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Studies of the pH Dependence of the Formation of Binary and Ternary Complexes with Liver Alcohol Dehydrogenase†

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ABSTRACT: The association of the coenzyme NAD^+ to liver alcohol dehydrogenase (LADH) is known to be pH dependent, with the binding being linked to the shift in the pK of some group on the protein from a value of 9–10, in the free enzyme, to 7.5–8 in the LADH–NAD^+ binary complex. We have further characterized the nature of this linkage between NAD^+ binding and proton dissociation by studying the pH dependence (pH range 6–10) of the proton release, Δn, and enthalpy change, $\Delta H°$(app), for formation of both binary (LADH–NAD^+) and ternary (LADH–NAD^+–I, where I is pyrazole or trifluoroethanol) complexes. The pH dependence of both Δn and $\Delta H°$(app) is found to be consistent with linkage to a single acid dissociating group, whose pK is perturbed from 9.5 to 8.0 upon NAD^+ binding and is further perturbed to ~6.0 upon ternary complex formation. The apparent enthalpy change for NAD^+ binding is endothermic between pH 7 and pH 10, with a maximum at pH 8.5–9.0. The pH dependence of the $\Delta H°$(app) for both binary and ternary complex formation is consistent with a heat of protonation of −7.5 kcal/mol for the coupled acid dissociating group. The intrinsic enthalpy changes for NAD^+ binding and NAD^+ plus pyrazole binding to LADH are determined to be ~0 and −11.0 kcal/mol, respectively. Enthalpy change data are also presented for the binding of the NAD^+ analogues adenosine 5′-diphosphoribose and 3-acetylpyridine adenine dinucleotide.

Horse liver alcohol dehydrogenase (LADH)[1] is an enzyme whose catalytic and structural properties have been much studied (Brändén et al., 1975; Eklund & Brändén, 1983). There appears to be no cooperative interaction between the two active sites of this dimeric protein (Anderson & Dahlquist, 1982). For each individual active site there is positive heterotropic cooperativity between the binding of coenzyme and substrate (or substrate analogue/inhibitor), as evidenced by the ordered bireactant kinetics of the enzyme (Sund & Theorell, 1963), as well as by direct, equilibrium studies of

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[1] Abbreviations: ADP-ribose or ADPR, adenosine 5′-diphosphoribose; $APAD^+$, 3-acetylpyridine adenine dinucleotide; LADH, horse liver alcohol dehydrogenase; NAD^+, oxidized β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; TFE, trifluoroethanol.

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ligand binding to the enzyme (Dahlbom et al., 1974; Theorell & McKinley-McKee, 1961; Theorell & Yonetani, 1963; Anderson & Dahlquist, 1982). Crystallographic studies with LADH and its various binary and ternary complexes indicate that a conformational change is induced by coenzyme binding (Brändén et al., 1975; Brändén & Eklund, 1978; Eklund et al., 1982a,b, 1984). This conformational change includes a rearrangement of the protein's peptide backbone near the coenzyme binding site, resulting in a closing of the active site.

Besides the coupling between coenzyme and substrate/inhibitor binding, there is apparently also a linkage between coenzyme binding and the acid dissociation of some functional group on the enzyme. For example, the binding of nicotinamide adenine dinucleotide (NAD^+) to LADH appears to be controlled by a group having an acid dissociation pK of about 9 in the free enzyme. The pK of this group is decreased by 1.5 to 2 pH units in the binary LADH–NAD^+ complex (Kvassman & Pettersson, 1979, 1980; Shore et al., 1974; Theorell & McKinley-McKee, 1961).

In this paper we report systematic studies of the pH dependence of the free energy change, $\Delta G°$, enthalpy change, $\Delta H°$, and proton release, Δn, for the formation of binary complexes between LADH and NAD^+, as well as other coenzyme analogues. Also we report studies of the pH dependence of $\Delta H°$ and Δn for the formation of ternary complexes with the specific inhibitors pyrazole and trifluoroethanol (TFE). The goal of these studies is (a) to further characterize the nature of the coupling between coenzyme and substrate analogue binding, as well as the coupling between coenzyme binding and the protonic equilibrium, and (b) to obtain intrinsic thermodynamic information regarding coenzyme and substrate analogue binding equilibria.

Experimental Procedures

Materials. Lyophilized horse liver alcohol dehydrogenase was obtained from Sigma Chemical Co. The percent active protein of the sample batches received ranged from 40% to 86%, as determined by the enzymatic assay and formula of Dalziel (1957). The lyophilized protein was heat treated as described elsewhere (Eftink & Selvidge, 1982). The resulting samples possessed higher percent active enzyme (70–100%) and were not as prone to aggregation as the initial lyophilized protein samples. The molar concentration of LADH was determined spectroscopically with a molar extinction coefficient of 3.74×10^4 M^{-1} cm^{-1} at 280 nm. In our microcalorimetric and proton release determinations the concentration of active enzyme was used (i.e., the enzyme concentration times the percent active protein, as determined by the assay of Dalziel (1957)). On a few occasions the concentration of active enzyme was determined by fluorometric titration with NADH in the presence of isobutyramide (Theorell & McKinley-McKee, 1961). In such cases, the concentrations of active enzyme were found to agree within 5% of values determined by the assay of Dalziel (1957). After the heat treatment, the protein was dialyzed overnight at 4 °C vs. the buffer to be used.

NAD^+ was obtained as a lithium salt and as a free acid from Calbiochem-Behring and Sigma Chemical Co., respectively. The lithium salt was used in microcalorimetric and fluorescence studies; the free acid was used in enzymatic assays and proton release studies. Adenosine 5′-diphosphoribose (ADP-ribose), 3-acetylpyridine adenine dinucleotide ($APAD^+$), NADH, pyrazole, and TFE were obtained from Sigma Chemical Co. LADH was also obtained as a crystallized suspension in 10% ethanol from Calbiochem-Behring. These protein samples were dialyzed exhaustively vs. the buffer to be used in order to remove ethanol. Such samples consistently showed near 100% active protein by the assay of Dalziel.

Methods. (*A*) *Batch Microcalorimetry.* Approximately 1.5–2.0 mL of protein solution (active-site concentration of $\sim 1 \times 10^{-4}$ M) and approximately 3.0 mL of ligand solution were added to separate compartments in an LKB batch microcalorimeter. The protein and ligand solutions were prepared in identical buffers and the pH of the solutions was matched to within 0.01 pH unit. To the reference side of the calorimeter was added ligand and buffer solutions in order to eliminate the heat of dilution of the ligand solution. After 2–4 h of thermal equilibration of the samples (experiments done at 25 °C) the protein and ligand solutions were mixed by rotation. The resulting heat burst signals were recorded on a strip chart and integrated by cutting and weighing the paper under the peak. The concentration of the ligand was usually more than 10 times greater than the reciprocal of its association constant, K_L(app). From the observed heat signal, V, the moles of active LADH subunits, m_p, the free ligand concentration, [L] (which was taken as being the total ligand concentration since this was always in great excess of the active-site concentration), and a calibration constant, ϵ, the apparent, uncorrected molar enthalpy change, $\Delta H°'$(app), was determined as $\Delta H°'(\text{app}) = \epsilon V(1 + [L]K_L(\text{app}))/(m_p[L]K_L(\text{app}))$. By this equation the $\Delta H°'$(app) are corrected for the fractional degree of saturation of the binding sites by ligand. The $\Delta H°'$(app) values are further corrected for contributions due to proton transfer to the buffer species by the relationship $\Delta H°(\text{app}) = \Delta H°'(\text{app}) - \Delta n \Delta H°_{\text{buffer}}$, where $\Delta H°_{\text{buffer}}$ is the heat of protonation of the buffer, Δn is the proton release per subunit upon ligand binding (protons which are transfered to buffer), and $\Delta H°$(app) is the apparent, corrected enthalpy change (per subunit) for ligand binding. As will be discussed under Results, $\Delta H°$(app) may be pH dependent due to contribution (when $\Delta n \neq 0$) from the heat of proton dissociation from a functional group on the protein (see Scheme I and eq 1). Buffer solutions used and their $\Delta H°_{\text{buffer}}$ were as follows: pH 6–8, a mixture of 0.04 M NaH_2PO_4 and 0.0132 M Na_2HPO_4, $\mu = 0.1$, $\Delta H°_{\text{buffer}} = -1.0$ kcal/mol; pH 8–9.3, 0.01 M $Na_4P_2O_7$ buffer, $\mu = 0.1$, $\Delta H°_{\text{buffer}} = -1.2$ kcal/mol; pH 9.3–10, mixture of 0.04 M $NaHCO_3$ and 0.0132 M Na_2CO_3, $\mu = 0.1$, $\Delta H°_{\text{buffer}} = -0.9$ kcal/mol. The Δn values used to determine $\Delta H°$(app) were taken from independent proton release measurement, as described below.

(*B*) *Fluorometric Binding Studies.* The pH dependence of the binding of NAD^+ and ADP-ribose was studied by the competitive displacement of bound NADH with the fluorescence method of Theorell and McKinley-McKee (1961). A Perkin-Elmer MPF-44 spectrophotofluorometer, with its cell holder thermoregulated at 25 °C, was used. Aliquots of a NADH solution were added by means of a Gilmont precision syringe to the protein and buffer solutions in separate fuorescence cells. The relative fluorescence of NADH in the presence and absence of LADH was then measured with excitation and emission wavelengths of 330 and 410 nm, respectively. The LADH solutions were then titrated with NADH in the presence of the competitive ligands, NAD^+ or ADP-ribose, in order to determine the association constant of the latter (the concentration of the competitive ligands was chosen to be approximately equal to the reciprocal of their association constant). The buffers used for these studies were the same as those used in the microcalorimetric studies.

(*C*) *Proton Release Measurements.* The proton release, Δn, from LADH upon adding ligands was measured with a Brinkman titrator, equipped with a stirred, temperature-

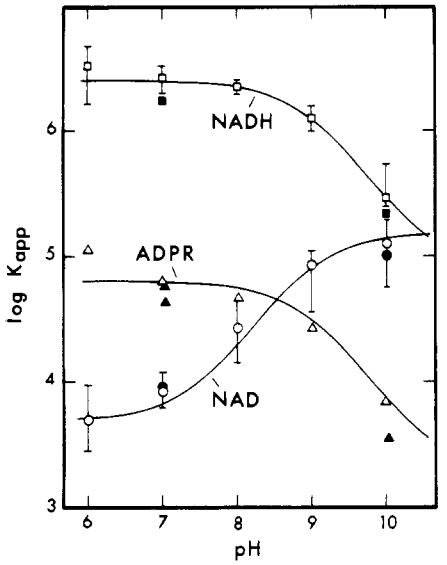


FIGURE 1: pH dependence of the apparent association constants for NADH (□, ■), ADP-ribose (△, ▲), and NAD^+ (○, ●) binding to LADH. The open symbols for NADH and NAD^+ are average values (and the vertical bars indicate the range of values) taken from the following literature sources: for NADH, Theorell and Winer (1959), Kvassman and Pettersson (1979), Winer and Theorell (1960), Theorell and McKinley-McKee (1961), and DeTaglia et al. (1977); for NAD^+, the above five references plus Taniguchi et al. (1967), Dalziel (1963), and Theorell et al. (1955); for ADP-ribose , Yonetani (1963a,b). All log K(app) values are for nearly identical conditions of 0.1 M ionic strength and 23–25 °C. The closed symbols for each ligand represent values determined in the present study using the method of competitive displacement of NADH (see Methods). The solid lines through the data are fits of eq 1 as follows: for NAD^+, $K_{L,H} = 4.5 \times 10^3$ M^{-1}, $pK_H = 9.0$, and $pK_{H,L} = 7.5$; for ADP-ribose, $K_{L,H} = 6.3 \times 10^4$ M^{-1}, $pK_H = 9.0$, and $pK_{H,L}$ 10.5; for NADH, $K_{L,H} = 2.52 \times 10^6$ M^{-1}, $pK_H = 9.0$, and $pK_{H,L} = 10.5$.

jacketed (25 °C) reaction vessel. A 5-mL sample of protein ($\sim 1 \times 10^{-4}$ M in active sites), prepared in a 0.1 M NaCl solution containing about 0.001 M sodium phosphate, was introduced into the reaction vessel. Nitrogen gas was passed over the solution and the pH was adjusted to the desired value. Aliquots (5–10 μL) of a concentrated ligand solution were then added. A NaOH solution (7.5×10^{-3} M) or HCl solution (1×10^{-2} M) was then added by means of the titrator until the initial pH was reestablished. Control experiments, in which ligand was added to the salt solution in the absence of protein, were also performed. From the volume of base (or acid) added and the concentration of active LADH subunits, the value of Δn was determined. The pH of the concentrated ligand solutions (1 M pyrazole or 0.1 M NAD^+) was adjusted to be near the experimental pH before mixing. TFE was added as a pure liquid.

(D) Zinc Determinations. The amount of zinc per protein subunit was determined with a Varian Model 1200 atomic absorption spectrophotometer. The enzyme was prepared in 12.5% trichloroacetic acid to a concentration of about 5×10^{-5} M subunit.

RESULTS

LADH–Coenzyme Association Constants. In Figure 1 is shown a compendium of data from the literature for the pH dependence of the apparent association constant for the binding of NAD^+, NADH, and ADP-ribose to LADH. The open symbols in this figure are averaged association constant values obtained from the references listed in the legend of Figure 1. The closed symbols are values we have determined with the fluorescence titration method of Theorell and McKinley-McKee (1961). The affinity of LADH for NAD^+ is greatest

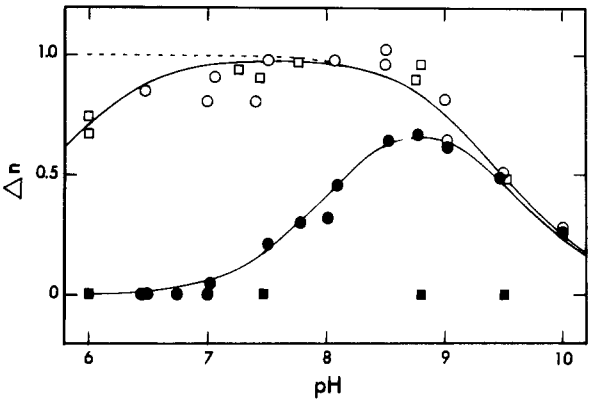
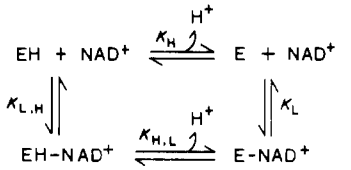


FIGURE 2: pH dependence of the proton release, Δn, for the binding of NAD^+ (●) to LADH and the formation of the LADH–NAD^+–pyrazole (□) and LADH–NAD^+–TFE (○) ternary complexes. The solid lines are fits of eq 2: for the NAD^+ binary complex, $pK_H = 9.5$ and $pK_{H,L} = 8.0$; for the ternary complex, $pK_H = 9.5$ and $pK_{H,L} = 6.0$ (the dashed line is for $pK_{H,L} = 0$). Also shown are Δn values for adding pyrazole (■) to LADH alone. Conditions: 25 °C, 0.1 M NaCl.

Scheme I



at high pH, and the pH dependence of NAD^+ binding can be described by a thermodynamic scheme (see Scheme I). In Scheme I the ligand, NAD^+, is able to interact wiht either the protonated (EH) or unprotonated (E) form of the protein. Accordingly, eq 1 describes the pH dependence of the apparent association constant for NAD^+.

$$K_L(\text{app}) = \frac{K_{L,H}(1 + K_{H,L}/[H^+])}{(1 + K_H/[H^+])} \quad (1)$$

The solid line in Figure 1 is a fit of eq 1 to the data for NAD^+ binding to LADH. The fit is with $K_{L,H} = 4.5 \times 10^3$ M^{-1}, $K_L = 1.58 \times 10^5$ M^{-1}, $pK_H = 9.0$, and $pK_{H,L} = 7.5$.

The coenzyme fragment, ADP-ribose, shows a decreased affinity for the protein at high pH (see Figure 1), similar to the case for NADH (Yonetani, 1963a,b). The solid line through the ADP-ribose data is a fit to Scheme I with $K_{L,H} = 6.3 \times 10^4$ M^{-1}, $K_L = 2.0 \times 10^3$ M^{-1}, $pK_H = 9.0$, and $pK_{H,L} = 10.5$ (i.e., stronger binding to the protonated form of the enzyme). Also shown is a similar fit for the pH dependence of the binding of NADH to LADH. We note that Andersson et al., (1981a) have presented evidence that a second ionization above pH 10 also affects NAD^+ and NADH binding. Even if this is true, the above fits are adequate for the pH range studied here (6–10).

Proton Release Data. On mixing NAD^+ with LADH a proton release is observed with reaches a maximum of 0.7 (mol of H^+)/(mol of protein subunit) at pH ~8.7. Shown in Figure 2 are proton release data obtained for NAD^+ binding between pH 6–10. According to Scheme I the proton release upon NAD^+ binding should follow eq 2. Shore et al. (1974) have

$$\Delta n = \frac{[H^+]}{K_H + [H^+]} - \frac{[H^+]}{K_{H,L} + [H^+]} \quad (2)$$

previously reported proton release data for NAD^+ binding in the pH range 5.5–8.5. Our data are in general agreement with their work and shows the complete bell-shaped pattern, ex-

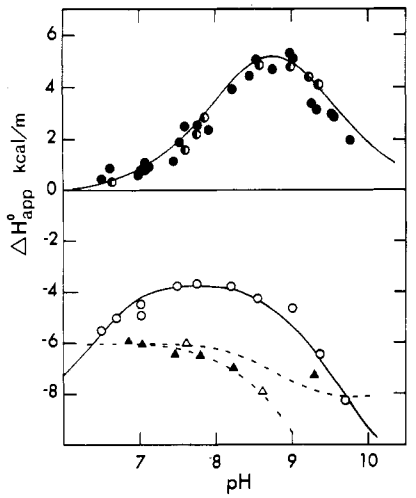


FIGURE 3: (Top) pH dependence of the apparent enthalpy change, ΔH°(app), for NAD^+ (●) binding and $APAD^+$ (◐) binding to LADH. The solid line is a fit of eq 3 with $\Delta H^\circ_L = \Delta H^\circ_{L,H} = -0.1$ kcal/mol. $\Delta H^\circ_H = -7.5$ kcal/mol, $pK_H = 9.5$, and $pK_{H,L} = 8.0$. (Bottom) pH dependence of the ΔH°(app) for ADP-ribose (▲, △) binding to LADH and for forming the LADH–NAD^+–pyrazole ternary complex (○). The solid line through the LADH–NAD^+–pyrazole data is for $\Delta H^\circ_L = \Delta H^\circ_{L,H} = -11.0$ kcal/mol, $\Delta H^\circ_H = 7.5$ kcal/mol, $pK_H = 9.5$, and $pK_{H,L}$ 6.0. For the ADP-ribose data, the open triangles represent data points for Subramanian and Ross (1979) and the dashed lines are two possible fits with $\Delta H^\circ_L = \Delta H^\circ_{L,H} = -6.0$ kcal/mol, $pK_H = 9.0$, $pK_{H,L} = 10.5$, and $\Delta H^\circ_H = 3.0$ kcal/mol (top) and $\Delta H^\circ_H = -7.5$ kcal/mol (bottom).

tending to pH 10, as expected from the above equation. The solid line through the NAD^+ binding data in Figure 2 is for a fit of eq 2 for $pH_H = 9.5$ and $pK_{H,L} = 8.0$. These pK values are similar to the values used in the above fit of the pH dependence of the NAD^+ association constant. Together the data in Figure 1 and 2 and the fits obtained strongly argue that the binding of NAD^+ is coupled to the downward shift of the pK of a *single* ionizable group on the protein. (Andersson et al. (1981a) have presented evidence that a second ionizing group with a pK of 10.4 affects NAD^+ binding as well. In the pH range we have studied the existence of such a group will have little effect on our data, and due to the instability of LADH above pH 10, we were unable to extend our studies to higher pH.)

The proton release resulting from the formation of the ternary complexes LADH–NAD^+–pyrazole and LADH–NAD^+–TFE was also studied. As shown in Figure 2 the formation of these complexes releases ~1.0 (mol of H^+)/(mol of protein subunit) in the pH range 7–9. The broad plateau in the Δn plot is consistent with a shift in the pK of the coupled/ionizing group from 9.5 on the free enzyme to ≤6 in the ternary complex. The pattern for both the pyrazole and TFE ternary complexes is indistinguishable.

The addition to LADH of pyrazole alone, up to a concentration of 0.02 M, does not produce a proton uptake or release between pH 6 and pH 9.5.

Enthalpy Change Measurements. The pH dependence of the apparent standard enthalpy change, ΔH°(app), for the association of NAD^+ to LADH is presented in the top portion of Figure 3. A bell-shaped pattern, similar to that for the pH dependence of Δn, is observed. The binding is endothermic over the pH range 6.5–10 and shows a maximum of 4 kcal/mol (of subunit) at pH 8.5–9.0. The ΔH°(app) values plotted in Figure 3 have been corrected for the heat of proton transfer to the buffer species, using the Δn values as determined in Figure 2. Above pH 10 very large exothermic microcalorimetric signals were found for NAD^+ binding to LADH. It is known that LADH loses Zn^{2+} above pH 10 (DeTraglia et al., 1977; Andersson et al., 1981a). Also LADH slowly loses activity, presumably due to loss of Zn^{+2}, below pH 6.0 (Vallee & Hoch, 1957). We have limited our report to the pH range 6.5–10.0, where the protein is stable during the time needed for the microcalorimetric measurements. The solid line through the ΔH°(app) data is a fit to eq 3, which derives from

$$\Delta H^\circ(\text{app}) = \frac{\Delta H^\circ_{L,H}[H^+]}{K_H + [H^+]} - \frac{(\Delta H^\circ_L + \Delta H^\circ_H - \Delta H^\circ_{H,L})[H^+]}{K_{H,L} + [H^+]} \quad (3)$$

Scheme I. This fit is obtained with $pK_H = 9.5$, $pK_{H,L} = 8.0$, $\Delta H^\circ_L = \Delta H^\circ_{L,H} = -0.1$ kcal/mol, and ΔH°_H (heat of protonation of the ionizing group on the free enzyme) of −7.5 kcal/mol.

The ΔH°(app) for the binding of $APAD^+$, a coenzyme analogue, is found to have a similar pH dependence, as shown in Figure 3. The fitting parameters are found to be virtually identical with those for NAD^+. Shore and Gilleland (1970) have previously reported that the association constant for $APAD^+$ to LADH is the same as that for NAD^+ at pH 7.0.

In contrast, the ΔH°(app) for ADP-ribose binding is found to be exothermic, with a value that varies only slightly from −6 to −8 kcal/mol as the pH increases from 6.5 to 10 (see the lower portion of Figure 3). These ΔH°(app) values have also have corrected for the proton uptake upon ADP-ribose binding (Andersson et al., 1980). In view of the pH dependence of the apparent association constant and Δn for ADP-ribose binding, eq 3 was fitted to the ΔH°(app) data with $pK_H = 9.0$ and $pK_{H,L} = 10.5$ (i.e., ADP-ribose binds better to the state of the protein having the controling group protonated). Two possible fits are shown in the figure; one for $\Delta H^\circ_H = \Delta H^\circ_{H,L} = -3.0$ kcal/mol and the other for $\Delta H^\circ_H = \Delta H^\circ_{H,L} = -7.5$ kcal/mol. A value of $\Delta H^\circ_{L,H} = -6.0$ kcal/mol is used in each fit. The poor quality of the data at high pH does not enable a good fit (and hence a determination of ΔH°_H) to be made, but it seems possible that the group whose pK is increased upon ADP-ribose binding may be the same as the group whose pK is decreased upon NAD^+ binding.

The ΔH°(app) for forming the ternary LADH–NAD^+ pyrazole complex is also exothermic, as shown in the lower portion of Figure 3. A plateau in the pH dependence is seen between pH 7.5 and 8.5; above and below this range, the ΔH°(app) is more exothermic. With eq 1 a fit is obtained with $pK_H = 9.5$, $pK_{H,L} = 6.0$, $\Delta H^\circ_L = \Delta H^\circ_{L,H} = -11$ kcal/mol, and $\Delta H^\circ_H = -7.5$ kcal/mol.

Zinc Analyses. Thermodynamic Studies with LADH from Different Sources. The ΔH°(app) for NAD^+ binding to LADH near neutral pH has previously been measured calorimetrically by Schmid et al. (1978) and by Subramanian and Ross (1979). Schmid et al., and Subramanian and Ross reported ΔH°(app) of −0.9 and −2.1 kcal/mol, respectively, for NAD^+ binding at pH 7.0. In contrast, our ΔH°(app) values are between 0.5 and 1 kcal/mol at pH 7. Also, Subramanian and Ross reported exothermic ΔH°(app) values below pH 7, whereas our values are near zero below this pH (our values agree with those of Subramanian and Ross above pH 8). Schmid et al. used crystallized LADH, Subramanian and Ross used lyophilized LADH (Sigma Chemical Co.), and we have used lyophilized LADH that was heat treated before use to remove inactive protein. To determine if the difference in protein samples can account for these discrepancies, we measured the ΔH°(app) for NAD^+ binding at pH 7 to crystallized LADH (from Calbiochem Chemical Co.) and to lyophilized, non-heat-treated LADH (Sigma). For the crys-

Table I: Comparison of Zinc Content and Activity of LADH Samples[a]

	non-heat-treated	heat-treated
Zn^{2+}/protein subunit		
batch 1	1.2	2.05
batch 2	1.8	1.95
% active protein		
batch 1	35%	68%
batch 2	86%	96%

[a] The percent active protein was determined by the assay and relationship of Dalziel (1957).

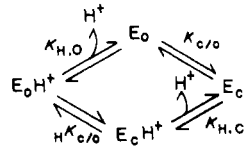
tallized sample, which was 98% active, an endothermic $\Delta H°$(app) of 1.1 kcal/mol was found. This value is in agreement with values we have measured for heat-treated, lyophilized LADH (see Figure 3). The non-heat-treated, lyophilized sample, which was only 60% active, showed an exothermic $\Delta H°$(app) of −2 kcal/mol. The latter value is in agreement with the value reported by Subramanian and Ross and indicates, at least in the neutral pH range, that the $\Delta H°$(app) is sample dependent. The percent active protein of a sample seems to be important, not whether the sample was crystallized or lyophilized. Subramanian and Ross found that the binding of NAD^+ to apo-LADH (having zinc removed according to the method of Iweibo and Weiner (1971)) produced an exothermic $\Delta H°$(app) equal to −6 kcal/mol. It seems likely that the non-heat-treated, lyophilized sample may contain an impurity of apo-LADH. This would account for its low percent activity and its exothermic $\Delta H°$(app) for NAD^+ binding.

To investigate this possibility, we determined the zinc content of heat-treated and non-heat-treated, lyophilized LADH by atomic absorption spectroscopy (see Table I). We found the zinc content of the non-heat-treated protein to be 1.2 zinc/subunit (this is for one batch of Sigma LADH, other batches gave higher zinc contents). After the heat treatment, the zinc content of the remaining soluble protein increased to 2.05 zinc/subunit. The expected zinc content for holo-LADH is 2.0 zinc/subunit. The increase in percent active protein following the heat treatment parallels the increase in zinc content, as can be seen in Table I. Data for other commercial batches are included to illustrate that their activity and zinc content may vary but that both properties of the enzyme are improved by the heat treatment process. This suggests that the material that is percipitated by the heat treatment is zinc-depleted protein.

Discussion

Our studies further characterize the linkage between NAD^+ binding to LADH and the acid dissociation of some group on the enzyme with a pK of around 9 (in the free enzyme). Previous equilibrium and transient kinetics studies of NAD^+ binding (Theorell & McKinley-McKee, 1961; Taniguchi et al., 1967; Shore et al., 1974; Kvassman & Pettersson, 1979) and steady-state enzyme kinetics studies (Dalziel, 1963) have implicated the coupling to some ionizing group. The present studies reinforce this conclusion and through the excellence of the fits of Scheme I to the data in Figures 1–3 indicate that a single ionizing group is involved (at least between pH 6 and pH 10). The same coupling is seen for the coenzyme analogue, $APAD^+$. The pK of the coupled/ionizing group decreases by about 1.5 pH units upon NAD^+ or $APAD^+$ binding. This corresponds to a free energy change of coupling, $\Delta G_{coupling}$, of 2.05 kcal/mol (i.e., $\Delta G_{coupling} = -2.303RT(pK_{H,L} - pK_H)$) between the binding of coenzyme and dissociation of a proton. From our data it appears that the enthalpy change of coupling

Scheme II



is approximately zero (i.e., $\Delta H_{coupling} = \Delta H°_L - \Delta H°_{L,H} = \Delta H°_H - \Delta H°_{H,L} \approx 0$). This indicates that the coupling is based on a favorable entropy change of coupling. A simple view is that the coupling involves electrostatic repulsion between the positively charged nicotinamide group and the positively charged, protonated state of the group on the protein (or electrostatic attraction to the negatively charged, unprotonated state of the group), which is minimized (enhanced) by a decrease in the pK of this ionizing group.

Formation of the ternary LADH–NAD^+–pyrazole complex results in a further decrease in the pK of the coupled/ionizing group to a value of 6 or below. Since the protein slowly loses activity below pH 6 (Vallee & Hoch, 1957), we were unable to obtain data below this pH and therefore the $pK_{H,L}$ of 6.0 for ternary complex formation is not well-defined. Others who have studied ternary complexes with TFE have presented evidence that $pK_{H,L}$ may be as low as 4.3 (Shore et al., 1974; Kvassman & Pettersson, 1980). There may be a difference in the degree to which TFE and pyrazole binding shifts the pK of the coupled/ionizing group. Nevertheless, the free energy change of coupling between ternary complex formation and the acid dissociation of the group on the protein is at least 4.8 kcal/mol for the pyrazole ternary complex. This $\Delta G_{coupling}$ is a quite large value (Weber, 1975).

In contrast to NAD^+ and $APAD^+$, the binding of ADP-ribose to the enzyme results in an increase in the pK of some group on the protein, as is also shown to be the case for NADH by the work of Andersson et al. (1981).

Both the rate constants for NAD^+ association, k_{on}, the dissociation, k_{off}, have been found to decrease dramatically with increasing pH (Kvassman & Pettersson, 1979). This observation indicates that the NAD^+ binding process and the coupling to the ionizing group are more complicated than explained above in terms of an electrostatic interaction between the coenzyme and a group on the protein. At neutral pH, where the association constant for NAD^+ is low, the binding and dissociation steps proceed relatively rapidly (k_{on} being one-thousandth of the diffusion-controlled limit). At high pH, where the association constant is higher, k_{on} and k_{off} are small (k_{off} decreases with pH faster than k_{on}). This strongly suggests that access to the NAD^+ binding site is controlled by a conformational change in the protein and that this conformational change is linked to the acid dissociation of a group on the protein with an apparent pK of 9.5 (in the free protein). If such coupling exists, then the apparent thermodynamic parameters (i.e., pK_H and $\Delta H°_H$) describing the ionization process will not be intrinsic values characteristic of the ionizing side chain. The apparent thermodynamic parameters will include a contribution from the induced conformational change. Consider Scheme II to illustrate this point. Let the subscript "o" and "c" refer to open and closed conformations of the enzyme and consider proton dissociation to occur from either state of the protein. The open state is assumed to be the one to which the ligand NAD^+ can most readily associate and dissociate. If the conformational transition $E_o \rightleftharpoons E_c$ lies greatly in favor of E_c when the group is unprotonated and the $E_oH^+ \rightleftharpoons E_cH^+$ equilibrium lies in favor of E_oH^+ when the group is protonated, then the apparent pK_H of the ionizing group in the free enzyme will be

$$pK_H(app) = pK_{H,O} - \log [(1 + K_{c/o})/(1 + {}_HK_{c/o})]$$

For example, if $K_{c/o} = 10$ and ${}_HK_{c/o} < 1$, then $pK_H(app)$ will be approximately 1 pH unit less than the intrinsic $pK_{H,0}$.

The identity of this coupled, ionizing group on the protein has been a matter of much discussion. A water molecule coordinated to the active-site zinc has frequently been suggested to be this group (Taniguchi et al., 1967; Theorell & McKinley-McKee, 1961; Kvassman & Pettersson, 1979, 1980; Brändén et al., 1975; Eklund et al., 1976, 1982a,b; Dworschak & Plapp, 1977; Dunn, 1974). But there have also been arguments that this coupled, ionizing group is Tyr-286, Cys-46, Lys-228, or His-51 (Parker et al., 1978; Laws & Shore, 1978; Reynolds & McKinley-McKee, 1975; Dworschack & Plapp, 1977). In forming a ternary complex, there is reason to believe that the additional proton release may come from the ligand itself (i.e., ionization of the hydroxyl group proton of TFE or loss of a proton from pyrazole upon forming a bound pyrazole–NAD^+ adduct) (Kvassman & Pettersson, 1980a,b; Theorell & Yonetani, 1963; Eklund et al., 1982a).

While there are a number of candidates for the coupled, ionizing groups, there is not, in our opinion, compelling evidence for any particular candidate at this time. The zinc-bound water is often cited as being this group, but the work of Dietrich et al. (1983) comparing the kinetics of NAD^+ binding to holo- and apo-LADH (active-site zinc depleted) casts serious doubt on the presumed role of this water molecule. The cases for Lys-228 and Cys-46 do not seem particularly strong, and the case for Tyr-286 is weakened by our argument, in the previous article (Eftink, 1986), that the fluorescence alkaline transition is not identical with the ionization that is linked to NAD^+ binding. Regarding the possible loss of a proton directly from the ligand in forming a ternary complex, NMR studies are consistent with this possibility for pyrazole (Becker & Roberts, 1984) but discount this possibility for TFE (Anderson & Dahlquist, 1982). The remaining candidate for the coupled, ionizing group, His-51, deserves support due to a lack of negative evidence regarding this group. Hennecke and Plapp (1983), using selective chemical modification, found LADH to be inactivated by modification of His-51. They found this inactivation to be governed by a pK of 9.6. They chose to interpret this result as reflecting the pK of the nearby zinc-bound water, but it seems possible that the pK they determined reflects that of His-51 itself. X-ray crystallographic studies show that His-51 forms a hydrogen bound with the 2′-hydroxyl group of the nicotinamide ribose ring (Eklund et al., 1982a). Thus the imidazole ring of His-51 is so close to the positively charged nicotinamide ring that it is reasonable that the pK of the former may be decreased upon NAD^+ binding.

To summarize, the identity of the coupled, ionizing group remains in question, but we believe the case for His-51 is strongest (by default perhaps). We note that Eklund et al. (1982) and Hennecke and Plapp (1983) describe a hydrogen bond network involving His-51, Ser-48, and the zinc-bound water in the protein. The acid dissociation of His-51 (and/or the zinc-bound water) may be a complex process that results in the shuffling of hydrogen bonds within this network. Regardless of the identity of the coupled, ionizing group, our studies describe the thermodynamic linkage between NAD^+ ($\Delta G_{coupling} = 2.05$ kcal/mol) and ligand ($\Delta G_{coupling} = 4.8$ kcal/mol for the pyrazole ternary complex) binding and the acid dissociation of this group.

Thermodynamic Parameters for Ligand Binding. The intrinsic $\Delta H°$ for the binding of NAD^+ to the protonated form of the enzyme (EH in Scheme I) is approximately zero; the $\Delta H°$ for the binding of NAD^+ to the high pH, unprotonated form of the enzyme is also approximately zero, although this value is not well-defined by our data. The near-zero intrinsic $\Delta H°$ indicates that the binding of NAD^+ to LADH is primarily driven by a large positive $\Delta S°$, at both low ($\Delta S°_{L,H} = 16.8$ cal/(mol·deg)) and high ($\Delta S°_L = 24$ cal/(mol·deg)) pH. It is interesting that the $\Delta H°$ for NADH binding to LADH has also been found to be approximately zero (Schmid et al., 1978; Subramanian & Ross, 1979). The nicotinamide ring of NADH is uncharged and therefore is not expected to lower the pK of the ionizing group. (In actuality, the binding of NADH is known to increase the pK of some ionizing group from a value of ~9 in the free enzyme to >10 in the binary complex (Andersson et al., 1981a) (see the fit in Figure 1).) X-ray crystallographic studies of LADH complexes with NAD^+ and NADH show the two coenzymes to bind in a nearly identical manner (Plapp et al., 1978). Thus it is understandable that the intrinsic, pH-independent $\Delta H°_{L,H}$ and $\Delta H°_L$ values for NAD^+ binding are virtually the same as the $\Delta H°$ for NADH binding at pH 7. That is, the two ligands interact similarly with the protein, except for the Coulombic perturbation of the pK of the ionizing group by NAD^+, and, therefore, the intrinsic thermodynamic parameters for coenzyme binding should also be similar (Andersson et al., 1981). Differences in $\Delta H°$(app) within the pH range 7–10 simply reflect differences in the way the coenzymes perturb the pK of the ionizing group.

The thermodynamic parameters and pH dependence of $APAD^+$ binding to LADH are the same as those for NAD^+. This is in contrast to the study of $APAD^+$ and NAD^+ binding to pig muscle lactate dehydrogenase, in which marked differences were found between the binding of the coenzyme and its analogue (Niekamp et al., 1980). This indicates that substitution of an acetyl group for the carboxamide group on the nicotinamide ring of the coenzyme does not alter the way in which the ligand binds. In particular, this suggests that the $-NH_2$ portion of the carboxamide group does not donate or accept a hydrogen bond in the binary complex.

The fact that the intrinsic $\Delta H°_{L,H}$ for NAD^+ binding is ca. zero is surprising in view of the fact that the binding of ADP-ribose (and also ADP and AMP (Schmid et al., 1978; Subramanian & Ross, 1979)) is exothermic. This indicates that interactions between the nicotinamide ring of NAD^+ (and dihydronicotinamide ring of NADH) and the protein result in an endothermic contribution to the $\Delta H°$ that cancels the exothermic contribution from interaction with the ADP-ribose portion of the coenzyme (Schmid et al., 1978). Also, the $\Delta H°$(app) for NAD^+ and NADH binding to LADH has been noted to be much smaller in magnitude than the $\Delta H°$(app) values for the binding of these coenzymes to several other types of dehydrogenase enzymes (Subramanian & Ross, 1978). These observations have led to the speculation that the interaction of the nicotinamide group of NAD^+ with the binding site of LADH is needed to induce a change in the conformation of this protein (hence the endothermic contribution). This proposal is supported by X-ray studies, which show a realignment of certain portions of the active-site region on NAD^+ or NADH binding. These structural changes do not appear to occur upon binding ADP-ribose (Brändén et al., 1975).

The formation of the LADH–NAD^+–pyrazole ternary complex is an exothermic process. Analysis of the data in Figure 3 in terms of a scheme similar to Scheme I yields an intrinsic $\Delta H°_{L,H} = -11$ kcal/mol, with a pK shift of 9.5 to 6.0 and $\Delta H°_H = -7.5$ kcal/mol. The $\Delta H°$(app) of ca. −4 kcal/mol between pH 7 and 8.5 is misleading, since this value

is the difference between the intrinsic $\Delta H°_{L,H}$ of ligand binding and the heat of protonation of the coupled/ionizing group. It is difficult to define the value of the intrinsic enthalpy change at high pH, $\Delta H°_{L,H}$, and $pK_{H,L}$ in our fit, but, nevertheless, the fit shown in Figure 3 illustrates that Scheme I adequately describes the data.

The moderately large, exothermic, intrinsic $\Delta H°_{L}$ for the simultaneous binding of both NAD^+ and pyrazole is interesting and suggests that a conformational change in the protein is induced by ternary complex formation. Alternatively, the exothermic $\Delta H°_{L}$ may possibly be due to covalent bond formation between pyrazole and C-4 of the nicotinamide ring of NAD^+, as suggested by X-ray structural studies (Eklund et al., 1982b). However, in work to be published, we have found that the binding of pyrazole alone to LADH produces a large, exothermic $\Delta H°$(app), which can account for most of the magnitude of $\Delta H°_{L,H}$.

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Registry No. LADH, 9031-72-5; TFE, 75-89-8; NAD, 53-84-9; ADPR, 20762-30-5; $APAD^+$, 86-08-8; NADH, 58-68-4; pyrazole, 288-13-1.

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