Investigation of the pH Dependencies of Coenzyme Binding to Liver Alcohol Dehydrogenase Lacking Zinc Ion at the Active Sites†

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ABSTRACT: The absorbance and fluorescence properties of horse liver alcohol dehydrogenase (LADH) from which the active site zinc ions have been specifically removed are shown to be in many respects similar to those of the native enzyme. The kinetics of coenzyme binding studied by stopped-flow, rapid-mixing fluorescence techniques establish that NAD^+ association and dissociation and NADH association are pH-dependent processes for this apoenzyme derivative. Comparisons with the data for the native enzyme [Kvassman, J., & Pettersson, G. (1979) *Eur. J. Biochem. 100*, 115–123] indicate the apparent pK_a values which govern coenzyme association and dissociation to apoenzyme and to native enzyme exhibit interesting similarities. The association rates for NADH binding to apoenzymes and native enzymes are dependent upon ionization processes with apparent pK_a values of 8.8 and 9.2, respectively. The maximum (pH-independent) rate constants are 4.5×10^6 M^{-1} s^{-1} (apoenzyme) and 1.2×10^7 M^{-1} s^{-1} (reported by Kvassman and Pettersson for the native enzyme). The rate of NADH dissociation from the apoenzyme ($\sim 2 \times 10^{-3}$ s^{-1}, pH 6–10) is considerably slower than the value reported for the native enzyme (5 s^{-1}). Due to the combination of relatively rapid binding and dissociation rates and a weak affinity, we were unable to determine the kinetics of NAD^+ binding to apoenzyme below pH 8.5 by stopped-flow rapid-mixing methods. In the region above pH 8.5, the pH dependencies of the association and dissociation rates for NAD^+ binding to the apoenzyme appear qualitatively similar to the behavior of the native enzyme. While it is recognized that in the apoenzyme one of the freed zinc ligands could become a surrogate for the zinc-bound water in the native enzyme, these findings bring into question the previous assignment of the apparent pK_a values which control coenzyme binding to the ionization of a zinc-coordinated water molecule.

The preparation of crystalline liver alcohol dehydrogenase specifically lacking any metal ion at the active sites makes possible new approaches to the investigation of the roles played by zinc ion in the catalytic mechanism. This zinc-deficient enzyme (apoenzyme)[1] may be converted back to native enzyme in high yield by diffusion of zinc ion into the crystals; alternatively site-specific metal ion substitutions are possible by addition of the appropriate metal ion. In this way, the Co(II)-, Ni(II)-, Cd(II)-, Cu(II)-, and Fe(II)-enzyme derivatives[1] have been prepared (Maret et al., 1979; Dietrich et al., 1979; Andersson, 1980; Dietrich, 1980), and detailed mechanistic studies of some of these derivatives have been initiated (Dunn et al., 1982; Koerber et al., 1983; M. F. Dunn, A. K. H. MacGibbon, S. C. Koerber, H. Dietrich, and M. Zeppezauer, unpublished results).

Since this apoenzyme lacks only the active site zinc ion and is reconstitutable, the properties of this derivative vis-à-vis native enzyme are potentially of interest. The pH dependencies of coenzyme binding to the native enzyme have been subjected to careful study (Kvassman & Pettersson, 1979). They found the apparent rate constants for the association of both NAD^+ and NADH decreased with increasing pH. Analysis of the pH dependencies for both compounds indicates that the rate of association is dependent upon the state of ionization of a single group with apparent $pK_a = 9.2$. The rate of dissociation of NAD^+ was found to depend on a group with apparent $pK_a = 7.6$, and the rate of NADH dissociation was found to be essentially independent of pH between pH 4 and pH 10. Recent work (Andersson et al., 1981) indicates that the dissociation of NADH depends on a group with apparent $pK_a = 11.2$. Kvassman & Pettersson (1979) propose that these pH dependencies all involve the ionization of a single group on the enyzme surface, the water molecule coordinated to the active site zinc ion. It is proposed that the pK_a of the coordinated water molecule is perturbed from 9.2 in the native enzyme to apparent values of 7.6 and 11.2 in the NAD^+ and NADH binary complexes, respectively.

In view of the above proposed role for the zinc-bound water molecule, we anticipated that an investigation of the coenzyme binding properties of the apoenzyme could provide new insight into the roles played by zinc ion and the coordinated water molecule in the structure and catalytic function of LADH. As will be shown, the pH dependencies of coenzyme binding to the apoenzyme are remarkably similar to some of the above described properties of the native enzyme.

Experimental Procedures

Materials. Buffer solutions were prepared from crystalline salts with doubly distilled water. 2-[[Tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid (Tes)[1] analytical grade was obtained from Serva, Heidelberg. The buffers *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) (Pipes), and *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes) were obtained

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[1] Abbreviations: E, LADH, horse liver alcohol dehydrogenase; apoenzyme or apoE, enzyme lacking metal ion at the active sites; Co(II)E, Zn(II)E, Cd(II)E, and Ni(II)E, specific active site substituted alcohol dehydrogenase species; O or NAD^+ and R or NADH, respectively oxidized and reduced nicotinamide adenine dinucleotides; DACA, *trans*-4-(dimethylamino)cinnamaldehyde; pyr, pyrazole; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; H_2NADH, reduced 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid.

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from Sigma Chemical Co. or Calbiochem Corp.

The phosphate and pyrophosphate salts were obtained from Mallinckrodt Chemical Co. Other buffer salts and 2,6-dipicolinic acid were from Sigma Chemical Co. As chloride ion may interfere with coenzyme binding and dissociation steps (Sund & Theorell, 1963; Shore & Gutfreund, 1970), the buffer solutions were either made up by using the acid form of the salt and adjusting to the required pH with sodium hydroxide or, in the case of the pyrophosphate buffer, adjusted to the required pH with phosphoric acid. The coenzymes NADH and NAD^+ were obtained from Sigma Chemical Co. or Boehringer Mannheim as the highest purity grades. Pyrazole (Aldrich) was purified by vacuum sublimation prior to use.

Preparation of Apoenzyme. The apoenzyme was prepared from crystalline native enzyme by the method of Maret et al. (1979). The catalytic zinc atoms were removed from crystals grown in 2-methyl-2-propanol (25% v/v) in 0.05 M Tes buffer at pH 6.9 by exhaustive dialysis against the same buffer containing 2,6-dipicolinic acid. The resultant enzyme species, which is depeleted of zinc at the active site but still retains zinc at the noncatalytic site, will henceforth be termed the apoenzyme. Prior to use, the crystalline apoenzyme was exhaustively dialyzed against changes of 25 mM Tes buffer, pH 7.0, containing 20% (v/v) 2-methyl-2-propanol to remove the 2,6-dipicolinic acid ($\epsilon_{270} = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$). The crystals were centrifuged and redissolved in 0.15 M Tes buffer at pH 7.0 overnight. The solution was recentrifuged, and the resultant supernatant of apoenzyme stock was used in the reported experiments. All preparation, storage, and manipulation of enzyme solutions were carried out under a N_2 atmosphere. The apoenzyme was used within 3 days of dissolution.

Analysis of the Apoenzyme. Metal analyses were performed on a Perkin-Elmer 400 atomic absorption spectrophotometer as described by Maret et al. (1979). According to these metal analyses, the apoenzyme contained 1.9–2.0 mol of Zn^{2+}/80 000-dalton molecule. The apoenzyme used in the fluorometric studies, when assayed by the method of Dalziel (1957), was found to retain 0.2–to 0.3% activity (with respect to the same concentration of native enzyme). The residual activity increased from 0.3% to 2% within 8 days. This could reflect a migration of noncatalytic zinc (about 2%) to the catalytic zinc, or zinc contamination. The increase could also be explained by denaturation of about 2% of the apoenzyme and uptake of the zinc released from denatured apoenzyme. The apoenzyme in the NADH binding studies had 1.8–3.3% residual activity. Apoenzyme used in the NAD^+ binding studies retained 5% activity.

Assay of the Apoenzyme. The protein concentration was determined spectrophotometrically by using a value of $A_{280} = 0.45\ mg^{-1}\ cm^2$ for apoenzyme and a value of $A_{280} = 0.455\ mg^{-1}\ cm^2$ for the native enzyme (Maret et al., 1979; Sund & Theorell, 1963). The concentration of coenzyme binding sites in an apoenzyme sample was determined fluorometrically by NADH titration (see Results). Generally the concentration of coenzyme binding sites was 90–98% of that predicted by the protein concentration.

Instrumentation. Unless otherwise, stated, a Hitachi Perkin-Elmer MPF 44 spectrofluorometer was used in the determination of fluorescence spectra. A Farrand Model M-II spectrofluorometer was used for the routine titration of coenzyme binding sites. Transient kinetic studies were carried out and analyzed on a computerized Durrum-Gibson Model D-110 stopped-flow spectrophotometer equipped with a Kel-F flow system and 2-cm excitation light path. The characteristics of the computer system and data analysis program have been described previously (Dunn et al., 1979). For very slow reactions the changes were recorded on a strip chart recorder attached to the amplified signal from the stopped-flow apparatus. Prior to use the instrument was flushed with 1 mM EDTA followed by distilled water to minimize metal ion contamination. When the fluorescence mode was used, the light from a 75-W xenon lamp was passed through the prism monochromator and the fluorescence was detected at 90° to the exciting light through the appropriate filters. Protein fluorescence was carried out by exciting at 285 nm (15-nm bandwidth) and detecting through Schott Glass filters UG-5 and WG-320 which together have a maximum transmission at about 350 nm. Fluorescence of NADH bound to the apoenzyme was studied by exciting at 325 nm (8-nm bandwidth) and detecting through Schott Glass UG-5 and WG-360 filters (combined maximum transmission at 372 nm). The fluorescence of NADH bound to native enzyme was excited at 328 nm (24-nm bandwidth) and detected through Schott Glass BG24 and GG395 filters (maximum transmission 420 nm).

Coenzyme Binding Experiments. The effect of pH on the coenzyme association rates was studied via a "pH-jump" technique in which apoenzyme in dilute Tes buffer (pH 7.15) was mixed in the stopped-flow apparatus with coenzyme premixed in concentrated buffer (0.05 M after mixing). The pH after mixing was determined by measuring the pH of a 1:1 mixture of the two buffers. The apoenzyme solution was prepared by dilution of concentrated stocks (200–800 μN) containing 0.15 M Tes buffer (pH 7.15), giving, after mixing, a Tes buffer concentration of $\leq$3.15 mM. Unless otherwise stated, concentrations of apoenzyme in the kinetic experiments refer to the concentration of coenzyme binding sites after mixing, and all other concentrations reported refer to concentrations after mixing. In all these experiments the coenzyme was in sufficient excess over apoenzyme to ensure that the kinetic processes are pseudo first order with respect to the concentration of coenzyme. The superscripts R and O^1 in the reaction parameters refer to the reduced and oxidized forms of the coenzyme, respectively.

Results

Spectroscopic Studies. The absorption spectrum of the apoenzyme (data not shown) is remarkably similar to that for the native enzyme (Theorell & Yonetani, 1963; Abdallah et al., 1978), exhibiting shoulders at 298.5, 270, 266, 260, and 253, nm; however, the maximum is shifted from 280 (for the native enzyme) to 277 nm. The protein fluorescence spectrum (data not shown) is also very similar to the spectrum reported by Laws & Shore (1978) for the native enzyme. The apparent emission maximum depends on the wavelength of excitation (data not shown); when the fluorescence intensities are arbitrarily normalized at 330 nm, excitation at 280 nm exhibits a longer wavelength maxima than does excitation at 300 nm. The spectra also exhibit the lower wavelength shoulders described for the native enyzme.

The effect of apoenzyme on the fluorescence of NADH is shown in Figure 1A. The NADH fluorescence is enhanced and blue shifted, indicating that coenzyme binds to the apoenzyme. The emission λ_{max} for bound NADH is 415–416 nm, and the enhancement factor at 410 nm is $Q_{410} = 5.2$. Comparison with the zinc enzyme (Figure 1B) shows that fluorescence enhancement on binding NADH to apoenzyme is not as large as for the native enzyme ($Q_{410} = 13.5$) and the maximum is blue shifted about 10 nm. While the fluorescence of NADH bound to native zinc enyzme is enhanced 2.9-fold by the formation of a ternary complex with isobutyramide,

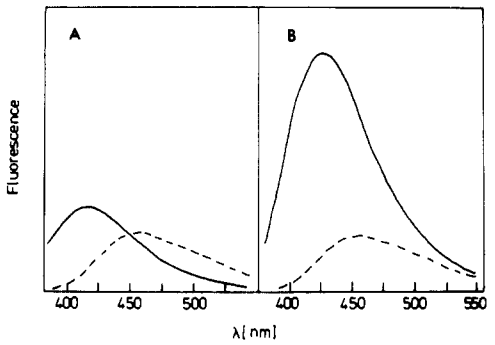

FIGURE 1: Comparison of the enhancement of NADH fluorescence upon binding to (A) apoenzyme and (B) native zinc enzyme. (A) The fluorescence emission spectrum of 3.9 μM NADH in 215 mM Tes buffer, pH 7.0, before (---) and after (—) the addition of 19 μN apoenzyme. (B) The fluorescence spectrum of 3.9 μM NADH before (---) and after (—) the addition of 19.4 μN native zinc enzyme. The fluorescence spectra are uncorrected. Instrument settings: λ_{ex} 330 nm; excitation slit width 6 nm; emission slit width 4 nm.

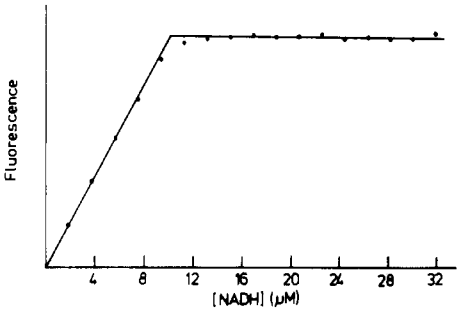

FIGURE 2: Titration of coenzyme binding sites with NADH. The excitation wavelength was 330 nm (6 nm slit), and the emission wavelength was 390 nm (4 nm slit). NADH was titrated into a solution of 10.3 μN apoenzyme in 25 mM Tes buffer, pH 7.0. The equivalence point corresponds to 10.1 μM NADH.

10 mM isobutyramide had no effect on the fluorescence of apoenzyme-bound NADH (data not shown). Thus while the properties of the two enzyme forms are similar, they are not identical.

The enhancement of NADH fluorescence on binding to the apoenzyme was used in Figure 2 to titrate the NADH binding sites. As a significant signal could be obtained at 390 nm from the fluorescence of bound NADH with minimal contribution from free NADH, the fluorescence change was monitored at this wavelength. The titration shows that 98% of the apoenzyme protein molecules (as determined from extinction coefficient) contain high-affinity NADH binding sites. This procedure was routinely used to determine the concentration of apoenzyme capable of binding NADH. The excellent agreement between total protein and NADH binding sites testifies to the homogeneous and distinctive nature of the apoenzyme. NADH is also able to quench the apoenzyme protein fluorescence (Figures 3 and 4). When monitored at 325 nm, the fluorescence is quenched 66% on excitation at 280 nm and 73% on excitation at 295 nm. The extent of quenching is virtually identical with that of the native enzyme–NADH binary complex. The shift in wavelength of the fluorescence maximum due to NADH quenching and the increased extent of quenching on excitation at higher wavelength (295 nm) is similar to the behavior of the native enzyme (Abdallah et al., 1978). While both the "red" and "blue" tryptophans are involved in NADH quenching, the blue tryptophan is quenched to a larger extent.

The quenching of apoenzyme fluorescence by NAD^+ is more difficult to determine because upon addition of NAD^+, traces of NADH are formed in a slow, "blank" reaction. This blank

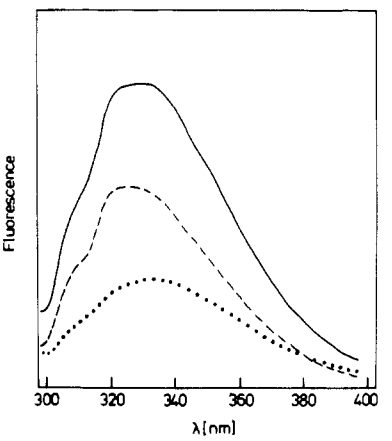

FIGURE 3: Quenching of the apoenzyme fluorescence with NADH. (—) The fluorescence emission spectrum of 5 μN apoenzyme in 25 mM Tes, pH 7.0; (···) the spectrum after addition of 15 μM NADH. The difference between these two spectra is shown as the dashed line. The maximum difference occurs at 325 nm. Instrument settings: λ_{ex} 280 nm; excitation slit 5 nm; emission slit 2 nm.

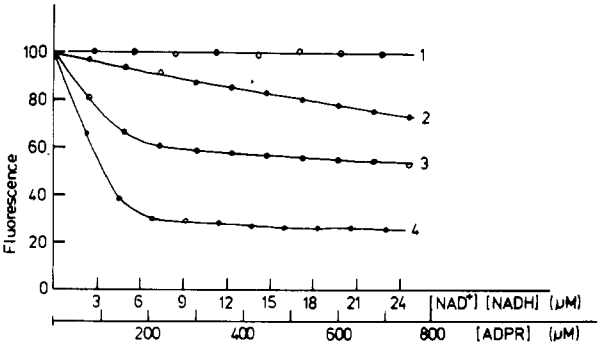

FIGURE 4: Quenching of apoenzyme fluorescence. Apoenzyme, 5.3 μM in 25 mM Tes buffer (pH 8.0), was excited at 295 nm, and fluorescence was measured at 325 nm in the presence of various coenzymes and analogues. Traces: (1) ADP-ribose; (2) NAD^+; (3) NAD^+ in the presence of 10 mM pyrazole; (4) NADH.

reaction is presumably catalyzed by the small, contaminating amount of native zinc enyzme. Fluorescence spectra show that the NADH produced became apoenyzme bound and thus contributes to the observed quenching. The extent of quenching for NAD^+ is approximately 40% (Figure 4, trace 2). In the presence of pyrazole, NAD^+ binds more tightly to the apoenzyme, and the blank reaction is negligible (Figure 4, trace 3). The fact that pyrazole binds in the absence of zinc at the active site is reasonable if, as Theorell & Yonetani (1963) have suggested, one of the nitrogens of pyrazole is covalently bound to the C_4 of the nicotinamide ring. The recent X-ray structure of the complex formed between pyrazole and NAD^+ at the active site of the native zinc enzyme is consistent with this bonding arrangement (Eklund et al., 1982). On the addition of pyrazole, the apoenzyme–NAD^+ complex shows an increase in absorbance in the range 290–300 nm (as found for the native enzyme). Thus the dissociation constant was determined by adding pyrazole (0.17–10 mM) to a solution containing apoenzyme (30 μM) and NAD^+ (3 mM) and observing the change at 300 nm. This procedure gave a dissociation constant for pyrazole and the apoenzyme–NAD^+ complex of 7.8×10^{-4} M at pH 7.4 in 0.05 M Tricine buffer. This dissociation constant is much weaker than the 10^{-7} M value reported for the native enzyme under similar conditions by Theorell & Yonetani (1963). The apoenzyme–NAD^+ complex binds and forms a covalent adduct with at least one other nucleophile, the *Z* isomer of *trans*-4-(dimethylamino)-cinnamaldoxime (H. Dietrich et al., unpublished results). As

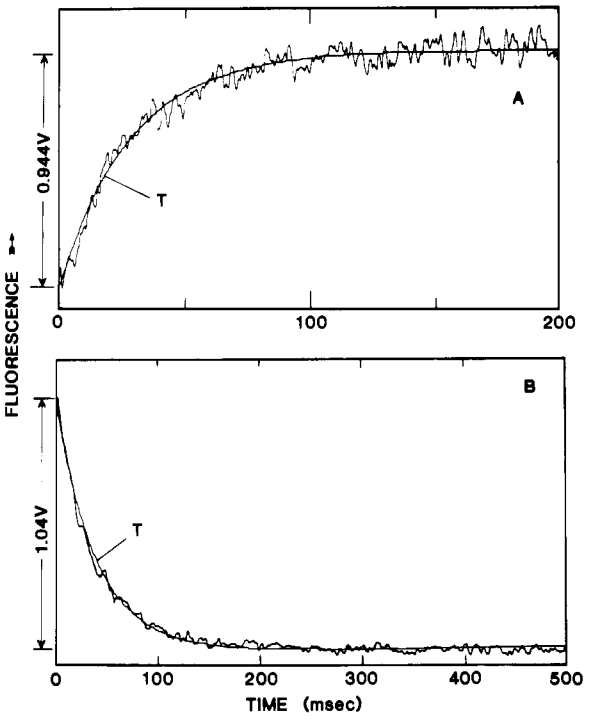

FIGURE 5: Representative stopped-flow rapid-mixing time courses for the association of NADH (A) and NAD^+ (B) with the apoenzyme. (A) Enhancement of NADH fluorescence on binding to apoenzyme. Conditions after mixing: [apoenzyme] = 5 μN; [EDTA] = 10 μM; [NADH] = 25 μM; final pH 9.1. Apoenzyme and EDTA were preincubated prior to mixing with NADH. Under the assumption that the time course is a single exponential, the observed trace is overlaid with the computer-generated, best-fit, theoretical time course, T, with k_{app} = 34 s^{-1}. (B) Quenching of apoenzyme fluorescence on mixing with NAD^+. Conditions after mixing: [apoenzyme] = 9.6 μN; [NAD^+] = 100 μM; final pH 9.1. See Experimental Procedures for the instrument conditions. Under the assumption that the time course is a single exponential, the observed trace is overlaid with the computer-generated, best-fit theoretical time course, T, with k_{app} = 23.6 s^{-1}.

was reported by Theorell & Tatemoto (1971) for the native enzyme, ADP-ribose does not quench the apoenzyme fluorescence (Figure 4, trace 1).

Kinetics of NADH Binding. Fluorescence stopped-flow kinetics were used to determine the rate constant for dissociation of NADH from the apoenzyme (k_{off}^R). In studying the dissociation, it was found that the obvious displacing agent, NAD^+, produced no release of NADH because NADH binds rather tightly to the apoenzyme. Hence instead the rate constant was determined in the presence of excess native enzyme and isobutyramide by following the increase in the fluorescence at 420 nm due to the binding of NADH to native enzyme. Competition for the NADH resulted in NADH dissociation from the apoenzyme and formation of the very tight ternary complex with the native enzyme and isobutyramide. Sund & Theorell (1963) have reported an equilibrium constant for NADH dissociation from the ternary complex of 5×10^{-9} M for the native enzyme. The fluorescence of NADH bound to native enzyme is enhanced 2-fold by formation of the ternary complex with isobutyramide, while we have shown above that isobutyramide does not alter NADH binding to the apoenzyme. In the stopped-flow instrument, apoenzyme (3.7 μN) and a small excess of NADH were mixed with native enzyme (15 μN) and isobutyramide (5 mM) in pH 6.3 sodium cacodylate buffer (50 mM). The resultant slow fluorescence increase (data not shown) was monitored by a strip chart recorder, and graphical analysis of the trace yielded a rate constant of about 2×10^{-3} s^{-1}. Thus the dissociation of NADH

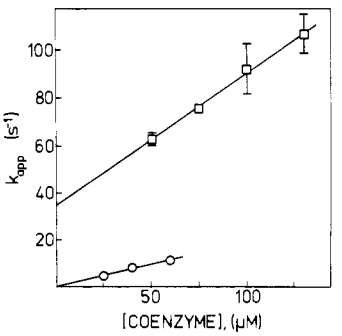

FIGURE 6: Dependence of k_{app} on coenzyme concentration at 25.0 ± 0.5 °C. NAD^+ data (□): 10 μN apoenzyme was mixed with NAD^+ at pH 8.2. The least-squares analysis gave k_{off}^O = 35 ± 7 s^{-1} and k_{on}^O = (5.6 ± 0.8) × 10^5 M^{-1} s^{-1} (see eq 4). NADH data (○): 5 μN apoenzyme was mixed with NADH at pH 10.3. Least-squares analysis gave k_{on}^R = (1.9 ± 0.2) × 10^5 M^{-1} s^{-1}. Each data point represents the mean value determined from at least three stopped-flow kinetic traces (typical time courses are shown in Figure 5).

from the apoenzyme–NADH complex is very much slower than the 5-s^{-1} rate constant found for the native enzyme (Shore & Gutfreund, 1970).

The NADH association rate constant (k_{on}^R) of Scheme I was studied by following the increase in the fluorescence of NADH bound to apoenzyme (Figures 5A and 6). Due to the small fluorescence enhancement on binding (Figure 1A), high apoenzyme concentrations (5 μN) were required, and EDTA (10 μM) was added to ensure no metal was scavenged during the experiment. To be sure that the observed fluorescence changes were due to the apoenzyme and not to the small native enzyme impurity, the kinetics of these two species were compared. At pH 10.3, the observed rate for the association of NADH (25 μM) to apoenzyme was 4.8 s^{-1}, yet a sample of native enzyme at a concentration equal to the impurity in the apoenzyme (0.17 μN, 3.3%) gave no detectable fluorescence change. At a much higher native enzyme concentration (1.2 μN) the observed rate (25 s^{-1}) agreed with the value calculated from the data of Kvassman & Pettersson (1979) and could not be confused with the apoenzyme rate. Thus the observed fluorescence change is indeed attributable to the apoenzyme and not native enzyme impurities. The detection filters used (maximum transmission at 372 nm) produced similar molar fluorescence responses for the apoenzyme and the native enzyme.

Scheme I

$$\text{apoE} + \text{NADH} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{apoE(NADH)}$$

The study of NADH association to apoenzyme was limited by the necessity to maintain an NADH concentration that was high enough to ensure the kinetics were pseudo first order with respect to the concentration of NADH. Yet because high concentrations of NADH diminished the excitation light intensity (via inner-filter effects) and the observed rates were very fast, the kinetics of NADH association could only be observed over a narrow range of concentrations (Figure 6). At all pH conditions investigated, the apparent rate of formation of the apoE(NADH) complex of Scheme I was found to be a single, pseudo-first-order process described by the relationship of eq 1. Since the NADH dissociation constant

$$k_{app}^R = k_{off}^R + k_{on}^R[\text{NADH}] \quad (1)$$

(k_{off}^R) is very slow (viz., Figure 6) and hence may be neglected, eq 1 may be rewritten as eq 2. Above pH 9 the observed rates

$$k_{on}^R = k_{app}^R/[\text{NADH}] \quad (2)$$

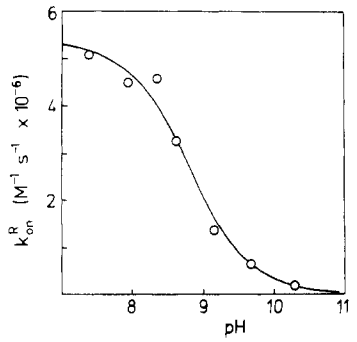

FIGURE 7: Effect of pH on the rate of NADH association. The theoretical dissociation curve (solid line) is derived from the best-fit parameters $pK_1^R = 8.8$ and $k_1^R = 5.4 \times 10^6$ M^{-1} s^{-1} (calculated from eq 3).

were relatively slow, and a series of NADH concentrations could be used to determine k_{on}^R (Figure 6). However, at lower pH values the rates were relatively fast, and so measurements were made at a single NADH concentration (25 μM). Figure 7 shows the effect of pH on the NADH association rate constant calculated from eq 2. The data can be described by a single apparent ionization constant with a dependency of the form given by

$$k_{on}^R = (k_1^R[H^+])/(K_1^R + [H^+]) \quad (3)$$

where K_1^R and k_1^R are the ionization constant and the maximum value of k_{on}^R at low pH, respectively. The values obtained from a plot of k_{on}^R vs. $k_{on}^R/[H^+]$ (Jencks, 1969) were (apparent) $pK_1^R = 8.8$ and $k_1^R = 5.4 \times 10^6$ M^{-1} s^{-1}. The buffers used to cover the pH range were Tricine, glycine and carbonate (see Appendix).

Kinetics of NAD$^+$ Binding. The NAD$^+$ binding characteristics were studied by following the quenching of protein fluorescence in the stopped-flow fluorometer (Figure 5B). The NAD$^+$ blank reaction, wherein NAD$^+$ is slowly converted to NADH in the presence of LADH (Sund & Theorell, 1963), was not a problem as the binding of NAD$^+$ is a relatively rapid process. Typical conditions employed 10 μN apoE, 10 μM EDTA, and 50–500 μM NAD$^+$. Figure 5B shows a time course for the quenching of the apoenzyme fluorescence due to NAD$^+$ binding. As judged by the overlaid theoretical time course generated from best-fit values of the computer analysis, the decay time courses are well described by a single, pseudo-first-order process. At a given pH, the rate of fluorescence decay was measured as a function of NAD$^+$ concentration (Figure 6), and the specific rate constants were determined from the relationship

$$k_{app}^O = k_{off}^O + k_{on}^O[NAD^+] \quad (4)$$

From this experiment the association and dissociation rate constants cannot be determined independently but, rather, must be determined from the slope and intercepts of a plot of k_{app} vs. [NAD$^+$] (viz., Figure 6). This presented experimental problems at low pH; the k_{app} values were very fast (100–200 s^{-1}), and the contribution by the k_{off} term was large, thus making accurate determinations difficult. A high apoenzyme concentration (i.e., 10 μM) was required to observe a satisfactory fluorescence signal, while the NAD$^+$ concentrations were restricted to a range which at the low values still maintained the pseudo-first-order relationship and at the high values did not completely quench the signal. Parts A and B of Figure 8 show the pH dependencies of the calculated association and dissociation rate constants. Though the data are scattered, the plots demonstrate that both the association and dissociation rate constants are extremely pH dependent.

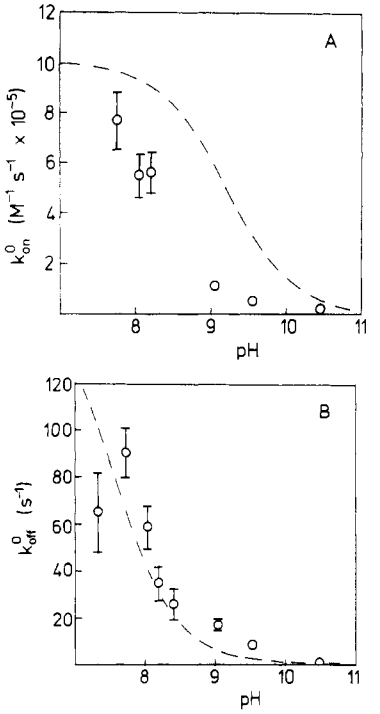

FIGURE 8: Effect of pH on the binding of NAD$^+$ to apoE. (A) Plot of the association rate constant (k_{on}^O) with respect to pH. Note that the dashed curve represents the theoretical best-fit pH dependence of k_{on}^O for the native enzyme (Kvassman & Pettersson, 1979), assuming the maximum association rate constant = 1×10^6 M^{-1} s^{-1} and $pK_a = 9.2$. (B) Plot of dissociation rate constant (k_{off}^O) with respect to pH. The dashed curve represents the theoretical, best-fit pH dependence of k_{off}^O for the native enzyme (Kvassman & Pettersson, 1979), assuming the maximum dissociation rate constant = 160 s^{-1} and $pK_a = 7.6$. The error bars in both plots indicate the standard deviation in the parameters of eq 4.

Comparison with the native enzyme shows distinct similarities in the shapes of these dependencies. Note that the buffers used for the apoenzyme study were Tricine, glycine, pyrophosphate, and carbonate (see Appendix).

Discussion and Conclusions

In contrast to previously described apo-LADH derivatives which lacked zinc ion at both the active sites and the structural sites (Coleman et al., 1972), the apoenzyme species described in this study lacks zinc ion only at the active sites and is fully reconstituted to native enzyme by the addition of zinc ion (Maret et al., 1979; Dietrich et al., 1979). The preparation of this apoenzyme species has made possible the active site specific replacement of zinc ion by other divalent metal ions, e.g., Co(II), Ni(II), Cd(II), Cu(II), and Fe(II) (Maret et al., 1979; Dietrich et al., 1979; Dunn et al., 1982; Koerber et al., 1983; M. Gerber, H. Dietrich, M. A. Abdallah, M. Zeppezauer, J.-F. Biellmann, S. C. Koerber, A. K. H. MacGibbon, and M. F. Dunn, unpublished results).

The fluorescence spectra of the apoenzyme and of the binary NAD$^+$ and NADH complexes (Figures 1–4) indicate the native enzyme and the apoenzyme have similar, but not identical, properties: both NAD$^+$ and NADH quench the intrinsic fluorescence of these two proteins; the fluorescence of bound NADH is blue shifted and enhanced; both proteins form tightly bound ternary complexes with NAD$^+$ and pyrazole. The differences between the two proteins are just as striking: the apoenzyme is catalytically inactive; isobutyramide does not enhance the fluorescence of apoenzyme-bound NADH; the fluorescence of bound NADH is not as blue shifted or as enhanced in the apoenzyme complex; NADH binds more tightly to apoenzyme than to native enzyme; in

Table I: Comparison of Kinetic Parameters for the Binding of NADH to Native Enzyme[a] and Apoenzyme

	apoenzyme	native enzyme[a]
k_1^R (M^{-1} s^{-1})	5.4×10^6	1.2×10^7
pK_1^R	8.8	9.2
k_{-1}^R (s^{-1})[d]	2×10^{-3} [c]	5[b]

[a] Data taken from Kvassman & Pettersson (1979). [b] The value for k_{-1}^R is virtually pH independent over the pH range 6–10. [c] Measured at pH 6.3 in sodium cacodylate buffer. [d] For pH-independent data, $k_{-1}^R = k_{off}^R$.

the presence of pyrazole, NAD^+ is bound less tightly to apoenzyme than to native enzyme. Consequently it is clear from these differences that in addition to functioning as a Lewis acid in catalysis (Dunn & Hutchison, 1973; Dunn et al., 1982), zinc ion influences the binding of both NAD^+ and NADH in heretofore unsuspected ways.

The pH dependencies of coenzyme binding to native enzyme have been carefully investigated by Kvassman & Pettersson (1979). They interpret the pH dependencies as resulting from the effects of a single, ionizable group with apparent pK_a = 9.2 in the native enzyme. It is proposed that both NADH and NAD^+ combine with the protonated form of the enzyme; ionization of this group interferes with binding; the pK_a of this group is perturbed to 7.6 in the binary E(NAD^+) complex and to 11.2 in the E(NADH) complex (Anderson et al., 1981). Kvassman & Pettersson (1979), Dworschack & Plapp (1977), and Dunn (1975, 1974) have suggested that the ionizable group involved in regulating the pH dependencies of coenzyme binding is the water molecule coordinated to the active site zinc ion. Consequently, we were surprised to find that the pH dependencies of the coenzyme association and dissociation processes are somewhat similar for the native enzyme and apoenzyme.

The data obtained for NADH binding to the apoenzyme (Figure 7 and Table I) are remarkably similar to those of the native enzyme. Not only are the apparent pK_a values quite close (8.8 and 9.2, respectively) but also are the magnitudes of the maximum second-order rate constants for NADH association (k_1^R) for the two enzyme forms. However, the removal of the active site zinc significantly lowers the dissociation rate constant of NADH, whereas the dissociation rate of NAD^+ appears to be only slightly affected. This illustrates the very different interactions which must be involved in the binary NADH and NAD^+ complexes. The NAD^+ binding data could not be expressed in quantitative terms, but comparisons with the native enzyme (Figure 8) show that the pH dependencies are qualitatively similar. Unfortunately we were unable to determine whether or not native enzyme and apoenzyme exhibit the same perturbation in the apparent pK_a upon binding NAD^+.

The mechanistic implications of these similarities are of considerable interest with respect to the mechanism of coenzyme binding to LADH. The pH dependencies which characterize coenzyme binding to the apoenzyme could arise from the ionization of one or more of those protein residues coordinated to zinc ion in the native enzyme (i.e., Cys-46, His-67, and Cys-174). Alternatively, it may be that the observed pH dependencies of coenzyme binding for both native enzyme and apoenzyme arise from pH-dependent protein conformation states with different affinities for NAD^+ and NADH and that the zinc-coordinated water plays no role in regulating coenzyme binding.

For both the native enzyme and the apoenzyme, the pH dependencies of the coenzyme association rates tend to be zero at high pH (viz., Figures 7 and 8), clearly implying that coenzyme binding is not just inhibited or slowed by the pH transition but in effect is forbidden. Any model for the pH dependence of coenzyme binding must incorporate this observation. Since the binding of anionic ligands [i.e., AMP, ADP-ribose, $Pt(CN)_4^{2-}$, chloride ion, and perhaps phosphate ion] is similarly regulated by the ionization of a group (or groups) presumed to be identical with that of the group (or groups) which regulate coenzyme binding, it appears that the affinity of the site for all these ligands is regulated by the same pH-dependent enzyme conformation change. Andersson et al. (1980) have speculated that the conformation change involves motion of the side-chain group of Arg-47. They propose that association of the guanidinium ion of Arg-47 with the zinc-bound hydroxyl group (at high pH) excludes ligand binding to the anionic site. However, it seems unlikely to us that this motion alone could completely exclude the binding of a molecule such as NADH with numerous other points of attachment. Whatever the nature of the detailed changes which account for the pH dependencies of the binding of coenzymes and anions, the similarities between the characteristics of native enzyme and apoenzyme bring into question the central role proposed by Andersson et al. (1980) for the zinc-coordinated water molecule. While it is recognized that a freed cysteine cysteine sulfhydryl group in the apoenzyme could become a surrogate for the zinc-bound water in the proposed interaction with Arg-47, it should be noted that this same thiol then must also affect the anion binding site to regulate coenzyme binding in the same manner as the zinc hydroxyl in the native enzyme and hence must be involved in the same set of ionic interactions. Determination of the plausibility of this explanation must await further experimentation.

If ionization of the zinc-bound water is not critical to the pH characteristics of ligand binding to alcohol dehydrogenase, then the possible involvement of alternative ionizable groups must be considered. The recently refined X-ray structure for the ternary complex formed between enzyme, H_2NADH, and *trans*-4-(dimethylamino)cinnamaldehyde (Cedergren-Zeppezauer et al., 1982) shows a new set of hydrogen-bonding interactions between the coenzyme analogue and ionizable residues at the site. These new interactions (which are also seen in ternary complexes with NADH) include an apparent hydrogen bond between the ϵ-ammonium ion of Lys-228 and the 2′-hydroxyl of the adenosine ribosyl moiety and an apparent hydrogen bond between the imidazolyl moiety of His-51 and the 2′-hydroxyl group of the nicotinamide ribosyl hydroxyl of the coenzyme. These interactions are not observed in the structure of the binary enyzme–Br–ADPR complex. It seems reasonable a priori to expect that the ionizations of Lys-228 and His-51 would alter both the protein conformation and electrostatic interactions with the coenzyme and therefore would influence the affinity of the enzyme for coenzyme. The work of Dworschack & Plapp (1977) very clearly shows that chemical modification of the ϵ-NH_2 group of Lys-228 drastically alters the affinity of the zinc enzyme for coenzyme.

We conclude that the microscopic events which are manifest in the pH dependencies of coenzyme binding are likely to be just as complex as the set of events which give rise to the Bohr effect in hemoglobin (Perutz, 1970). These events very likely include the ionizations of Lys-228 and His-51 coupled to a conformational transition which drastically alters the affinities of the coenzyme site for NAD^+ and NADH.

Appendix

Phosphate Inhibition. While the rate of association of

Table II: Variation of the Observed Rate of NADH Binding to Native Liver Alcohol Dehydrogenase as a Function of Ionic Strength and Buffer Ion at pH 7.4 and 25.0 °C

buffer	concentration (M)	ionic strength	observed rate (s^{-1})
phosphate	0.05	0.13	40 ± 1
cacodylate	0.01	0.009	57 ± 7
cacodylate	0.3	0.28	67 ± 4

NADH to the apoenzyme was being investigated, it was found that the rate constant at pH 7.4 in 0.05 M sodium phosphate was lower than that observed at pH 9 in 0.05 M sodium pyrophosphate buffer. Since glycine buffer gave observed rates similar to those measured in sodium pyrophosphate buffer, a comparison was made with a series of different buffers (all 0.05 M) at pH 7.4 under the conditions described under Results to investigate the origins of these effects. At 25 μM NADH, the association rate was found to be 30 s^{-1} for sodium phosphate buffer, while the other buffers studied showed substantially higher rates: Tricine (122 ± 15 s^{-1}), Tes (116 ± 9 s^{-1}), and Pipes (106 ± 10 s^{-1}). Obviously the phosphate buffer system behaves differently from the other buffers, reducing the binding rate under these conditions by a factor of 3–4. Similarly, the NAD^+ association rate constant at pH 7.4 was determined to be 1.2×10^5 M^{-1} s^{-1} in 0.05 M sodium phosphate buffer, whereas a value of at least 5×10^5 M^{-1} s^{-1} was found in 0.05 M Tricine buffer. The NAD^+ dissociation rate constant remained essentially unchanged.

This inhibitory effect of phosphate buffer is not restricted to the apoenzyme, but rather appears to be a general phenomenon of horse liver alcohol dehydrogenase. By use of the procedure of Kvassman & Pettersson (1979) in which the NADH association rate constant of the native enzyme is studied by the use of the chromophoric aldehyde *trans*-4-(dimethylamino)cinnamaldehyde (DACA), the observed rate for NADH association of pH 7.4 using 1 μM NADH and 100 μM DACA was found to be 40 ± 1 s^{-1} in 0.05 M sodium phosphate buffer and 80 ± 2 s^{-1} in 0.05 M Tricine buffer. These rates are equivalent to second-order rate constants of 8×10^6 M^{-1} s^{-1} and 1.6×10^m M^{-1} s^{-1}, respectively. That this difference is not due solely to ionic strength effects was shown by repeating the experiment in 0.01 and 0.3 M sodium cacodylate buffer. Table II shows that the large variation in ionic strength of these cacodylate buffers produces a much smaller effect than does substitution of phosphate buffer for cacodylate buffer of the same pH and ionic strength. The NAD^+ association rate constant of native enzyme was studied with a similar procedure. Subsequent to NAD^+ binding, the following rapid sequence of steps occur: propanol binding, reaction, and propanal release after which the chromophoric probe DACA is observed to bind to the E(NADH) complex (Kvassman & Pettersson, 1979). When enzyme (1 μN) was mixed with NAD^+ (10 μN), propanol (6 mM), and DACA (100 μM) in 0.05 M buffer, pH 7.4, the observed rate in Tricine was found to be 18 ± 3 s^{-1} (equivalent to a second-order rate constant of 1.8×10^6 M^{-1} s^{-1}), whereas in 0.05 M phosphate buffer, the observed rate was found to be 8.4 s^{-1} (equivalent to a second-order rate constant of 0.8×10^6 M^{-1} s^{-1}).

These results attest to the inhibitory effect of phosphate ion on the association rates of coenzymes to alcohol dehydrogenase. Since this is not an ionic strength effect, the decreased rates in phosphate buffer presumably result from a specific binding of phosphate ion to the enzyme and could involve the anion binding site in the coenzyme binding domain discussed by Brändén et al. (1975). This view has also been forwarded by Dahl & McKinley-McKee (1980), who found that phosphate ion protected alcohol dehydrogenase against the two affinity labels iodoacetic acid and bromoimidazolylpropionic acid. Consequently, the inhibitory effects of phosphate were taken into consideration when the buffers were chosen for the herein described study of coenzyme binding to the apoenzyme.

Registry No. NADH, 58-68-4; NAD, 53-84-9; alcohol dehydrogenase, 9031-72-5; zinc, 7440-66-6.

References

Abdallah, M. A., Biellmann, J.-F., Wiget, P., Joppich-Kuhn, R., & Luisi, P. L. (1978) *Eur. J. Biochem. 89*, 397–405.

Andersson, I. (1980) Dissertation, Universität des Saarlandes, Saarbrücken, West Germany.

Andersson, P., Kvassman, J., Lindström, A. Oldén, B., & Pettersson, G. (1980) *Eur. J. Biochem. 108*, 303–312.

Andersson, P., Kvassman, J., Lindström, A., Oldén, B., & Pettersson, G. (1981) *Eur. J. Biochem. 113*, 425–433.

Brändén, C.-I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 2*, 103–190.

Cedergren-Zeppezauer, E., Samama, J.-P., & Eklund, H. (1982) *Biochemistry 21*, 4895–4908.

Coleman, P. L., Iweibo, I., & Weiner, H. (1972) *Biochemistry 11*, 1010–1018.

Dahl, K. H., & McKinley-McKee, J. S. (1980) *Eur. J. Biochem. 103*, 47–51.

Dalziel, K. (1957) *Acta Chem. Scand. 11*, 397–398.

Dietrich, H. (1980) Dissertation, Universität, des Saarlandes, Saarbrücken, West Germany.

Dietrich, H., Maret, W., Wallén, L., & Zeppezauer, M. (1979) *Eur. J. Biochem. 100*, 267–270.

Dunn, M. F. (1974) *Biochemistry 13*, 1146–1151.

Dunn, M. F. (1975) *Struct. Bonding (Berlin) 23*, 61–122.

Dunn, M. F., & Hutchison, S. J. (1973) *Biochemistry 12*, 4882–4892.

Dunn, M. F., Bernhard, S. A., Anderson, D., Copeland, A., Morris, R. G., & Rogue, J.-P. (1979) *Biochemistry 18*, 2346–2354.

Dunn, M. F., Dietrich, H., MacGibbon, A. K. H., Koerber, S. C., & Zeppezauer, M. (1982) *Biochemistry 21*, 354–363.

Dworschack, R. T., & Plapp, B. V. (1977) *Biochemistry 16*, 2716–2725.

Eklund, H., Samama, J.-P., & Wallén, L. (1982) *Biochemistry 21*, 4858–4866.

Jencks, W. P. (1969) in *Catalysis in Chemistry and Enzymology*, pp 577–585, McGraw-Hill, New York.

Koerber, S. C., MacGibbon, A. K. H., Dietrich, H., Zeppezauer, M., & Dunn, M. F. (1983) *Biochemistry* (preceding paper in this issue).

Kvassman, J., & Pettersson, G. (1979) *Eur. J. Biochem. 100*, 115–123.

Laws, W. R., & Shore, J. D. (1978) *J. Biol. Chem. 253*, 8593–8597.

Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, R., & Zeppezauer, M. (1979) *Eur. J. Biochem. 98*, 501–512.

Perutz, M. F. (1970) *Nature (London) 228*, 734–739.

Shore, J. D., & Gutfreund, H. (1970) *Biochemistry 9*, 4655–4659.

Sund, H., & Theorell, H. (1963) *Enzymes, 2nd Ed. 7*, 25–83.

Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553.

Theorell, H., & Tatemoto, K. (1971) *Arch. Biochem. Biophys. 142*, 69–82.