pH-dependent Conformational States of Horse Liver Alcohol Dehydrogenase*

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The quenching of liver alcohol dehydrogenase protein fluorescence at alkaline pH indicates two conformational states of the enzyme with a pKᵢ of 9.8 ± 0.2, shifted to 10.6 ± 0.2 in D₂O. NAD⁺ and 2-p-toluidinonaphthalene-6-sulfonate, a fluorescent probe competitive with coenzyme, bind to the acid conformation of the enzyme. The pKᵢ of the protein-fluorescence quenching curve is shifted toward 7.6 in the presence of NAD⁺, and the ternary complex formation with NAD⁺ and trifluoroethanol results in a pH-independent maximal quench. At pH (pD) 10.5, the rate constant for NAD⁺ binding was 2.6 times faster in D₂O than in H₂O due to the shift of the pKᵢ. Based on these results, a scheme has been proposed in which the state of protonation of an enzyme functional group with a pKᵢ of 9.8 controls the conformational state of the enzyme. NAD⁺ binds to the acid conformation and subsequently causes another conformational change resulting in the perturbation of the pKᵢ to 7.6. Alcohol then binds to the unprotonated form of the functional group with a pKᵢ of 7.6 in the binary enzyme-NAD⁺ complex and converts the enzyme to the alkaline conformation. Thus, at neutral pH liver alcohol dehydrogenase undergoes two conformational changes en route to the ternary complex in which hydride transfer occurs.

Previous kinetic studies (1) showed that the rate of binding of NAD⁺ to liver alcohol dehydrogenase (EC 1.1.1.1.) depended on the existence of the protonated form of an enzyme functional group with a pKᵢ of approximately 9.6. These steady state kinetic studies and subsequent direct observation of proton release (2) demonstrated that NAD⁺ binding perturbs the pKᵢ of this group to 7.6. Perturbation of the pKᵢ indicates that oxidized coenzyme does not bind directly to this group. The effect of pH on the coenzyme binding rate is therefore probably due to pH-dependent conformational states of the enzyme.

It has been established that binding NAD⁺ to liver alcohol dehydrogenase quenches the enzyme's tryptophan fluorescence (3, 4). This fluorescence quenching could stem from singlet-singlet energy transfer to another chromophore (NAD⁺), from direct collisional quenching, or from nonradiative transitions perhaps involving solvent. The spectral properties of NAD⁺ preclude an energy transfer quenching mechanism, and the tryptophan residues are not near the active center (5) so that collisional quenching is impossible. The quenching due to oxidized coenzyme binding thus strongly implies a conformational change. A recent stopped flow study (6) indicates that this conformational change is very rapid.

The present study was undertaken to demonstrate directly the existence of pH-dependent conformational states of liver alcohol dehydrogenase. The effect of NAD⁺ binding on this conformational equilibrium and its relationship to the mechanism of action of the enzyme were evaluated.

MATERIALS AND METHODS

Alcohol dehydrogenase was prepared from horse livers by the method of Theorell et al. (7). Enzyme concentration was determined by assay (8) and by fluorescence titration of NADH binding sites in the presence of isobutyramide (9). NAD⁺, grade III, was purchased from Sigma Chemical Corp. and was purified on Dowex 1 by the method of Stinson and Holbrook (10) and crystallized by the method of Winer (11). The potassium salt of 2-p-toluidinonaphthalene-6-sulfonate (TNS) was obtained from Sigma and was used without further purification. Trifluoroethanol was purchased from Aldrich Chemical Corp. and deuterium oxide from General Dynamics Corp.

Fluorescence studies were performed on a Farrand spectrophotometer. The fluorescence level of 4.7 μM liver alcohol dehydrogenase was measured at various pH values in 0.1 ionic strength buffers. Sodium pyrophosphate buffers were used for pH 7 through 9.5; sodium carbonate buffers were used for pH 9.5 to 11. Measurements were made at 25°, using 290 nm excitation and 335 nm emission, in both H₂O and D₂O. pD was calculated as the pH reading + 0.41 (12). Additional protein fluorescence measurements, in the presence and absence of NAD⁺, were made on a rapid titration apparatus which has been described (13). The protein fluorescence was excited at 297 nm and was measured through a Kodak-Wratten filter 18A. This instrument permits continuous variation of pH from 7 to 11 within 1 min with simultaneous observation of the protein fluorescence. The enzyme was initially in 5 mM sodium phosphate buffer containing 45 mM sodium sulfate, pH 7.0. The pH was increased by titrating with sodium hydroxide.

TNS binding to enzymes was also studied in both instruments. Titrations were performed in the Farrand by adding TNS to enzymes at various pH values, using the same buffers as in the protein fluorescence quenching studies. An excitation wavelength of 385 nm was used to minimize inner filter corrections. The effect of pH on

*This research was supported by National Science Foundation Grant OD43507 and NATO Grant 760.
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The abbreviation used is: TNS, 2-p-toluidinonaphthalene-6-sulfonate.
TNS binding was also measured on the rapid titration apparatus, using 375 nm excitation and emission through a Kodak-Wratten 58 filter. The fluorescence polarization of the liver alcohol dehydrogenase-TNS complex was measured from pH 7 to 11 on an instrument similar to that described by Weber and Bablouzian (14) and Rosen (15).

The rate constant for NAD\(^+\) binding to enzymes was measured by protein fluorescence quenching in a Durrum-Gibson stopped-flow spectrophotometer with fluorescence attachment. Data were collected with a Transidyne neurograph model N-3 waveform recorder and pseudo-first order rate constants were computed using an analog device, designed by Dr. David Ballou of the University of Michigan, with the bimolecular rate constant calculated subsequently. Both the reaction trace and the first order plot were recorded on a Hewlett-Packard model 7044a recorder.

RESULTS

The quenching of protein fluorescence of liver alcohol dehydrogenase due to increasing pH is shown in Fig. 1. A theoretical curve, assuming a maximum quench of 40%, indicates a pK\(_a\) of 9.8 ± 0.2 in aqueous buffer. In D\(_2\)O solvent, the pK\(_a\) is shifted approximately 0.8 unit to a value of 10.6 ± 0.2. At pH or pD values above 10.5 it was extremely difficult to obtain accurate values due to a time-dependent additional quenching reaction related to alkaline denaturation of the enzyme.

The binding of TNS, a fluorescent probe competitive with NAD\(^+\) (16), was studied as a function of pH. Fig. 2 shows typical double reciprocal plots of titrations performed at pH 8.0 and 10.15, indicating that the affinity of enzymes for TNS is much weaker at alkaline pH, but that the quantum yield of bound probe is essentially unchanged. The pH-invariant quantum yield was reaffirmed by fluorescence polarization studies, which resulted in values of p = 0.34 ± 0.01 in the pH range 7.0 to 10.5. The diminished affinity of enzyme for TNS at alkaline pH was also determined kinetically by its inhibition competitive with NAD\(^+\). Dixon plots (17) at pH 8 and 10 yielded inhibition constants of 36 and 313 \(\mu\)M, respectively, in good agreement with enzyme-TNS dissociation constants calculated from fluorimetric titrations.

The pH independence of the quantum yield of bound TNS and the increasing dissociation constant with increasing pH enabled direct determination of the pK\(_a\) of the TNS binding reaction in the rapid titration apparatus. The lower limit of the dissociation constant of the enzyme-TNS complex, obtained between pH 6.0 and 7.5, was 25 \(\mu\)M. Consequently, if a concentration of TNS well below this value is added to the enzyme and the quantum yield of bound TNS is pH-independent, a pH titration will indicate the pK\(_a\) of the group involved in binding. This occurs because whenever probe concentrations of TNS are used, the concentration of bound TNS will be proportional to the association constant of the enzyme-TNS complex. Fig. 3 shows the result of a titration of the complex from pH 7 to 10.5 in the rapid titration apparatus, indicating a pK\(_a\) of 9.7 ± 0.2 for the binding constant.

Fig. 4 shows a typical oscilloscope trace from the rapid titration apparatus in which liver alcohol dehydrogenase tryptophan fluorescence in the presence of 100 \(\mu\)M TNS is measured as a function of pH. The effect of NAD\(^+\) binding on the conformational equilibrium is shown in Fig. 5. With increasing NAD\(^+\) concentration, the pK\(_a\) shifts to a lower value. A ternary complex can be formed between the binary enzyme-NAD\(^+\) complex and trifluoroethanol, an inhibitor competitive with ethanol. When the fluorescence of this complex is measured as a function of pH, as shown in Fig. 5, two effects are seen. The protein fluorescence level corresponds to the maximal quench obtained at alkaline pH with free enzyme or binary complex, and it is relatively invariant with pH.

Stopped flow studies showing the rate of NAD\(^+\) binding are given in Fig. 6. The second order rate constants are 0.12 \(\mu\)M\(^{-1}\) s\(^{-1}\) in water and 0.31 \(\mu\)M\(^{-1}\) s\(^{-1}\) in deuterium oxide at pH or pD 10.5. These data reflect a difference in proportion of protonated (deuterated) enzyme at pH (pD) 10.5. The rate of the pH-dependent protein fluorescence quenching of the free enzyme was estimated using a pressure-jump fluorimeter, in the laboratory of Professor H. Gutfreund of the University of Bristol, and 0.1 M borate buffer so that the change in pressure resulted in a change in pH. At pH 9.5, a single process was observed with a relaxation time of 300 to 500 \(\mu\)s. Since a pH of 9.5 is close to the pK\(_a\) of the group involved in the alkaline fluores-
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... fluorescence quenching, it is reasonable to assign a value of 1000 to 1500 s⁻¹ to the conformational change. This rate is too rapid to be observed in stopped flow studies of NAD⁺ binding. Attempts were made to determine the rates of quenching or fluorescence increase by pH jump studies in both directions in a stopped flow fluorimeter. However, even at 8° the reaction was too rapid to be observed.

**DISCUSSION**

The results of this study can best be interpreted in terms of two separate linked ligand-mediated conformational transitions; one associated with the deprotonation of an enzyme functional group and the other with the binding of NAD⁺. Regarding the alkaline quenching of tryptophan fluorescence (pKₐ = 9.8), structural considerations eliminate energy transfer to tyrosinate anion as a plausible explanation. Each subunit of the dimeric enzyme contains 2 tryptophan residues, Trp-15 and Trp-314 (5). Trp-15, located at the surface of the molecule, would already be extensively quenched by solvent. Trp-314, buried at the subunit-subunit interface, probably provides most of the protein fluorescence. There are 4 tyrosine residues within 30 Å of Trp-314:Tyr-180, Tyr-246, and Tyr-286 of each subunit. Estimates of R₀, the distance for 50% efficiency of energy transfer from tryptophan to tyrosinate, range from 8.4 Å (18) to 11.2 Å (19). These lead to expectations of 2 and 12% quenching, clearly inconsistent with the observed 40% quench. In fact, energy transfer to tyrosinate is quite consistent with the small additional quench at alkaline pH of the already extensively quenched ternary complex E·NAD⁺·trifluoroethanol (Fig. 5e). With energy transfer ruled out, the inescapable conclusion is that the alkaline quench is brought about by a change in the environment of Trp-314, i.e., a pH-dependent conformational change.

It is already clear that the quenching concomitant with NAD⁺ binding is due to a conformational change (6). This study also demonstrates that, although most of the fluorescence quenching is associated with the preceding transition, NAD⁺ binding promotes an additional conformational change. The evidence for linkage between proton binding and NAD⁺ binding is straightforward. NAD⁺ binding shifts the apparent pKₐ to a lower value, or, equivalently, NAD⁺ binds more tightly to the unprotonated form of the enzyme. The intrinsic pKₐ (in the absence of NAD⁺) is 9.8. Although saturating amounts of NAD⁺ could not be achieved at low pH because of trace alcohol contaminants, the limiting perturbed pKₐ is consistent with the value of 7.6 obtained in earlier direct studies of proton liberation (2). The pKₐ, perturbation means that NAD⁺ binds to the unprotonated form of the enzyme 10⁻³ = 160 times more tightly than to the protonated form, yet kinetically the reaction is the protonated form which preferentially binds NAD⁺ (1). Additional support for this kinetic route (and for the equilibrium linkage scheme) comes from measurements of NAD⁺ binding kinetics in H₂O and in D₂O (Fig. 6). The pKₐ for the alkaline quench shifts to 10.6 in D₂O (Fig. 1). The enzyme is 17% protonated at pH 10.5, whereas it is 56% deuterated at pH 10.5. Accordingly, the rate constant for NAD⁺ binding should be 3.3 times greater in D₂O. The experimental ratio of 2.6 is remarkably close, considering the inaccuracy of the pKₐ estimates.

The studies with the hydrophobic fluorescent probe, TNS, provide further substantiation of the pH-dependent conformational equilibrium. This work confirms the previous report that TNS competes with NAD⁺ for the binding site (16) and extends the validity of the observation to alkaline pH. The titrations imply that the relative quantum yield of bound TNS is invariant with pH (Fig. 2). This was corroborated by a similar invariance in the fluorescence polarization. Fluorescence polarization, related to the fluorescence lifetime of the chromophore and the rotational relaxation time of the protein, would change significantly if either parameter changed. The absence of a change argues for a constant fluorescence lifetime, hence a constant quantum yield. Changes in polarization would be detected for changes in rotational relaxation time associated with gross unfolding of the protein (20) or dissociation of the dimer to monomers (21). Although fluorescence of TNS is normally interpreted as being due to binding through hydrophobic interactions, the dye is negatively charged. The binding of ADP-ribose, a fragment of the NAD⁺ molecule, also becomes weaker with increasing pH (22). It is conceivable that TNS interacts with Arg-47, the group responsible for binding the pyrophosphate of ADP-ribose (5). The guanidine moiety of arginine has a pKₐ of 12.5, making that an unlikely candidate for explaining the pH dependence of binding.

The most plausible single explanation for the common pH dependence of protein fluorescence, ADP-ribose binding, TNS binding, and NAD⁺ binding rate is a pH-dependent conformational change which simultaneously exposes Trp-314 to solvent and covers the hydrophobic coenzyme binding site. Scheme 1...
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The alkaline conformation of the enzyme is represented by \( *E^p \). \( E^{p'} \) is the form that can bind \( \text{NAD}^+ \) or its analogs. When \( \text{NAD}^+ \) is the ligand does \( E_{XAD} \) proceed to \( *E_{XAD} \), a new conformation. The rationale for the existence of this state proceeds as follows. First, since \( \text{NAD}^+ \) binds 160 times more tightly to the unprotonated form of the enzyme, some explanation must be sought for the exclusive role of the protonated form in the kinetic route. The failure of \( E^{X'} \) to proceed directly to \( E_{XAD} \) suggests that an essential intermediate is formed only with great difficulty (if at all). No such difficulty is encountered when Group X is protonated. The simplest candidate for the intermediate is the direct addition product of enzyme with \( \text{NAD}^+ \) (or analog). A subsequent conformational change suffices to make the enzyme in the liganded unprotonated state \( (*E^{Xp}_{XAD}) \) distinct from the unliganded unprotonated state \( (*E^p) \), a necessary corollary. Second, it is difficult to explain the \( pK_a \) perturbation of 2.2 pH units by simple electrostatic interaction between \( \text{NAD}^+ \) and the proton. The distances required for coulombic interaction are not unreasonable, but if such forces dominated the perturbation, it might be involved in an acid-base catalysis mechanism for the hydride transfer. Considering the central role of Group X in the conformational equilibrium and in alcohol binding, it might be involved in an acid-base catalysis mechanism for the hydride transfer.

To recapitulate, the transition \( E \leftrightarrow *E \) involves the ionization of Group X with a \( pK_a \) of 9.8, the quenching of tryptophan fluorescence, and the masking of the ADP ribose binding site. The transition \( *E \leftrightarrow *E \) differs in having a \( pK_a \) of 7.6. The transition \( E \leftrightarrow *E \) perturbs the \( pK_a \) of Group X and probably involves binding the nicotinamide moiety of \( \text{NAD}^+ \). The species to which alcohol binds, \( *E_{XAD} \), thus has undergone two distinct conformational changes from the free enzyme which exists at neutral pH. Concurrent with or immediately subsequent to the hydride transfer step, the \( pK_a \) of Group X returns to 9.8 and the enzyme reverts to the \( *E^{Xp} \) conformation (at neutral pH). Considering the central role of Group X in the conformational equilibrium and in alcohol binding, it might be involved in an acid-base catalysis mechanism for the hydride transfer.

REFERENCES

pH-dependent conformational states of horse liver alcohol dehydrogenase.
J K Wolfe, C F Weidig, H R Halvorson and J D Shore


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