Liver Alcohol Dehydrogenase-Coenzyme Reaction Rates*

(Received for publication, September 20, 1976)

MICHAEL C. DETRAGLIA, JACK SCHMIDT, MICHAEL F. DUNN, and JAMES T. MCFARLAND
From the Department of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53201

Specific rate constants for the association and dissociation of liver alcohol dehydrogenase-coenzyme complexes have been measured within the pH range 6 to 10.5. NADH and NAD+ association rates were measured under pseudo-first order conditions by monitoring changes in enzyme and coenzyme fluorescence. Dissociation rates were measured by replacing bound NADH or NAD+ with tight binding tertiary complex inhibitors (NAD+, pyrazole) and (NADH, isobutyramide), respectively.

Results indicate that the rate constant for NAD+ and NADH association show the same pH rate profile with pKₐ = 9.5. Furthermore, the pKₐ for o-phenanthroline association to liver alcohol dehydrogenase is 8.1. Since x-ray studies indicate that o-phenanthroline displaces the water bound to Zn²⁺ at the active site (Branden, C.-I., Jornvall, H., Eklund, H., and Furugaen, G. (1975) in The Enzymes (Boyer, P., ed) p. 133, Academic Press, New York (1)), the pKₐ of 8.1 is most likely associated with ionization of this water molecule. Thus ionization of water bound to zinc apparently does not control the rate of nucleotide association as previously suggested (Taniguchi, S., Theorell, H., and Åkeson, A. (1967) Acta Chem. Scand. 21, 1903 (2)).

The dissociation rate constant for E-NAD⁺ shows a pKₐ = 8.9. By contrast, the rate constant for E-NADH dissociation exhibits a complex behavior not interpretable as associated with ionization of a single group. Agreement between equilibrium-binding constants for nucleotide and kₐ(association)/kₐ(dissociation) ratios is quite good. Furthermore, agreement with rate constants determined from steady state kinetic experiments is good except for the rate of NADH association. Mechanistic consequences of the nucleotide kinetic results are discussed.

We have also discovered that liver alcohol dehydrogenase rapidly loses Zn²⁺ at pH values >10; this finding makes previous studies at these very basic pH values suspect.

Liver alcohol dehydrogenase catalyzes the interconversion of aldehydes and alcohols by a compulsory ordered mechanism in which coenzyme binding precedes substrate binding, and product release precedes coenzyme release as shown in Scheme 1. The dissociation of the E(NADH) complex (k₋) is thought to be the rate-limiting step for aliphatic alcohol oxidation, and the dissociation of the E(NAD⁺) complex (k₋) is believed to be the rate-limiting step for aliphatic aldehyde reduction (3-6). However, the direct measurement of coenzyme association and dissociation rates as a function of pH have not been reported.

Two major proposals for the catalytic action of LADH have been suggested. One mechanism (Scheme 2) involves the active site zinc ion as a Lewis acid catalyst in the activation of the aldehyde carbonyl for reduction by NADH via inner sphere coordination of the carbonyl oxygen (7-9) and the subsequent acid catalyzed breakup of the zine alcoholate product via the involvement of a protonic amino acid side chain residue (10). The second mechanism (Scheme 3) involves acid catalysis of aldehyde reduction by a water molecule bound to the active site zinc ion (11).

The apparent enzyme pKₐ values which regulate the association and dissociation of NAD⁺, as determined from the analysis of the steady state kinetic behavior of liver alcohol dehydrogenase are pKₐ = 9.5 for association and pKₐ = 8.0 for dissociation (6). The apparent pKₐ values which regulate the affinity of the site for NAD⁺ and NADH (2) have been reported as pKₐ = 8.5 for NAD⁺ and pKₐ = 10.5 for NADH. The pKₐ values for the enzyme group controlling NAD⁺ binding are perturbed from pKₐ = 8.5 to pKₐ = 6.5 in the presence of NAD⁺. If a single ionizable group controls coenzyme binding, then sizeable pKₐ perturbations must accompany the association and dissociation steps for coenzyme binding to account for the apparent pKₐ values. The occurrence of such perturbations has been suggested to explain the release of protons to solution during NAD⁺ binding (12, 13).

In view of the importance of protonic equilibria on the rate-limiting steps for aldehyde reduction and alcohol oxidation, we have investigated the pH dependencies of NAD⁺ and NADH binding to liver alcohol dehydrogenase as determined by direct measurement of the specific association and dissociation rate constants for these processes.

**Experimental Procedures**

Preparation of Reagents—The preparation of enzyme has been reported previously (14, 15). Liver alcohol dehydrogenase was obtained from C. F. Boehringer and Söhne and purified by gel filtration chromatography at pH 8.75 (0.05 M pyrophosphate) on Bio-Gel P-30. Enzyme protomer molarity was determined by spectrophotometric measurement at 280 nm, ε₂₈₀ = 17,700 M⁻¹ cm⁻¹ per protomer. Active

---

* The abbreviation used are: LADH, liver alcohol dehydrogenase; IBA, isobutyramide; ppy, pyrazole.
site normality was based on a titration of enzyme with NAD⁺-pyrazole (16).

NADH and NAD⁺ were "Chromato-pure" grade from P-L Biochemicals, used without further purification. Isobutryramide was purchased from Aldrich Chemical Co. and doubly recrystallized from water. Pyrazole was purchased from Aldrich Chemical Co. and used without further purification. o-Phenanthroline was obtained from Eastman Organic Chemicals and used without further purification.

Buffer used during enzyme purification and activity assay was 0.05 M pyrophosphate (Na₂P₂O₇·10H₂O, Mallinckrodt analytical reagent (AR) grade) adjusted to pH 8.75 with HCl. Buffers for pH dependence kinetics were 0.1 M phosphate prepared by mixing 0.1 M solution of mono-, di-, and tribasic sodium phosphates (Mallinckrodt AR grade) in the proper proportions to achieve the desired pH. Since we have employed phosphate buffers at pH values where the buffer capacity is low, pH values were measured before and after each experiment.

Standard zinc solutions [Zn²⁺] = 10 to 80 μM were prepared by dilution of a 15.3 mM stock solution of zinc chloride in double glass-distilled water. The stock solution of zinc chloride was prepared by dissolution of zinc metal (20-mesh Mallinckrodt AR grade) in 10 ml of 12 N HCl followed by addition of distilled water.

Stopped Flow Kinetic Experiments — All rapid kinetic experiments were carried out on a Durrum stopped flow spectrophotometer using a pneumatic air-actuated pushing device. Absorption measurements were made with a linear optical configuration utilizing a tungsten lamp source and a 2-cm light path. The fluorescence configuration utilized a Xenon lamp source and a monochromator with filtered emission optics (cutoff < 340 nm). Phototube placement was 90° to the excitation beam for fluorescence detection. Enzyme fluorescence changes were measured with the use of an excitation wavelength of 280 nm with emission measurement through a Corning 0-54 filter (0% transmittance below 300 nm). Changes in the NADH fluorescence signal were measured with the excitation wavelength at 330 nm; fluorescence emission was observed through a Corning 0-52 filter (0% transmittance below 340 nm). Phototube voltages were stored in a Northern Scientific NS-560 time-averaging computer modified to collect data at two scanning speeds in each of two 1024-
Liver Alcohol Dehydrogenase-Coenzyme Reaction Rates

3495

channel storage units. The data were then plotted on a Houston Instruments Div., Bausch & Lomb omniographic 2000 X-Y recorder. Data were treated by regression analysis of the linear form of the first order rate law. Second order (association) rates were determined under pseudo-first order conditions using a series of increasing coenzyme concentrations.

NAD⁺ Dissociation Rates – Dissociation rates for NAD⁺ were measured for the pH range 6.2 to 10.0 by mixing liver alcohol dehydrogenase (5 μM) preincubated with NAD⁺ (70 μM) in one syringe, with NADH (15 μM) and isobutramide (1.3 mM) in a second syringe. The increase in fluorescence of E-NAD⁺-IBA complex (λex = 330, λem > 340 nm) was followed in the stopped flow spectrometer. Identical results were obtained when measuring complex formation either by fluorescence or absorbance spectroscopy (absorbance measurements were made at 555 nm). Table I shows the concentration dependence of the observed single exponential formation of E-NAD⁺-IBA; concentration independence of the rate indicates that we are measuring the dissociation rate of E-NAD⁺.

NAD⁺ Association Rates – Association rates for NAD⁺ between pH 7.9 and 10.5 were measured by mixing liver alcohol dehydrogenase (4 μM) with a series of NAD⁺ solutions (10 to 60 μM) in a stopped flow experiment. Measurement was made of the decrease in enzyme fluorescence on formation of E-NAD⁺ complex (λex = 280 nm, λem > 340 nm); data were treated as described below.

NADH Dissociation Rates – NADH dissociation rates were determined over the pH range 6.0 to 10.5 by mixing liver alcohol dehydrogenase (6.5 μM) preincubated with NADH (21 μM) in one syringe, with NAD⁺ (2.3 mM) and pyrazole (9.4 mM) in a second syringe. The decrease in fluorescence of the E-NAD⁺ complex (λex = 330 nm, λem > 340 nm) was followed in the stopped flow spectrometer. Experiments at several NAD⁺ and pyrazole concentrations (Table II) confirmed that the observed process was the first order E-NADH dissociation.

NADH Association Rates – NADH association rates were measured over the pH range 6.2 to 10.3 by mixing liver alcohol dehydrogenase (3 μM) with varying concentrations of NADH (10 to 100 μM) in the stopped flow spectrometer. Immediately prior to use, NADH solution concentrations were determined by spectrophotometric reading at 340 nm (εmax = 6290 M⁻¹ cm⁻¹) on the Cary-16 spectrophotometer. Measurements of NADH fluorescence enhancement (λex = 330, λem > 340 nm) or enzyme fluorescence quenching (λex = 280, λem > 300 nm) gave identical results. Determination of rate constants at pH values above 10 were carried out by observation of NADH quenching of enzyme fluorescence only. Pseudo-first order rates were treated as described below to determine the second order rate constants.

-o-Phenanthroline Association Rates – o-Phenanthroline association rates were measured over the pH range 7.0 to 10.0 by mixing liver alcohol dehydrogenase (4 μM) with varying concentrations of o-phenanthroline (10 to 50 μM). The decrease in enzyme fluorescence was a pseudo-first order single exponential process; data were analyzed as described previously for NAD⁺.

-o-Phenanthroline Dissociation Rates – o-Phenanthroline dissociation rates were measured at pH 7.0, 8.3, and 10.0 by displacement of phenanthroline from the liver alcohol dehydrogenase-o-phenanthroline complex with NADH, IBA. Liver alcohol dehydrogenase (10 μM) and phenanthroline (40 μM) were mixed in the stopped flow spectrophotometer with NADH (30 μM) and IBA (100 μM). Increase in fluorescence due to E-NADH-IBA complex formation was a single exponential process.

Zinc Determination – Zinc content of LADH at pH 8.7 and 11.0 was determined on an Instrumentation Laboratory, Inc. model 151 atomic absorption (AA) spectrometer. Concentration calibration curves were made using zinc standards prepared as described above. Liver alcohol dehydrogenase solutions (3 ml of 50 μM enzyme) were incubated for 2 h at pH 8.7 and 11.0 at room temperature. The solutions were chromatographed at their respective pH values on Bio-Gel P-30 (1 x 20 cm), and the fractions with highest optical density at 280 nm were assayed for catalytic activity. The control (pH 8.7) lost no more than 5% activity during this treatment, but the more basic enzyme solution had lost all catalytic activity. Protein determination by the biuret method confirmed that the 280 nm extinction coefficient (εmax = 17,700 M⁻¹ cm⁻¹) of native enzyme was unaffected by incubation at pH 11.0. Zinc content per protomer of LADH was determined by comparing the concentration of zinc determined by AA with the enzyme protomer molarity determined from enzyme absorption at 280 nm.

RESULTS

Steady state kinetic analyses of the liver alcohol dehydrogenase catalytic cycle indicate that the dissociation of the E(NAD⁺) and E(NADH) binary complexes are relatively slow processes exhibiting specific first order rate constants in the range ~100 s⁻¹ to 1 s⁻¹ over the pH range 6 to 10 (3–6). The specific second order association constants for binary complex formation fall in the range 10⁶ M⁻¹ s⁻¹ to 10⁴ M⁻¹ s⁻¹ (3–6, 17). Hence, it is possible to measure directly dissociation and association rate constants for both oxidation states of coenzyme by utilizing rapid mixing stopped flow kinetic techniques and the chromophoric or fluorophoric (or both) properties of various enzyme binary and ternary complexes.

Assuming that the ternary complex involving E, NADH, and IBA is formed by replacement of NAD⁺ from the E(NAD⁺) complex via the mechanism of Equation 1, the rate of NAD⁺ dissociation (k₄) will be the rate-limiting step if k₄ << k₂[NADH] ≫ k₁[NAD⁺]. If these conditions do not hold, then the apparent rate of complex formation will depend on the concentration of one or more of the ligands involved (e.g. NAD⁺ or NADH). The data presented in Table I demonstrate that the rate of formation of the ternary E(NADH, IBA) complex on mixing the E(NAD⁺) complex with a solution of NADH and IBA is insensitive to 10-fold changes in concentrations of either NAD⁺ or NADH. Fig. 1 shows a typical stopped flow trace of the single exponential increase in fluorescence at ~390 nm (λex = 330 nm) associated with formation of the E(NADH, IBA) complex. The data in Table I were obtained from similar traces by monitoring the decrease in optical density at 355 nm (the maximum in the difference spectrum between free and bound NADH).

Since the observed single exponential process is independent of ligand concentration (and is, therefore, not mixed with a component) the rate equation consistent with the mechanism of Equation 1 is

k₄ = k₄[NAD⁺][IBA] = k₄[NAD⁺][IBA] + k₄[NAD⁺]k₄[NAD⁺]

This equation was derived by making the steady state approxima-

TABLE I

Concentration dependence of NAD⁺ dissociation rate constant

| Enzyme | NAD⁺ | NADH | Isobutramide | k₄ | s⁻¹ |
|--------|------|------|--------------|----|-----|
| μM     | μM   | μM   | μM           |    |     |
| 10     | 50   | 10   | 100          | 7.5 | 0.5 |
| 50     | 10   | 100  | 5            | 5.4 | 0.8 |
| 10     | 500  | 50   | 100          | 7.7 | 1.0 |

TABLE II

Concentration dependence of NADH dissociation rate constant

| Enzyme    | NADH | Pyr | k₄ | s⁻¹ |
|-----------|------|-----|----|-----|
| μM        | μM   | mM  |    |     |
| 6.61      | 21.1 | 2.3 | 9.35| 4.6 |
| 8.54      | 21.9 | 5.0 | 5.0 | 4.4 |
| 8.54      | 21.9 | 10  | 8.20| 4.7 |
| 8.54      | 21.9 | 15  | 8.20| 4.6 |
second order rate process), and since rates measured both by fluorescence and by absorbance are identical, the observed rate process must be limited by the specific first order dissociation of E(NAD+)\( ^{+}\). Fig. 2 shows the results of this same experiment over the pH range 6.2 to 10.0. Note that the solid line represents the best fit theoretical protonic dissociation curve, calculated for a rate process dependent on the ionization of a group with \( pK_a = 8.0 \). The precision of the fit at high pH is good; however, at low pH values the observed rates reflect a response to pH which is slightly greater than first order in [H\( ^+ \)].

Formation of the E(NAD\( ^+ \)) binary complex results in a significant quenching of the enzyme tryptophan fluorescence. Under the pseudo-first order condition [NAD\( ^+ \] > [enzyme], quenching is a single exponential rate process when the tryptophan fluorescence is monitored at -340 nm (\( \lambda_{ex} = 280 \) nm). The dependence of the observed pseudo-first order rate constant on NAD\( ^+ \) concentration at pH 9.1 is shown in Fig. 3. Since the approximate equation for the single step reversible binding of NAD\( ^+ \) to liver alcohol dehydrogenase is as follows

\[
k_{390} = k_{a1} \text{ (NAD}^{+}\text{)} + k_{-1}
\]

for the mechanism

\[
E + \text{NAD}^{+} \overset{k_{1}}{\underset{k_{-1}}{\rightleftharpoons}} E - \text{NAD}^{+}
\]

the second order rate constant for association of NAD\( ^+ \), \( k_{1} \), is obtained as the slope of the plot in Fig. 3. In calculating association rate constants for NAD\( ^+ \) and NADH we have always assigned intercepts as the measured dissociation rate constants, \( k_{-1} \). Linear regression analysis was employed to determine the "best fit" line to experimental data. Fig. 4 shows the pH dependence of the specific second order association constants for NAD\( ^+ \)-liver alcohol dehydrogenase binary complex formation for the pH range 7.9 to 10.5. At more acidic pH values the binding constant of NAD\( ^+ \) is not sufficiently large to yield suitably large amounts of fluorescence quenching. Over the limited pH range amenable to investigation, the values of the association rates can be fit to a theoretical acid dissociation curve with an apparent \( pK_a \) of 9.5 (Fig. 4).

Measurement of the rate of NADH dissociation from the binary E(NADH) complex was accomplished by a replacement technique similar to that employed for the E(NAD\( ^+ \)) system. NAD\( ^+ \) and pyrazole form a ternary complex (15, 16) in which NAD\( ^+ \) is bound much more tightly than is NADH in the E(NADH) binary complex. Hence, formation of the E(NAD\( ^+ \), pyr) complex can be used to replace NADH at the enzyme site with NAD\( ^+ \).

\[
E(NADH) \overset{k_{1}}{\underset{k_{-1}}{\rightleftharpoons}} E + \text{NADH} \overset{k_{3}}{\underset{k_{1}}{\rightleftharpoons}} E(\text{NAD}^{+}, \text{pyr})
\]

if \( k_{-1} \ll k_{3} \) NAD\( ^+ \gg k_{-1} \) [NADH] (Equation 2). As explained previously, deviation from these conditions will result in concentration dependence of the observed rate of E-\text{NAD}-pyrazole complex formation.

The data presented in Table II show that the observed rate of NADH dissociation, as measured by the decrease in fluorescence for enzyme-bound NADH, is independent of the concentrations of NAD\( ^+ \) and pyrazole when the concentrations used are sufficiently high to insure that the rate of E(NAD\( ^+ \), pyr) complex formation (the rate constant at saturating [NAD\( ^+ \)] is 100 s\( ^{-1} \) at pH 7.0 and pH 8.75) is rapid relative to E(NADH) dissociation (15). The pH dependence of the specific E(NAD\( ^+ \)) dissociation rate over the pH range 6.0 to 10.5 is shown in Fig. 5. In contrast to the behavior of the E(NAD\( ^+ \)) complex, the rate of dissociation of the E(NADH) complex varies only slightly as a function of pH, showing a small, nearly linear increase in rate constant with increasing pH.
Formation rate constants for the \( E(NADH) \) complex were obtained either by measuring the increase in NADH fluorescence, or by measuring the quenching of the intrinsic enzyme tryptophan fluorescence on forming a complex of NADH with the enzyme site. Unlike our measurement of off rates which would be expected to reflect only coenzyme dissociation from the active site, our on rate experiments might also reflect binding to the loose ancillary coenzyme binding sites (19). In the case of NADH binding the ancillary binding sites are not observed to enhance NADH fluorescence and contribute only 20% to tryptophan fluorescence quenching; therefore, these sites could not interfere with our measurement of binding to the active site. Second order rate constants were evaluated by plotting \( k_{on} \) against \([NADH]\) as explained for NAD+. Rate measurements utilizing either NADH fluorescence enhancement or tryptophan quenching were found to yield identical second order rate constants. The pH dependence of the NADH association rate constants for the pH range 6.2 to 10.3 is shown in Fig. 6.

In order to determine the pK\(_{a}\) of Zn-O at the active site,

\[
\text{FIG. 4. pH dependence of the NAD}^+ \text{ association rate constant (} k_a \text{). The curve corresponds to a theoretical dissociation process with pK\(_{a}\) = 9.5. Temperature = 25\(^\circ\), 0.1 M phosphate buffer. In all cases error limits were assigned from the range of values determined from the slope of a plot similar to that shown in Fig. 3.}
\]

we have measured the pH dependence of the rate of association and dissociation of phenanthroline and LADH. Since x-ray investigation has shown that phenanthroline displaces the water molecule bound to the active site Zn\(^{2+}\) (1), we expected to find that the ionization state of this water molecule would directly influence the association rate of phenanthroline and LADH. That this process is indeed dependent on pH can be seen in Fig. 7. Phenanthroline binding was studied by following the rate of enzyme fluorescence quenching as previously described for coenzyme. We have also measured the rate of dissociation of phenanthroline by means of displacement by NADH-IBA. The rate of dissociation of phenanthroline was monitored by observing the increase in fluorescence due to:

\[
E \rightarrow \text{NADH complex formation as outlined above; values of}
\]

\[
\begin{align*}
0.21, & 0.20, \\
0.25 & \text{s}^{-1}
\end{align*}
\]

were determined at pH 7.0, 8.3, and 10.0, respectively. The dissociation rate constant is seen to be pH-independent consistent with the loss of the ionizable water molecule bound to Zn\(^{2+}\) upon displacement by phenanthroline.

The measured equilibrium dissociation constants for the \( E(NAD^+) \) (2) and \( E(NADH) \) complexes (20) are compared with values calculated as the ratio of the specific dissociation and association rate constants in Table III. Where necessary the theoretical best fit line was used to interpolate individual rate
constants not available from our data at a given pH value. At all pH values agreement is within a factor of 3, and at most pH values agreement is even better. This is an indication that over the range in which we are able to compute these calculated values, binding is a single step process and that the equations used in our calculations are adequate to fit the binding mechanism. Table IV shows a comparison of our directly measured rate constants (identified by the constants in Scheme 1) with those derived from steady state rate experiments. Fig. 8 also shows graphically the steady state results and pH dependence for various rate parameters. Agreement is generally good, although the pKₐ for rate of NADH association is different from ours, and we are unable to compare our results for the rate of NAD⁺ association below pH 8.0 because of experimental difficulties. At pH values above 9, earlier researchers have reported that either the NADH binding stoichiometry or the enzyme affinity is drastically altered (20-22). Because the decreased fluorescence enhancement on NADH binding below pH 10 is similar to the reported behavior of apoazinc LADH (23), we investigated the hypothesis that enzyme loses Zn²⁺ at basic pH. This would not be unexpected since competition with OH⁻ at basic pH is often seen with complexes of Zn²⁺. Atomic absorption measurements comparing enzyme handled identically at pH 8.7 versus pH 11.0 shows the enzyme species at pH 8.7 contains 2.0 ± 0.3 g atom of zinc per 40,000 daltons, whereas at pH 11.0, the zinc content decreases to 1.1 ± 0.2 g atom of zinc per 40,000 daltons. Thus, above pH 10, the binding and kinetic experiments may be erroneous due to the loss of zinc ion from the active site. Where we have reported rate experiments at pH > 10, these pH values were achieved by “jumping” the pH to basic values in the stopped flow spectrometer, i.e. enzyme was never allowed to stand at pH > 10. The absence of an enhanced fluorescence emission spectrum for bound NADH is consistent with the properties of a zinc-deficient enzyme (23).

DISCUSSION

Several interesting conclusions can be drawn from this study of the kinetics of dinucleotide association and dissociation. Steady state kinetic studies (3-6) have suggested that the LADH reaction both in the direction of aldehyde reduction and in the direction of alcohol oxidation is a compulsory ordered mechanism in which coenzyme dissociation is the rate-limiting process. Our data are quite consistent with this conclusion. The rate constants for dissociation of NAD⁺ (Fig 2) correlate well with the turnover numbers for acetaldehyde reduction at all pH values (Table IV, Fig. 8). Likewise, our measured dissociation rate constants for NADH (Fig 5) correlate well with turnover numbers for ethyl alcohol oxidation (Table IV, Fig. 8). For aromatic substrates which exhibit smaller turnover numbers than the acetaldehyde turnover number (e.g. acetaldehyde, kₕ = 9.72 s⁻¹, while for benzaldehyde, kₕ = 4.0 s⁻¹, and for β-napthaldehyde, kₕ = 0.4 s⁻¹ at pH = 8.75) (6, 14), NAD⁺ dissociation is not rate-limiting. It is likely that alcohol dissociation becomes rate-limiting for these tightly binding aromatic substrates.

The good agreement between our measured dissociation rates for NAD⁺ and NADH and the kₕ values for acetaldehyde and ethanol turnover has interesting consequences relative to subunit interactions between protomers. It has been suggested that in oligomeric enzymes showing half-of-the-sites reactivity, liganding by substrates at the inactive subunit may cause a reduction in the energy of activation of the rate-limiting step (24). If this were the case with liver alcohol

![A](image1.png)

![B](image2.png)

**Table III**

Comparison of measured equilibrium values for coenzyme binding with values calculated from rate data

Where values from the present study are not available for comparison at a given pH, we have reported values interpolated from our current data. All rate constants are identified in Scheme 1.

| pH | kₐ/kₐ⁺ | kₐ⁺ NAD⁺ | kₐ⁻/kₐ⁺ | NAD⁺ |
|----|--------|---------|---------|------|
| 10.5 | 5.7    | 5.0     | 1.9     | 5.5  |
| 10   | 1.8    | 2.4     | 6.5     | 3.9  |
| 9.5  | 0.63   | 0.45    | 0.43    | 9.3  |
| 9    | 0.63   | 0.45    | 0.43    | 9.3  |
| 8.5  | 0.27   | 0.45    | 0.43    | 9.3  |
| 8    | 0.47   | 0.45    | 0.43    | 9.3  |
| 7.5  | 0.47   | 0.45    | 0.43    | 9.3  |
| 7    | 0.48   | 0.45    | 0.43    | 9.3  |
| 6.5  | 0.63   | 0.45    | 0.43    | 9.3  |
| 6    | 0.62   | 0.45    | 0.43    | 9.3  |

* Data from Ref. 20.
* Data from Ref. 2.

**Table IV**

Comparison of rate parameters calculated from steady state kinetic data with those directly measured in current study

Where values from the present study are not available for comparison at a given pH, we have reported values interpolated from our current data. All rate constants are identified in Scheme 1. d = directly measured in current study; s = steady state data, Ref. 6.

| pH   | kₐ⁺ (µM⁻¹ s⁻¹) | kₐ⁻ (µM⁻¹ s⁻¹) | kₐ⁺ NAD⁺ s⁻¹ | kₐ⁻ NAD⁺ s⁻¹ |
|------|----------------|----------------|--------------|--------------|
| 10.5 | 1.7            | 1.0            | 4.7          | 5.2          |
| 10   | 0.63           | 0.45           | 0.27         | 0.44         |
| 9.5  | 0.63           | 0.45           | 0.27         | 0.44         |
| 9    | 0.63           | 0.45           | 0.27         | 0.44         |
| 8.5  | 7.6            | 7.6            | 3.5          | 3.2          |
| 8    | 7.6            | 7.6            | 3.5          | 3.2          |
| 7    | 7.6            | 7.6            | 3.5          | 3.2          |
| 6    | 4.1            | 9.0            | 2.4          | 1.6          |

FIG. 8. Parameters derived from steady state relations. A, NAD⁺ association rate constants (µM⁻¹ s⁻¹), PKₐ = R 4 and 9 for NAD⁺ dissociation rate constants (s⁻¹), ○, PKₐ = 8.1. B, NADH association rate constants (µM⁻¹ s⁻¹), ○, PKₐ = 8.7; NADH dissociation rate constants (s⁻¹), ○. From Ref. 6.
Liver Alcohol Dehydrogenase-Coenzyme Reaction Rates

Liver alcohol dehydrogenase, it might be expected that turnover numbers should be much higher than the measured dissociation rate constants. Because the turnover numbers are measured under conditions where inactive subunits have aldehyde and NADH bound (14, 15) while the rate constants for coenzyme dissociation show a good correlation with the acetaldehyde-ethanol turnover numbers, we presume that ligand binding at the putative "inactive" subunit can account for no more than a 2- to 3-fold increase in the rate of coenzyme dissociation.

Though turnover numbers and our measured dissociation rate constants ($k_{on}$ values) show satisfactory agreement, the NADH association rate constants ($k_{on}$ values) calculated from steady state kinetic observations do not correlate well with our directly measured rate constants. Values for the NADH association rate constants calculated from steady state parameters show pH dependence with a more acidic $pK_a$ than our data. The apparent $pK_a$ from our NADH association rate constant data is 9.4 to 9.6 while the $pK_a$ for the steady state data is ~8.7 to 8.9 (Table IV, Fig. 8). The absolute values of the association rate constant for NADH measured directly are in fair agreement with the steady state data; however, we see a decrease in this rate constant at pH values <6.8 not apparent in the steady state data.

Even though our $k_{on}$ values for NADH do not correlate well with steady state results, the calculated equilibrium dissociation constants, $K_{eq} = k_{off}/k_{on}$ for NADH binding compare well with the reported binding constants (20) (Table III).

The largest discrepancies between equilibrium-binding rate constants calculated from our rate data and the directly measured values occur at basic pH = 9.5 to 10.0. Since we see reduced fluorescence enhancement of NADH at these pH values (an observation also reported in the equilibrium-binding experiments) and since apoenzyme also shows no fluorescence enhancement on NADH binding, we have investigated the hypothesis that LADH loses Zn$^{2+}$ at basic pH. This would not be surprising from a chemical standpoint since OH$^-$ competes effectively for Zn$^{2+}$ in a variety of other Zn$^{2+}$ ligand systems at pH > 9.0. Indeed, our atomic absorption studies confirm that zinc ion is lost at pH > 10; therefore, great care must be taken in handling LADH at these basic pH values if valid data are to be collected.

The directly measured NAD$^+$ association rate constants (Fig. 4) are in much better agreement with rates calculated from steady state kinetic data (Table IV, Fig. 8) than are NADH association rates. The absolute rate constants and the observed $pK_a$ for our directly measured data are within experimental error of the values calculated from steady state kinetic measurements. Over the pH range we are able to study the agreement between the measured $K_a$ for NAD$^+$ binding (Table III), and the values calculated from our rate data is good. This is consistent with a single step binding mechanism.

The most striking feature of the binding association rate constants is the pH dependencies for both NAD$^+$ and NADH are similar (Figs. 4, 6). This indicates that the ionization of the same enzyme group is responsible for the pH dependence of these processes. On the other hand the pH dependencies of the dissociation rate constants for NAD$^+$ and NADH are very different (Figs. 2, 5), and the pH dependence for the rate of NADH dissociation does not appear to be controlled by ionization of a single group. The dissociation rate constant for NAD$^+$ appears to reflect ionization of a group with $pK_a = 8.0$.

There are two possibilities for the assignment of the ionizing group controlling nucleotide association: (a) Zn$-$OH as previously suggested (2), or (b) a basic group necessary for nucleotide binding such as arginine 47 or lysine 228 (both of which have been implicated in nucleotide binding (1, 25).

In order to test the first hypothesis we have investigated the pH dependence of the rate of phenanthroline binding (Fig. 7). The $pK_a$ for this process is 8.1 ± 0.1 with rates being slower at basic pH values. Since x-ray crystallography has shown that phenanthroline displaces the water molecule bound to zinc, the rate of formation of the phenanthroline complex with LADH should be pH-dependent with displacement of Zn$-$OH$^+$ being slower than displacement of Zn$-$OH$_2^{2+}$. Our data at each pH were consistent with a bimolecular process for formation of the complex. This would indicate either an SN$_2$ mechanism for complexation at Zn$^{2+}$ or a unimolecular dissociation of water and OH$^-$ which is much faster than complexation of phenanthroline. Since we see the profound pH effect noted above and OH$^-$ should dissociate more slowly than H$_2$O$_2$, we favor the SN$_2$ process. In any case the $pK_a$ of Zn$-$OH = 9.4 to 9.6 would appear to be 8.1 on the basis of our rate experiments.

Further confirmation of the hypothesis that Zn$-$OH controls the rate of association of phenanthroline with liver alcohol dehydrogenase is that there is no pH dependence of phenanthroline dissociation. Since x-ray crystallography indicates that phenanthroline displaces water from Zn$^{2+}$, the lack of pH dependence for dissociation of phenanthroline is consistent with the assignment of Zn$-$OH$^-$ as the ionic species causing the pH dependence of the rate constant for phenanthroline association.

Another interesting conclusion is that the group controlling the association of nucleotides to the enzymes is not the same group controlling the association of phenanthroline. Therefore, it seems unlikely that Zn$-$OH is responsible for the pH dependence of nucleotide binding. This conclusion has interesting consequences concerning the catalytic mechanism. One of the proposals for catalysis by liver alcohol dehydrogenase involves protonation of aldehyde by Zn$-$O (Scheme 3); however, there is no pH dependence of aldehyde reduction which would be required on passing through the form of the enzyme containing Zn$-$OH but not to Zn$-$

\[
\text{H} \quad \text{H} \\
\text{Zn} \quad \text{O} \\
\text{H} \quad \text{H}
\]

\[
\text{pK}_a = 10.
\]

However, if NADH bound only to that
OH⁻, the NADH-binding constant but not the rate of aldehyde reduction would be pH-dependent. Our experiments strongly suggest that if the water molecule bound to zinc is the acid catalytic group, NADH binding would not affect the ionization state of Zn—OH. Since Zn—OH does not control NADH binding. Furthermore since our experiments seem to indicate that the pKₐ of Zn—OH is 8.1, and our previous experiments covered the pH range 6 to 10, it seems unlikely that Zn—OH is the acid catalyst for aldehyde reduction.

In conclusion our studies indicate: (a) that the pKₐ of Zn—OH in liver alcohol dehydrogenase is 8.1; (b) that both NAD⁺ and NADH show the same pH rate profile for the association rate constant (pKₐ = 9.5); (c) that the dissociation rate of E—NAD⁺ complex depends on a group with pKₐ = 8.1 (it has previously been suggested that NAD⁺ perturbs the pKₐ of 9.5 for E—NAD⁺ association to this latter value); (d) that dissociation of E—NADH complex shows a complicated pH rate profile; (e) that enzyme loses Zn⁺⁺ at pH > 10.0 making studies at pH > 10.0 suspect. These data impose constraints on any model for catalysis since the pKₐ for ionization of Zn—OH falls within the pH range of previous experiments on pH dependence of aldehyde reduction. This in turn makes it less likely that Zn—OH is acting as an acid catalyst for hydride transfer to the aldehyde carbonyl group.

Acknowledgments — We would like to thank Dr. P. L. Luisi, Dr. K. L. Watters, Dr. D. H. Petering, and Dr. D. T. Minkel for helpful discussions.

REFERENCES

1. Branden, C.-I., Jornvall, H., Eklund, H., and Furugren, G. (1975) in The Enzymes (Boyer, P., ed) p. 133, Academic Press, New York
2. Taniguchi, S., Theorell, H., and Åkeson, A. (1967) Acta Chem. Scand. 21, 1903
3. Theorell, H., and Chance, B. (1951) Acta Chem. Scand. 5, 1127
4. Wratten, C. C., and Cleland, W. W. (1967) Biochemistry 2, 935
5. Wratten, C. C., and Cleland, W. W. (1965) Biochemistry 4, 2442
6. Dalziel, K. (1965) J. Biol. Chem. 238, 2850-2858
7. Abeles, R. H., Hutton, R. F., and Westheimer, F. (1957) J. Am. Chem. Soc. 79, 712
8. Dunn, M. F., and Hutchison, J. S. (1973) Biochemistry 12, 4882
9. Dunn, M. F., Biellmann, J.-F., and Branlant, G. (1975) Biochemistry 14, 3176
10. McFarland, J. T., and Chu, Y.-H. (1975) Biochemistry 14, 1140
11. Sloan, D., Young, M., and Midvan, A. S. (1975) Biochemistry 14, 1988
12. Dunn, M. F. (1974) Biochemistry 13, 1146
13. Shore, J. D., Gutfreund, H., Brooks, R. L., Santiago, D., and Santiago, P. (1974) Biochemistry 13, 4185
14. Bernhard, S. A., Dunn, M. F., Luisi, P. L., and Schack, P. (1970) Biochemistry 9, 227
15. McFarland, J. T., and Bernhard, S. A. (1972) Biochemistry 11, 1486
16. Theorell, H., and Yonetani, T. (1963) Biochim. Biophys. Acta 75, 237
17. Geraci, G., and Gibson, Q. H. (1967) J. Biol. Chem. 242, 4275
18. Wilkins, R. G. (1974) in The Study of Kinetics and Mechanism of Reactions of Transition Metal Complexes, p. 17, Allyn and Bacon, Inc., Rockleigh, N. J.
19. Iwebo, I., and Weiner, H. (1975) J. Biol. Chem. 250, 5967-5971
20. Harada, K., and Wolfe, R. G. (1968) as cited in Bernhard, S. A., and Seydoux, J. (1974) Crit. Rev. Biochem. 227
21. Shore, J. D., and McKinley-McKee, J. S. (1961) Acta Chem. Scand. 15, 2
22. Gunnarsson, P. O., and Petterson, C. (1974) FEBS Lett. 44, 289
23. Hoagstrom, C. W., Iwebo, I., and Weiner, H. (1969) J. Biol. Chem. 244, 5967-5971
24. Harada, K., and Wolfe, R. G. (1968) as cited in Bernhard, S. A., and Seydoux, J. (1974) Crit. Rev. Biochem. 227
25. Lange, L. G., Riordan, J. F., and Vallee, B. L. (1974) Biochemistry 13, 4861
Liver alcohol dehydrogenase-coenzyme reaction rates.
M C DeTraglia, J Schmidt, M F Dunn and J T McFarland

J. Biol. Chem. 1977, 252:3493-3500.

Access the most updated version of this article at http://www.jbc.org/content/252/10/3493.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/10/3493.citation.full.html#ref-list-1