculated pH profile and fit to the data will be provided upon written request.
* From Ref. 11. The second set of entries indicated for this substrate are the ionization constants governing the substrate-activated pathway. In this study, zwitterionic buffers were employed in the absence of added chloride.
* From Ref. 39. The pK, values governing kcat are not reported. In this study, zwitterionic buffers were employed as in Ref. 11. Values in parentheses refer to measurements in D2O.
results of Theorell et al. (32) yield clear identification of two ionizations governing $k_{cat}/K_M$ in the oxidation of ethanol that correspond to $pK_1$ and $pK_1'$ (cf. Table I), as do the results of Dalziel (40).

**Influence of Temperature on Ionizations Governing Substrate Oxidation**

The temperature dependences of the graphically determined values of $pK_1$ and $pK_1'$ are shown in Fig. 5. These plots yield estimates of 8.8 and 9.9 kcal/mol for the enthalpies of ionization associated with $pK_1$ and $pK_1'$, respectively. The circumstance that hydride transfer is rate-limiting in the oxidation of 2-propanol (31) ensures that conformational changes in the protein during coenzyme release should not contribute significantly to these estimates. These results agree with calorimetric estimates (8.8–9.8 kcal/mol) for the enthalpy of ionization of the group with $pK_1$ (cf. Table I), as do the results of Dalziel (40).

In Fig. 6, we have plotted the pH dependence of the enthalpy of activation associated with $k_{cat}$. We have noted previously (45) that the enthalpy of activation associated with $k_{cat}$ can be separated into a pH-independent term ($\Delta H^0_{cat}$) associated with $k_{cat,\text{cat}}$ and the enthalpy of ionization ($\Delta H_{\text{ion}}$) associated with the ionization constant $K_c$. The difference of 8 kcal/mol in Fig. 6 between the limiting values of $\Delta H^0$ at low pH (16.1 kcal/mol) and at high pH (8.1 kcal/mol) is in good agreement with the value of $\Delta H_{\text{ion}}$ estimated at pH 7.1 on the basis of the temperature dependence of the burst reaction for hydride transfer in the oxidation of ethanol by transient-state kinetic methods (46).

The results in Fig. 6 are also in good agreement with the value of $\Delta H^1$ of $\sim 13$ kcal/mol estimated at pH 7.1 on the basis of the temperature dependence of the burst reaction for hydride transfer in the oxidation of ethanol by transient-state kinetic methods (46). Pocker and Page (47) have reported a value of 9.7 kcal/mol for the enthalpy of activation in ethanol oxidation at pH 7 and have assigned this to the ionization of the hydroxyl proton of metal-bound ethanol. These results determined only at pH 7 cannot be meaningfully compared with the results reported in this study or with those reported by Brooks et al. (46) on the basis of transient-state methods. As shown through Fig. 6, the enthalpy of activation associated with $k_{cat}$ is pH-dependent. Since ethanol oxidation under these conditions is heavily governed by rate-limiting coenzyme release (40, 42, 46), it is doubtful that the result of Pocker and Page obtained under steady-state conditions can be associated with only one chemical process.

In our studies, it has not been feasible to determine the values of the corresponding thermodynamic potentials associated with the ionization constants $pK_2$ and $pK_2'$ by steady-state methods since the half-life of the enzyme is <1 min in solutions of pH >12 (48).

**DISCUSSION**

The **pH Dependence of Kinetic Parameters Governing the Oxidation of Alcohols**

**Assignment of $pK_1$ and $pK_2$ to the Ternary Enzyme-NAD$^+$-Alcohol Complex**—In determining the pH rate profiles of enzyme-catalyzed reactions, the assumption is often made that ionizations governing kinetic parameters behave as unperturbed equilibria. However, this is frequently not the case since the observed ionizations may be shifted from their true
Mechanism of Liver Alcohol Dehydrogenase

Fig. 4. Comparison of the pH dependence of the inhibitor binding constant of TFE in the oxidation of BzOH catalyzed by ZnLADH and CoLADH. To estimate the pH dependence of the inhibitor binding constant of TFE, only zwitterionic buffers were employed. The inhibitor constants were determined on the basis of initial velocity data collected at the following concentrations of TFE: 1.3 × 10^{-6}, 4.3 × 10^{-6}, 8.6 × 10^{-6}, and 17.2 × 10^{-6} M. The values of $K_I$ are plotted as (○) for ZnLADH and (△) for CoLADH. The inhibitor constants are indicated by arrows (↑ for ZnLADH and ↓ for CoLADH). The values of the inhibitor constants are $pK_a = 7.5$ and $pK_a = 8.1$ for ZnLADH. For CoLADH, the only one ionization constant can be determined because of the closely overlapping curves, and we estimate $pK_a = 7.5 = pK_b$. Identical results were obtained with zwitterionic buffers, as discussed in the text.

Under conditions of saturating concentrations of NAD$^+$, the two-substrate compulsory order, ternary complex mechanism of LADH (38, 49) can be treated as a one-substrate reaction represented in Equation 1.

$$E\cdot NAD^+ + \text{alcohol} \rightleftharpoons E\cdot NAD^+-\text{alcohol} \rightleftharpoons E$$

$$+ \text{NADH} + \text{aldehyde} + H^+$$

For this reaction, the kinetic parameters retain their definitions according to theory developed for one-substrate reactions with the understanding that the enzyme-NAD$^+$ complex corresponds to the free enzyme. The pH dependence of the kinetic parameter $k_{cat}$ then depends only on ionizations in the reactive enzyme-substrate complex, i.e., the $E\cdot NAD^+-\text{alcohol}$ complex, under the condition that the reactants and the enzyme-substrate intermediate are in equilibrium (50). Since hydride transfer responsible for ternary complex interconversion is the rate-limiting step in the oxidation of 2-propanol (31), application of the theory (50) to Equation 1 then requires that the ionization $pK_i$ governing the oxidation of 2-propanol belongs to the enzyme-NAD$^+$-alcohol complex. For the oxidation of BzOH, the values of $pK_i$ agree directly with values of 6.4 in ZnLADH (9) and 6.0 in CoLADH (51) for the ionization governing ternary complex interconversion determined by transient-state methods. Since the values of $pK_i$ are similar for both BzOH and 2-propanol despite different rate-limiting steps at higher pH (8, 31, 52), we conclude that the ionization $pK_i$ belongs to the ternary enzyme-NAD$^+$-alcohol complex in the oxidation of both BzOH and 2-propanol.

In the alkaline pH range, coenzyme release is slower than hydride transfer in the oxidation of BzOH (9, 52), and the theory for a simple two-step reaction cannot be applied. The ionization $pK_i$ governing the oxidation of BzOH at high pH then could belong to the enzyme-NAD$^+$ complex, to the enzyme-NAD$^+$-aldehyde complex, or to the enzyme-NAD$^+$-alcohol complex. The very large value of the rate constant for aldehyde dissociation compared with that of coenzyme dissociation (8) rules out the enzyme-NAD$^+$-aldehyde complex. Furthermore, since no ionization with $pK_i < 11.2$ governing dissociation of NADH from the enzyme-NAD$^+$ complex is observed (48), this complex can be also ruled out. We conclude that $pK_i$ with an observed value of 10.7 for ZnLADH reflects an ionization in the enzyme-NAD$^+$-alcohol complex. Under conditions of rate-limiting coenzyme release, the value of $pK_i$...
may be modulated by the rate constants for hydride transfer and coenzyme release (37). Accordingly, the intrinsic value of pK₁ is expected to be ≤10.7.²

Assignment of pK₁' and pK₂' to the Enzyme-NAD⁺ Complex—The pH profile of log κₑₐ/Kₑₐ for both ZnLADH and CoLADH is bell-shaped with an approach to a plateau at high pH, as shown in Figs. 1 and 2. Under conditions of saturating coenzyme as required for Equation 1, the approach to a plateau indicates an ionization in an enzyme-substrate species, whereas the bell-shaped part at lower pH is attributable only to molecular ionization constants of the free enzyme³ in the absence of substrate, i.e., the enzyme-NAD⁺ complex. The extent of kinetic modulation of pK₁' and pK₂' governing κₑₐ/Kₑₐ can be evaluated by determining their intrinsic values at thermodynamic equilibrium, i.e., through the pH dependence of the binding of the competitive inhibitor TFE under the condition that the ionizations are independent of substrate structure (36, 37). Since this criterion for thermodynamic equilibrium was satisfied in our studies for the binding of TFE, the values of the ionization constants pK₁ and pK₂ correspond to unmodulated values intrinsic to the enzyme-NAD⁺ complex. Comparison of the value of pK₂ (≈7.5) with that of pK₂' in Table I for the oxidation of BzOH by CoLADH reveals the presence of a kinetic shift. The limited data for ethanol oxidation show considerable kinetic modulation. On the basis of relationships derived by Cleveland (37) and estimates of rate constants determined by transient-state kinetic methods (9), we calculate pK₂' to be shifted to higher values by ≥0.4 pK units from its intrinsic value.³ The limited data for ethanol oxidation (32) (cf. Table I) suggest that ethanol behaves similarly to the primary alcohol BzOH.

In contrast, slow desorption of 2-propanol in the alkaline pH range would result in a large kinetic displacement of pK₂' to values >11, and indeed, pK₂' is not observed in the oxidation of 2-propanol (cf. Table I). A similar observation has been made for the secondary alcohol cyclohexanol (11).

A Minimal Reaction Scheme for LADH—Our assignment of two sets of prototropic equilibria to both the enzyme-NAD⁺ complex and to the enzyme-NAD⁺-alcohol complex can be employed to define the reaction scheme for the oxidation of alcohols catalyzed by LADH, shown as Scheme I. In this scheme, O represents the oxidized coenzyme, R the reduced coenzyme, and P the product of alcohol oxidation. The prototropic equilibria governing the initial binding of the coenzyme are drawn in a dashed box to emphasize that our results do not contribute directly to their description. We have indicated also that there are two pathways through which the enzyme-NAD⁺-alcohol complex can proceed to products because of our analysis of the alkaline region of the pH profile of κₑₐ/Kₑₐ in Figs. 1 and 2 and because of the pH profile of log Kₑₐ as discussed above.

Differentiation of Metal-induced Shifts in pKₗ Values from Kinetic Modulation Effects—To assign ionizations of the metal-bound ligands, we verify that changes in pKₗ values associated with metal ion substitution reflect properties at thermodynamic equilibrium. As discussed above, there is no significant kinetic modulation of the values of pK₁ and of pK₂' since pK₂ = pK₂'. Also, there is no metal effect on this ionization. On the other hand, a kinetic displacement of ≥0.6 dependent on the rate of hydride transfer is predicted in the value of pK₁ of the oxidation of BzOH catalyzed by Zn-LADH.² Correspondingly, the displacement for CoLADH is calculated to be ≥0.5. Since this kinetic effect would shift the ionization constant from its intrinsic value to higher pKₗ values and the observed shift in pK₁ for CoLADH to lower values is approximately 2-fold greater than the calculated kinetic shift, we conclude that the decrease in pK₁ to a value of ~9.7 in the Co⁺⁺-enzyme from that of 10.7 in the native enzyme reflects an intrinsic (equilibrium) property induced by metal substitution.

In the enzyme-NAD⁺ complex, the only metal-influenced ionization is pK₂'. The difference in measured values between pK₂ and pK₂' indicates considerable kinetic modulation in the enzyme-NAD⁺ complex for BzOH oxidation. Also, the modulations so severe in the oxidation of 2-propanol as to cause an apparent shift to very high pKₗ values. Nonetheless, even in the oxidation of 2-propanol, the onset of this ionization can be detected for CoLADH with an estimated pK₂' ~ 10.2, whereas the ionization is not detected for ZnLADH (cf. Table I). Most importantly, the intrinsic equilibrium value of the ionization pK₂ is not influenced by metal substitution, and the metal-induced shift in the constant is directly demonstrated, as shown in Fig. 4 for TFE inhibition.

Identification of Ionizing Groups Governing Oxidation of Alcohols

The Ionizing Group Responsible for pK₁ and pK₁': His-51—Pettersson and co-workers (8–10, 54, 55) have attributed an ionization corresponding to pK₁ to the metal-bound alcoholic hydroxyl group. We have previously pointed out that the free energy changes associated with the binding of alcohols at low and at high pH are not sufficient to account for a shift in the ionization of an alcohol substrate by ≥10 pKₗ units and that linkage relationships require that the ionizing group in the ternary complex responsible for pK₁ is the same as that in the tetracobalt-substituted enzyme (53).

³The rate constant governing hydride transfer has not been directly measured for CoLADH, in contrast to ZnLADH (19, 31, 52, 53). To provide an estimate of the kinetically induced shift in pK₂, we refer to the observation that the rate of hydride transfer for ethanol oxidation is decreased from 132 ± 1 in the native enzyme to 90 s⁻¹ in the tetracobalt-substituted enzyme (53).

¹The ionization constants governing kₑₐ/Kₑₐ may also belong to the free substrate. However, since the pKₗ of an alcoholic hydroxyl group is ≥14 (21), we need concern ourselves only with the enzyme-NAD⁺ complex. Furthermore, the pH profile of kₑₐ/Kₑₐ reflects ionizations on the free enzyme in the limit of low substrate (alcohol) concentration. Since all of our kinetic data were collected under saturating concentrations of NAD⁺, the possibility of contributions to kₑₐ/Kₑₐ from binary enzyme-alcohol complexes can be, therefore, directly disregarded.

²For ionizations observed in the pH profile of kₑₐ, the influence of kinetic modulation of pK₁ is estimated from the equation pK₁ = pK₁ + log(1 + k₉/k₈), where k₉ is the rate constant for dissociation of the product NADH, and pK₈ is the apparent ionization constant as measured through steady-state kinetic studies (37). The equation that applies to ionizations observed in the pH profile of kₑₐ/Kₑₐ is pK₂ = pK₂ + log(1 + k₉/k₈), where k₉ is the rate constant for alcohol desorption, k₈ is defined above, and pK₂ is correspondingly the apparent value of the ionization constant.

³In principle, ionizations governing kₑₐ/Kₑₐ may also belong to the free substrate. However, since the pKₗ of an alcoholic hydroxyl group is ≥14 (21), we need concern ourselves only with the enzyme-NAD⁺ complex. Furthermore, the pH profile of kₑₐ/Kₑₐ reflects ionizations on the free enzyme in the limit of low substrate (alcohol) concentration. Since all of our kinetic data were collected under saturating concentrations of NAD⁺, the possibility of contributions to kₑₐ/Kₑₐ from binary enzyme-alcohol complexes can be, therefore, directly disregarded.
binary complex observed as $pK_{1}'$ (56). In addition, the hypothesis of Pettersson and co-workers is now directly contradicted by mutagenesis studies. The ionization $pK_1$ is abolished by mutagenesis of His-51 to glutamine (liver enzyme sequence numbering) to glutamate (57). This ionization is similarly perturbed through mutagenesis of His-51 to glutamine in the human liver $\beta_1\beta_2$ alcohol dehydrogenase, which has 88% amino acid sequence identity with ZnLADH (58). We conclude that the ionization $pK_1$ must have origin in an amino acid residue of the protein.

For a catalytically productive pentacoordinate complex, we suggest that the neutral, metal-bound water molecule serves as a conduit through hydrogen bridging for proton abstraction. In multihydrate metal ion systems, covalent metal-ligand interactions polarize OH bonds and strengthen hydrogen bridging between the metal-bound ligands (62). These interactions can facilitate ionization of the proton from the hydroxyl group of the substrate. Moreover, it has been shown with model metal ion systems that a neutral metal-bound water molecule can have a functional role as a conduit for proton transfer (63). X-ray crystallographic studies (15, 23, 25) of a variety of binary and ternary enzyme-NADH complexes have shown an intricate network of solvent molecules together with the side chains of Ser-48 and His-51 hydrogen-bonded to the ribose OH substituents of the coenzyme. We suggest that the alcohol-metal-OH$_2$ complex in the active site remains tightly hydrogen-bonded to other nearby groups of the proton relay system for kinetically facile proton abstraction and transfer to bulk solvent. Although we have pointed out that there is no significant influence of the metal ion on $pK_1$, it is seen in Table I that the values of $pK_1$ for CoLADH are consistently slightly lower than those of ZnLADH, although the differences are essentially within experimental uncertainty. This observation supports the suggestion that His-51 at the solvent-protein interface "feels" the influence of the open-shell metal ion through a tightly coupled network of hydrogen-bonded residues. This tight coupling is absent in the binary enzyme-NAD$^+$ complex since no parallel metal effect on $pK_1'$ is detected.

It is of interest, furthermore, to point out that the metal-bound water with $pK_w \approx 10.7$ in the ternary enzyme-NAD$^+$-alcohol complex is ionized under conditions of high pH and that the pH profile of $k_{cat}/K_M$ shows a flattening in this region due to ionization of the alcohol-metal-OH$_2$ complex to an alcohol-metal-OH$^-$ species (cf. Figs. 1 and 2). This observation indicates that the ternary complex with a metal-bound hydroxide group must be also catalytically competent, although it is not operant under physiological conditions.

Acknowledgments—We thank Drs. F. J. Kezdy and J. Westley for helpful discussions.

REFERENCES

1. Maret, W., Anderson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, I., Zeppezauer, M. (1979) Eur. J. Biochem. 98, 501–512

2. Zeppezauer, M. (1983) in Coordination Chemistry of Metalloenzymes in Hydrolytic and Oxidative Processes (Bertini, L., and Drago, R. S., eds) pp. 99–122, D. Reidel Publishing Corp., Dordrecht, The Netherlands

3. Wolfe, J. K., Weidig, C. F., Halvorson, H. R., Shore, J. D., Parker, J. J. (1977) J. Biol. Chem. 252, 433–436

4. Coates, J. H., Hardman, M. J., Shore, J. D. & Guttridge, H. (1977) FEBS Lett. 84, 25–28

5. Schmidt, J., Chen, J., DeTraglia, M., Minkel, D. & McFarland, J. T. (1979) J. Am. Chem. Soc. 101, 5634–5640

6. Welsh, K. M., Creighton, D. J. & Klinman, J. P. (1980) Biochemistry 19, 20643

7. McFarland, J. T., Wong, R. K., Chen, J., DeTraglia, M., Minkel, D. & McFarland, J. T. (1979) J. Am. Chem. Soc. 101, 5634–5640

8. Bertini et al. (59) have attributed a pH-dependent decrease in intensity of an NMR band to ionization of the metal-bound imidazole group of His-67 in CoLADH with a $pK_w \approx 9.0$. The intensity of this band is shifted and does not disappear with increasing pH in the binary enzyme-NAD$^+$ complex, indicating that the metal-bound imidazole is not responsible for $pK_1'$.
Mechanism of Liver Alcohol Dehydrogenase

7. Morris, R. G., Salimian, G. & Dunn, M. F. (1980) Biochemistry 19, 725–731
8. Kvassman, J. & Pettersson, G. (1978) Eur. J. Biochem. 87, 417–427
9. Kvassman, J. & Pettersson, G. (1980) Biochemistry 19, 565–575
10. Pettersson, G. (1987) CRC Crit. Rev. Biochem. 21, 349–389
11. Cook, P. F. & Cleland, W. W. (1981) Biochemistry 20, 1805–1816
12. Dworschack, R. T. & Papp, B. V. (1977) Biochemistry 16, 2716–2725
13. Shore, J. D., Gutfreund, H., Brooks, R. L., Santiago, D. & Santiago, P. (1974) Biochemistry 13, 4185–4191
14. Evans, S. A. & Shore, J. D. (1980) J. Biol. Chem. 255, 1509–1514
15. Branden, C.-I., Jörnvall, H., Eklund, H. & Furugen, B. (1975) in The Enzymes (Boyer, P. D., ed) 3rd Ed., Vol. XI, pp. 103–190, Academic, New York
16. Klinnman, J. P. (1981) CRC Crit. Rev. Biochem. 10, 39–78
17. Dietrich, H., MacGibbon, A. K. H., Dunn, M. F. & Zeppezauer, M. (1983) Biochemistry 22, 3432–3438
18. Eftink, M. R. (1986) Biochemistry 25, 6620–6624
19. Sekhar, V. C. & Plapp, B. V. (1988) Biochemistry 27, 5082–5088
20. Schneider, G., Eklund, H., Cedergren-Zeppezauer, E. & Zeppezauer, M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5289–5293
21. Boyd, R. H. (1989) in Solvent-Solvent Interactions (Coetzee, J. P., and Ritchie, C. D., eds.) pp. 97–218, Marcel Dekker, New York
22. Makinen, M. W., Maret, W. & Yim, M. B. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2984–2988
23. Cedergren-Zeppezauer, E., Samama, J. P. & Eklund, H. (1982) Biochemistry 21, 4895–4908
24. Eklund, H., Samama, J. P., Brandén, C. I., Åkeson, Å. & Jones, T. A. (1981) J. Mol. Biol. 146, 561–587
25. Eklund, H., Plapp, B. V., Samama, J. P. & Brandén, C.-I. (1982) J. Biol. Chem. 257, 14349–14358
26. Beaucamp, K., Bergmeyer, H. U. & Beutler, H. O. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed.) 3rd Ed., p. 580, Verlag Chemie, Weinheim, Germany
27. Taniguchi, S., Theorell, H. & Åkeson, A. (1967) Acta Chem. Scand. 21, 1903–1920
28. Lowry, O. H., Passonneau, J. V. & Rock, M. K. (1961) J. Biol. Chem. 236, 2756–2759
29. Dixon, M. (1955) Biochem. J. 59, 161–170
30. Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
31. Brooks, R. L. & Shore, J. D. (1971) Biochemistry 10, 3855–3858
32. Theorell, H., Nygaard, A. P. & Bonnichsen, R. (1955) Acta Chem. Scand. 9, 1145–1165
33. Shore, J. D. & Theorell, H. (1966) Arch. Biochem. Biophysics 117, 375–380
34. Dalziel, K. & Dickinson, F. M. (1966) Biochem. J. 100, 491–501
35. Dalziel, K. & Dickinson, F. M. (1966) Biochem. J. 100, 34–46
36. Tipton, K. F. & Dixon, H. B. F. (1979) Methods Enzymol. 63, 183–234
37. Cleland, W. W. (1977) Adv. Enzymol. 45, 273–387
38. Watten, C. C. & Cleland, W. W. (1966) Biochemistry 4, 2442–2451
39. Taylor, K. B. (1983) Biochemistry 22, 1040–1045
40. Dalziel, K. (1963) J. Biol. Chem. 238, 2850–2856
41. Coleman, P. L. & Weiner, H. (1973) Biochemistry 12, 1705–1709
42. Shore, J. D. & Gutfreund, H. (1970) Biochemistry 9, 4655–4659
43. Anderson, D. C. & Dahlquist, F. W. (1982) Biochemistry 21, 3569–3578
44. Subramanian, S. & Ross, P. D. (1979) J. Biol. Chem. 254, 7827–7832
45. Makinen, M. W., Kuo, L. C., Dymowski, J. J. & Jaffer, S. (1979) J. Biol. Chem. 254, 356–366
46. Brooks, R. L., Shore, J. D. & Gutfreund, H. (1972) J. Biol. Chem. 247, 2382–2383
47. Pocker, Y. & Page, J. D. (1990) J. Biol. Chem. 265, 22101–22108
48. Anderson, P., Kwasman, J., Lindström, A., Olden, B. & Pettersson, G. (1961) Eur. J. Biochem. 113, 425–433
49. Cornish-Bowden, A. (1979) Fundamentals of Enzyme Kinetics, pp. 99–129, Butterworths, London
50. Brocklehurst, K. & Dixon, H. B. F. (1976) Biochem. J. 155, 61–70
51. Sartorius, C. (1987) Die Rolle der Proteinkonformation im Katalysezyklus der Pflanzenfermente. PhD thesis, Universität des Saarlandes, Saarbrücken, Germany
52. Weidig, C. F., Halvorson, H. R. & Shore, J. D. (1977) Biochemistry 16, 2916–2922
53. Shore, J. D. & Santiago, D. (1975) J. Biol. Chem. 250, 2008–2012
54. Kvassman, J. & Pettersson, G. (1979) Eur. J. Biochem. 100, 115–123
55. Kvassman, J., Larsson, A. & Pettersson, G. (1981) Eur. J. Biochem. 114, 555–563
56. Makinen, M. W. & Maret, W. (1986) in Zinc Enzymes (Bertini, I., Luchinat, C., Maret, W., Zeppezauer, M., eds.) pp. 465–470, Birkhäuser Verlag, Basel, Switzerland
57. Plapp, B. V., Ganzhorn, A. J., Gould, R. M., Green, D. W. & Hershey, A. D. (1987) Prog. Clin. Biol. Res. 232, 227–236
58. Ehrig, T., Hurley, T. D., Edenberg, H. J., and Bozon, W. F. (1991) Biochemistry 30, 1062–1068
59. Bertini, I., Gerber, M., Lanini, G., Luchinat, C., Maret, W., Rawer, S., and Zeppezauer, M. (1984) J. Am. Chem. Soc. 106, 1826–1830
60. Makinen, M. W. & Yim, M. B. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6221–6225
61. Yim, M. B., Wells, G. B., Kuo, L. C. & Makinen, M. W. (1986) Frontiers in Bioinorganic Chemistry (Xavier, A. V., ed.) pp. 562–570, VCH Verlagsgesellschaft, Weinheim, Germany
62. Zandell, G. (1989) Hydration and Intermolecular Interaction, pp. 76–81, Academic Press, New York
63. Kluger, R., Wong, M. K. & Dodds, A. K. (1984) J. Am. Chem. Soc. 106, 1113–1117