Quenching of the Intrinsic Fluorescence of Liver Alcohol Dehydrogenase by the Alkaline Transition and by Coenzyme Binding†

Maurice R. Eftink

Department of Chemistry, University of Mississippi, University, Mississippi 38677

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ABSTRACT: The fluorescence of alcohol dehydrogenase is quenched by the acid dissociation of some group on the protein having an apparent pK_a of 9.6 at 25 °C. The pK_a of this alkaline quenching transition is unchanged by the binding of trifluoroethanol or pyrazole to the enzyme or by the selective removal of the active site of Zn^{2+} ion. This indicates that the ionization of a zinc-bound water molecule is not responsible for the quenching. The binding of NAD^+ to the enzyme causes a drop in protein fluorescence and an apparent shift in the alkaline quenching transition to lower pH. In the ternary complex formed with NAD^+ and trifluoroethanol the alkaline transition is difficult to discern between pH 6 and pH 11. In the NAD^+–pyrazole ternary complex, however, a small but noticeable fluorescence transition is observed with a $pK_a(app) \approx 9.5$. We propose that the alkaline transition centered at pH 9.6 is not shifted to lower pH upon binding NAD^+. Instead, the amplitude of the alkaline quenching effect is decreased to the point that it is difficult to detect when NAD^+ is bound. We present a model that describes the dependence of the fluorescence of the protein on pH and NAD^+ concentration in terms of two independently operating, dynamic quenching mechanisms. Our data and model cast serious doubt on the identification, made previously in the literature, between the alkaline quenching pK_a and the pK_a of the group whose ionization is coupled to NAD^+ binding. Also, simulations demonstrate that the apparent pK_a values, obtained for LADH from plots of fluorescence intensity vs. pH, are underestimates of the true pK_a values. The true pK_a for the alkaline quenching transition is found to be 9.9 at 25 °C.

The intrinsic tryptophanyl fluorescence of horse liver alcohol dehydrogenase (LADH)[1] is quenched at basic pH as a result of the ionization of a group having a pK_a of about 9.8 (Wolfe et al., 1977; Parker et al., 1978). This alkaline quenching effect causes a selective loss of 50–80% of the fluorescence of the buried tryptophanyl residue, Trp-314, of this protein. LADH is a dimeric protein and possesses only two types of tryptophanyl residues, Trp-15, which lies at the surface of the enzyme, and Trp-314, which is buried in a hydrophobic environment at the intersubunit interface (Branden et al., 1975). The selective quenching of Trp-314 in the alkaline transition is indicated by a red shift in the emission spectrum as pH is increased and by solute quenching studies (Laws & Shore, 1978; Eftink & Selvidge, 1982; Eftink & Hagaman, 1986). The identity of the ionizable group responsible for this alkaline quenching and the mechanism of the selective quenching of Trp-314 are uncertain. Laws and Shore (1978) have proposed that the group may be a tyrosyl residue, Tyr-286. Ionization of the phenol side chain of this residue could cause quenching of Trp-314 by resonance energy transfer to the tyrosinate group.

The binding of NAD^+ to LADH is also linked to the ionization of some group on the enzyme having a pK_a of about 9, which is shifted to about 8 in the binary complex (Theorell & McKinley-McKee, 1961; Dalziel, 1963; Kvassman & Pettersson, 1979; Eftink & Bystrom, 1986). Besides the similarity of the alkaline quenhcing pK_a and the pK_a of the group coupled to coenzyme binding, the addition of NAD^+ also quenches the fluorescence of Trp-314 and results in an apparent shift in the fluorescence titration curve of LADH to lower pH (Wolfe et al., 1977; Parker et al., 1978). This is evidence that the alkaline quenching group and the ionizing group coupled to NAD^+ binding are the same and extends the list of candidates for this group to include those in the vicinity of the coenzyme binding site, namely Lys-228, His-51, Cys-46, and a zinc-bound water molecule (Parker et al., 1978).

Below we report a reinvestigation of the pH dependence of the fluorescence of LADH, in the absence and presence of NAD^+ and other ligands. We will present data and a model for the pH dependence of LADH fluorescence that show that it is quite doubtful that the alkaline quenching group and the group coupled to NAD^+ binding are the same. Instead there appears to be two separate ionizing groups on the protein.

Experimental Procedures

Materials. Crystallized LADH and NAD^+ (lithium salt) were obtained from Cal-Behring. The enzyme was dialyzed in the cold against several changes of 0.03 M sodium phosphate (pH 7.2) to remove as much ethanol as possible. Typically we found the enzyme to be 100 ± 10% active by the assay and formula of Dalziel (1957). Apo-LADH was prepared as described by Maret et al. (1979) using dipicolinic acid as a chelating agent to selectively remove the active-site zinc ions from LADH crystals formed in 25% *tert*-butyl alcohol. Immediately following such treatment we found a residual enzymatic activity of ~2%. Zinc determinations, using a Perkin-Elmer 2380 AA spectrophotometer with a HGA-400 graphite furnace (protein samples digested with 6 M HCl overnight at 90 °C), showed a ratio of 3.7 ± 0.3 zinc/subunit

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[1] Abbreviations: ADP-ribose, adenosine 5′-diphosphoribose; apo-LADH, LADH in which the two active-site zinc ions have been removed; LADH, horse liver alcohol dehydrogenase; NAD^+, oxidized β-nicotinamide adenine dinucleotide; TFE, trifluoroethanol.

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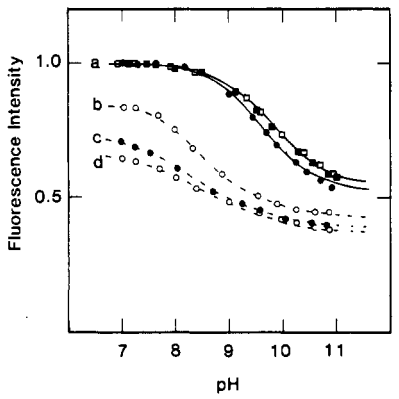

FIGURE 1: pH dependence of the fluorescence of LADH (solid squares) and apo-LADH (open squares) at 20 °C and of LADH (circles) in the absence (a) and presence of NAD^+ (b, 3×10^{-5} M; c, 7.5×10^{-5} M; d, 1.6×10^{-4} M) at 25 °C. Conditions: 0.1 M NaCl, λ_{ex} = 295 nm, λ_{em} = 323 nm. The solid line through the open and solid squares is a fit with pK_a(app) = 9.7 and ΔF = 0.45 and the solid line through curve a (closed circles) is a fit with pK_a(app) = 9.6 and ΔF = 0.46. The dashed lines through the other data have no theoretical significance.

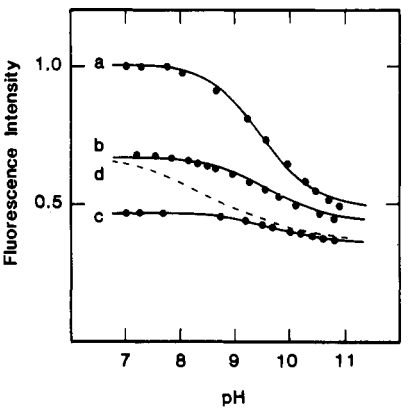

FIGURE 2: pH dependence of the fluorescence of LADH (a) and the ternary complexes LADH–NAD^+–pyrazole (b) and LADH–NAD^+–TFE (c). Conditions: 25 °C, 0.1 M NaCl, λ_{ex} = 295 nm, λ_{em} = 323 nm, [NAD^+] = 1.6×10^{-4} M in (b) and (c), [pyrazole] = 3.2×10^{-3} M in (b), and [TFE] = 42.7×10^{-3} M in (c). The solid line through (a) is a fit with pK_a(app) = 9.5 and ΔF = 0.50; the line through (b) is a fit with pK_a(app) = 9.5 and ΔF = 0.21; the line through (c) is a fit with pK_a(app) = 9.6 and ΔF = 0.10.

for the native protein and 1.7 ± 0.4 zinc/subunit for apo-LADH.

Dipicolinic acid, pyrazole, and trifluoroethanol (TFE) were obtained from Sigma Chemical Co. and were used without further purification.

Fluorescence pH Titrations. A Perkin-Elmer MPF 44 spectrophotofluormeter with a thermostated cell holder was used for fluorescence measurements. An aliquot (i.e., 200 μL) of a stock LADH solution was typically added to 2.0 mL of a 0.1 M NaCl solution in a fluorometric cell to give a final subunit concentration of about 1 μM. After temperature equilibration, fluorescence (323-nm emission, 295-nm excitation) and pH measurements were made. To minimize photolysis, a narrow (3-nm) excitation slit was used and the lamp shutter was closed during mixing and pH measurements. A London PHM 64 and microelectrode was used for measuring pH. Small aliquots of a 0.01 M NaOH solution were added via a Hamilton syringe to increase the pH. A titration from pH to near 11 was completed in about 15 min. The absorbance at 295 nm, A_{295}, of a LADH solution was measured at the initial and final pH, and the fluorescence values were corrected for absorptive screening by using the factor antilog ($\Delta A_{295}/2$) and assuming that the change in A_{295} is directly proportional to the volume of base added. This correction factor was always less than 1.08. Most studies were done at 20 or 25 °C. In cases where temperature was varied, the pH electrode was calibrated vs. standards at the temperature investigated.

Measurements of enzymatic activity, made within 15 min following titration of a sample to pH 11, showed loss of less than 10% activity. Similar measurements with the apo-LADH showed no discernable increase in activity, indicating that the protein is not regaining zinc ions (i.e., from the titrant or glassware) during the course of the experiment.

RESULTS

LADH and Apo-LADH. The pH dependence of the fluorescence of LADH in 0.1 M NaCl solution at 20 °C is shown as the closed squares in Figure 1. The observed alkaline quenching can be well described by a pK_a(app) of 9.7 and a fluorescence change, ΔF, of 0.45 relative units. No change (±0.1 pH unit) was seen in the apparent pK_a in the presence of 1.0 M NaCl. A moderate temperature dependence was observed for the pK_a(app). From data obtained at six temperatures between 8 and 35 °C, a van't Hoff enthalpy change, ΔH_{VH}, of −9.1 ± 1.5 kcal/mol was calculated for the heat of protonation of this group.

Removal of the active-site zinc ions from the enzyme resulted in no appreciable change in the pH dependence of the fluorescence. The open squares in Figure 1 show the alkaline quenching transition for apo-LADH.

Binary and Ternary Complexes. Upon the addition of 50 mM pyrazole, 34 mM TFE, or 0.22 mM ADP-ribose, the alkaline quenching of LADH at 25 °C was unchanged with an apparent pK_a of 9.6 ± 0.1. Also the addition of these ligands does not appreciably quench the fluorescence of the protein at neutral pH. At the concentrations added, the enzyme should be saturated to the extent of 85%, 96%, and 93% with pyrazole, TFE, and ADP-ribose, respectively (Anderson & Dahlquist, 1982; Gunnarsson & Pettersson, 1974).

The formation of a binary complex between LADH and NAD^+, on the other hand, results in a general loss of fluorescence and an apparent shift in the titration curve to lower pH, as previously reported by Wolfe et al. (1977). In Figure 1 are shown fluorescence titrations of 0.8 μM solutions of LADH at 25 °C as a function of the concentration of added NAD^+. At very high NAD^+ concentration the alkaline quenching pK_a appears to be shifted to a value near 8, although the shape of the curve becomes somewhat distorted.

The formation of a ternary complex with NAD^+ and TFE results in a relatively flat fluorescence titration curve that shows only a slight downward dip at high pH (see Figure 2). At all pHs, the fluorescence of this ternary complex is less than that of the binary LADH–NAD^+ complex or other forms of the enzyme.

In contrast to the TFE ternary complex, that formed with the inhibitor pyrazole shows a higher fluorescence than the LADH–NAD^+ binary complex (see Figure 2). Also a much more noticeable inflection is observed in this case at pH ≈9.5. This indicates that the alkaline quenching transition, observed for the free enzyme, remains in the LADH–NAD^+-pyrazole ternary complex. The amplitude (ΔF) of this transition is just reduced in this ternary complex. This suggests that in the TFE ternary complex, where the total fluorescence of the enzyme is quenched to a greater degree, the alkaline quenching transition still occurs in the range of pH 9–10 but that its amplitude is so small that it is difficult to recognize. Fits are shown in Figure 2 for pK_a(app) = 9.5 and ΔF = 0.21 for the

pyrazole ternary complex, and $pK_a(app) = 9.6$ and $\Delta F = 0.10$ for the TFE ternary complex.

Discussion

The alkaline quenching of LADH fluorescence is well described as being due to the ionization of a group on the enzyme having a $pK_a(app)$ of 9.7 at 20 °C. Steady-state acrylamide quenching studies at neutral pH and at pH 10.8 indicate that the alkaline quenching process results in a selective quenching of about 40% (at an emission wavelength of 323 nm) of the fluorescence of Trp-314, without appreciably affecting the fluorescence of Trp-15 (Eftink & Hagaman, 1986). Also, this selective quenching of Trp-314 is a dynamic process; the fluorescence lifetime of Trp-314 drops from ~3.6 to ~2.2 ns upon an increase in pH from 7 to 10.8 (Eftink & Hagaman, 1986). Apparently the quenching results from the ionization of a single group. If more than one group is involved, they must have nearly the same pK_a in order to account for the fit shown in Figure 1.

The pK_a of the group responsible for the alkaline quenching effect is insensitive to the ionic strength of the solution. This suggests that the pK_a of the ionizing group is not perturbed to an abnormally high or low value by electrostatic interaction with fixed charges in its microenvironment. The heat of protonation of the ionizing groups is found to be −9 kcal/mol. In previous studies, Parker et al. (1978) reported that the pK_a of the alkaline quenching group is increased by ca. 0.8 pH unit in D_2O.

The fact that apo-LADH shows the same alkaline transition as the native enzyme clearly indicates that the ionizing group causing the loss of fluorescence is not a zinc-bound water molecule. Additional evidence that discounts the involvement of a zinc-bound water is the observation that the addition of pyrazole or TFE does not change the apparent pK_a for the alkaline quenching effect. These ligands are believed to form binary complexes in which they (the ligands) interact at either the first or second coordination sphere of the catalytic zinc ion (Anderson & Dahlquist, 1982; Andersson et al., 1981; Makinen & Yim, 1981; Kvassman & Petterson, 1980). X-ray crystallographic studies show pyrazole to bind directly to the zinc ion (Eklund et al., 1982); this would result in either a displacement of the zinc-bound water molecule or a change in the zinc from being tetracoordinate to being pentacoordinate. Thus, the binding of pyrazole to LADH should either perturb the pK_a of the zinc-bound water or completely eliminate this ionization process (i.e., if pyrazole displaces the water molecule). The absence of a shift in the apparent pK_a for the alkaline quenching effect upon addition of pyrazole or TFE corroborates the study with apo-LADH and proves that the zinc-bound water is not the group responsible for the quenching effect. (Note that this does not discount the possibility that the zinc-bound water molecule has a pK_a near 9). Parker et al. (1978) have previously demonstrated that the addition of another inhibitor, imidazole, does not alter the alkaline quenching effect.

The remaining candidates for the ionizing groups responsible for the alkaline quenching effect are Tyr-286, Lys-228, Cys-46, and His-51. It is conceivable that each of these residues could have a pK_a of 9.7, which is insensitive to ionic strength and shows an increase of 0.8 pH unit in D_2O. The $\Delta H_{VH} = -9.1 \pm 1.5$ kcal/mol for protonation is within experimental error of the values normally expected for most of these residue side chains and does not allow us to distinguish between the possible candidates (i.e., heats of protonation of −11, −7, −6, and −8 kcal/mol for Lys, His, Tyr, and Cys side chains). Parker et al. (1978) have previously shown that selective carboxymethylation of Cys-46 and acetimidylation of most of the Lys residues, including Lys-228, does not change the apparent pK_a for the alkaline quenching effect. Of the possible candidates, the ionization of Tyr-286 provides a direct quenching mechanism, e.g., resonance energy transfer (Laws & Shore, 1978). The other candidates are located at the coenzyme's binding site, which is ~15 Å from Trp-314 (Eklund et al., 1976). The ionization of the other candidates, Cys-46 and His-51 (as well as Lys-228 and the zinc-bound water), must quench Tryp-314 indirectly through an induced change in the conformation of the protein (Wolfe et al., 1977). No obvious change in the microenvironment of Trp-314 is seen on comparison of the crystal structure of ternary complexes of LADH with the enzyme itself (Brändén et al., 1975; Brändén & Eklund, 1978). As pointed out by Abdallah et al. (1979), one cannot discount a small conformational change that brings a quenching group a fraction of an angstrom closer to Trp-314.

In considering the possible candidates for the alkaline quenching group, the most important question is whether this group is the same as the ionizing group that is coupled to NAD^+ binding (Wolfe et al., 1979; Parker et al., 1978). For the candidates located at the coenzyme binding site, this identification is implied. Our results in Figures 1 and 2 show that this identification is quite doubtful. In particular, the NAD^+–pyrazole ternary complex shows an alkaline quenching transition, centered at pH 9.5, with a small, but discernable amplitude. Below we present a model that can explain the shapes of the fluorescence titrations in terms of (a) the dynamic quenching of Trp-314 by the ionization of the alkaline transition group, (b) the dynamic quenching of Trp-314 by the binding of NAD^+, and (c) the pH dependence of the binding of NAD^+ to LADH. That is, in this model there are two separate quenching mechanisms acting on Trp-314.

The fluorescence lifetime of Trp-314 is known to decrease by about 40% on the binding of NAD^+ and pyrazole to LADH (Ross et al., 1981). Also we show (Eftink & Hagaman, 1986) that the fluorescence lifetime of Trp-314 is decreased by about 60% in the LADH–NAD^+–TFE ternary complex and by about 40% as a result of the alkaline transition. If there are two separate, dynamic mechanisms for quenching Trp-314 fluorescence (i.e., mechanism 1 involving the ionization of some group with $pK_a(app) = 9.7$ and mechanism 2 involving the binding of NAD^+), the result will be that the amplitude of the quenching effect by each mechanism will be decreased by the action of the other quenching mechanism. This is true not only because the action of quenching mechanism 1 decreases the amplitude of the remaining fluorescence to be quenched by mechanism 2 (and vice versa) but alo because mechanism 1 lowers the remaining fluorescence lifetime and thus decreases the effectiveness of the second dynamic quenching mechanism.

To illustrate this model, consider mechanisms 1 and 2 to involve energy-transfer quenching with rate constants, k_{T1} and k_{T2}, given as follows (Lakowicz, 1983):

$$k_{Ti} = (8.71 \times 10^{23})(r_i^{-6} J_i \kappa_i^2 n^{-4} k_r)\ s^{-1} \qquad (1)$$

where r_i is the distance in angstroms between the donor (assumed to be Trp-314 in this case) and the acceptor (assumed to be Tyr-286 for mechanism 1 and NAD^+ for mechanism 2), J_i is the spectral overlap integral between the donor emission and acceptor absorption, κ_i^2 is an orientation factor for the transition dipoles, n is the refraction index of the medium, and k_r is the radia ive rate constant of the donor. (Note that quenching mechanisms 1 and 2 need only be dynamic mechanisms for this model to hold; they do not necessarily need to be energy-transfer mechanisms. Also note that Ross et al. (1981) have calculated that, despite a small overlap integral,

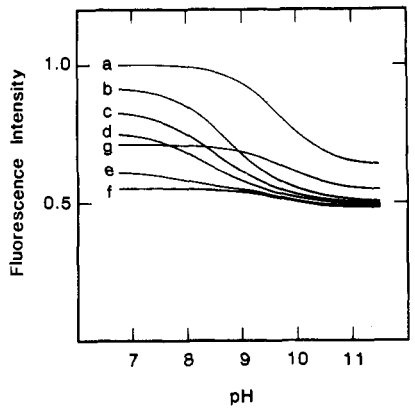

FIGURE 3: Simulated pH fluorescence titration curves for LADH, in the absence and presence of NAD^+, using the model described in the text. Curve a is for LADH in the absence of coenzyme. Curves b, c, d, and e correspond to $[NAD^+]$ of 2×10^{-5}, 5×10^{-5}, 1×10^{-4}, and 5×10^{-3} M, respectively. Curves f and g are simulated titration curves for the TFE and pyrazole ternary complexes, respectively. The simulated relative fluorescence intensity, F, is calculated from the relationship $F = 0.28 + 0.72(\phi/\phi_0)$, where ϕ is given by eq 2 and ϕ_0 is the yield when $f_1 = 0$ and $f_2 = 0$ (i.e., in the absence of quenching mechanisms 1 and 2). The value 0.28 is included in the simulation to represent the relative fluorescence of Trp-15 (Eftink & Selvidge, 1982), which is assumed to not be quenched by mechanisms 1 and 2. For curve g, that simulated for the LADH–NAD^+–pyrazole ternary complex, $k_{T2} = 2 \times 10^8$ s^{-1}. For both curves f and g (the TFE and pyrazole ternary complexes), f_2 is assumed to be equal to 1.0 at all pHs due to the tightness of binding for these complexes.

the total transfer efficiency between Trp-314 residues and bound NAD^+ is 38%. Thus energy transfer is a possible quenching mechanism for process k_{T2}). According to this model the fluorescence yield, ϕ, of the donor, Trp-314, will be given by the equation:

$$\phi = \frac{k_r}{k_r + k_{nr} + f_1 k_{T1} + f_2 k_{T2}} \quad (2)$$

where k_{nr} is a nonradiative rate constant and f_1 and f_2 are fractional degrees of progress of quenching mechanism 1 and 2. f_1 is determined by the pH and pK_a of Tyr-286 and f_2 is determined by the concentration of (NAD^+) and its apparent association constant, K_{app}.

$$f_1 = \frac{10^{-pK_a}}{10^{-pH} + 10^{-pK_a}} \quad (3)$$

$$f_2 = \frac{(NAD^+)K_{app}}{1 + (NAD^+)K_{app}} \quad (4)$$

As discussed in the following paper (Eftink & Bystrom, 1986), the value of K_{app} depends on pH as follows:

$$K_{app} = \frac{K_{L,H}(1 + 10^{-pK_H}/10^{-pH})}{(1 + 10^{-pK_{H,L}}/10^{-pH})} \quad (5)$$

The meaning of $K_{L,H}$, pK_H, and p$K_{H,L}$ is defined in the following paper. Note that pK_a and pK_H are not necessarily the same value, although they appear to be similar.

In Figure 3 is shown a simulation of eq 2 in terms of a plot of fluorescence intensity vs. pH for various concentrations of NAD^+. In this simulation the following parameters are selected: $k_r = 1 \times 10^8$ s^{-1}, $k_{nr} = 2 \times 10^8$ s^{-1}, $k_{T1} = 2 \times 10^8$ s^{-1}, $k_{T2} = 5 \times 10^8$ s^{-1}, p$K_a = 10.0$, $K_{L,H} = 4.0 \times 10^3$ M^{-1}, pK_H = 8.8, and p$K_{H,L}$ = 7.3. The latter three values are from a fit described in the following paper (Eftink & Bystrom, 1986). The selected values of k_{Ti} correspond to 40% and 62.5% quenching of Trp-314 fluorescence by the individual mechanisms 1 and 2. The selected values of k_r and k_{nr} are consistent with the measured fluorescence lifetime and quantum yield of Trp-314 (Eftink & Hagaman, 1986; Ross et al., 1981; Abdallah et al., 1978).

The simulation for LADH alone (curve a) depicts an "alkaline quenching transition" similar to that actually observed. Note that the apparent pK_a for the simulated transition is pK_a(app) $\approx$9.7, whereas a pK_a = 10.0 was used in the calculations. This somewhat surprising result is due to the fact that in Figure 3 we are actually presenting a simulation of $\phi/\phi_0 = 1/(1 + k_{Ti}\tau_0 f_1)$, where ϕ_0 and τ_0 are the fluorescence yield and lifetime in the absence of quenching mechanisms 1 and 2 (i.e., $\phi_0 = k_r/(k_r + k_{nr})$ and $\tau_0 = 1/(k_r + k_{nr})$). The inflection point of such a plot of ϕ/ϕ_0 vs. pH yields an apparent pK_a of pK_a(app) = pH − log $(1 + k_{Ti}\tau_0)$. If, instead, the reciprocal ratio $\phi_0/\phi = 1 + k_{Ti}\tau_0 f_1$ were plotted vs. pH, it would have an inflection point at a pH equal to the pK_a. Thus the simulation demonstrates that when dynamic fluorescence quenching occurs due to an acid–base dissociation, that plots of ϕ/ϕ_0 (or direct plots of relative fluorescence intensity) vs. pH will yield apparent pK_a values that differ from the true pK_a values. In the present study, the pK_a(app) of 9.7 found for LADH at 20 °C in Figure 1 is an underestimate of the true pK_a; from a reciprocal plot (not shown) of F_0/F vs. pH (where F_0 is the relative fluorescence of the unquenched state and F is the fluorescence as a function of pH), a pK_a value of 10.0 at 20 °C (9.9 at 25 °C) is determined, which should be the true value for the alkaline quenching acid dissociation constant.

Also, as can be seen in the simulation in Figure 3, there is a progressive shift in the fluorescence intensity vs. pH curve to lower pH as NAD^+ concentration increases and the alkaline transition at pH 9.7 becomes masked due to its decrease in amplitude. Also shown (curve g) is a simulation in which $k_{T2} = 2 \times 10^8$ s^{-1} and $f_2 = 1.0$. These parameters are chosen to simulate the pH dependence of the fluorescence of the NAD^+–pyrazole ternary complex (40% quenching of Trp-314 by NAD^+ binding instead of the 62.5% in the other simulations). For this case the amplitude of the alkaline effect is larger and a distinct transition can more easily be observed at pH $\approx$ 9.7.

These simulations, together with the titration curve in Figure 2 of the NAD^+–pyrazole ternary complex (which shows a transition with a pK_a(app) $\sim$9.5), strongly argue that the alkaline transition pK_a does not shift to lower pH upon NAD^+ binding. Thus, it appears that the group whose ionization is coupled to NAD^+ binding is a separate group on the protein. We conclude that inferences drawn regarding the coupling between NAD^+ binding and the ionization of the latter group for native or chemically modified enzyme derivatives (Wolfe et al., 1977; Parker et al., 1978), based on observations of apparent shifts in the alkaline transition, must be reconsidered.

In our above discussion we have implied that Tyr-286 is the alkaline quenching group. This identification is, of course, still putative. The ionization of Tyr-286 provides an alkaline quenching mechanism that does not require a significant change in the microenvironement of Trp-314, and ultraviolet difference spectroscopy studies confirm the presence of ionized tyrosine in LADH at pH 9.8, 23 °C (Laws & Shore, 1979). Our above studies eliminate a zinc-bound water and cast serious doubt on the assignment of Lys-228, Cys-48, and His-51 as the alkaline quenching group. Thus evidence greatly favors the assignments of Tyr-286 as this group.

Registry No. LADH, 9031-72-5; NAD, 53-84-9.

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

Maurice R. Eftink* and Katarina Byström‡
Department of Chemistry, University of Mississippi, University, Mississippi 38677
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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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‡ Present address: Thermochemistry, Chemical Center, University of Lund, S-220 07 Lund 7, Sweden.

[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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