pH-Dependent Changes of Intrinsic Fluorescence of Chemically Modified Liver Alcohol Dehydrogenases

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(Received 24 October 1977)

Horse liver alcohol dehydrogenase specifically carboxymethylated on cysteine-46 (a ligand to the zinc in the active site) or acetimidylated on 25 of the 30 lysine residues per subunit (including residue 228) was studied. The tryptophan fluorescence of these enzymes decreased by 35% as pH was increased, with an apparent pK_a of 9.8 ± 0.2 , identical with that of native enzyme. Native enzyme in the presence of 30 mM-imidazole, which displaces a water molecule ligated to the zinc, also had a pK_a of 9.8. The ionizable group is thus neither the water molecule nor one of the modified groups. Binding of NAD^+ shifted the pK_a for the fluorescence transition to 7.6 with native enzyme and to 9.0 with acetimidylated enzyme, but did not shift the pK_a of carboxymethylated enzyme. Binding of NAD^+ and trifluoroethanol, an unreactive alcohol, gave maximal fluorescence quenching at pH 7 with all three enzymes. The acetimidylated enzyme- NAD^+ -trifluoroethanol complex had an apparent pK_a of 5.0, but the pK of the native enzyme complex was experimentally inaccessible. The results are interpreted in terms of coupled equilibria between two different conformational states. On binding of NAD^+ , the modified enzymes apparently change conformation less readily than does native enzyme, but binding of alcohol can drive the change to completion.

The tryptophan fluorescence of horse liver alcohol dehydrogenase (EC 1.1.1.1) depends on the state of ionization of a group (or coupled system) on the enzyme with an apparent pK_a of 9.8 ± 0.2 , where the fluorescence of the alkaline form is quenched by about 35%. Binding of NAD^+ shifts the apparent pK_a of the quenching curve towards 7.6, and formation of the ternary complex with NAD^+ and trifluoroethanol quenches maximally at neutral pH (Wolfe *et al.*, 1977) and results in the release of one proton per active site (Shore *et al.*, 1974). Wolfe *et al.* (1977) concluded that conformational changes cause the quenching, but they did not identify the ionizable groups involved. Studies on the enzyme kinetics and the binding of NAD^+ previously revealed pK_a values of about 9 for free enzyme and 7 for the enzyme- NAD^+ complex (Dalziel, 1963; Taniguchi *et al.*, 1967). Theorell and co-workers (Theorell & McKinley-McKee, 1961; Taniguchi

et al., 1967) suggested that a water molecule ligated to the zinc ion at the active site (Eklund *et al.*, 1976) controls binding of NAD^+ . Another candidate for the ionizable group is the ϵ -amino group of lysine residue 228, which is close to the adenosine ribose (Eklund *et al.*, 1974). Chemical modification of this amino group can increase the enzyme activity up to 20-fold by increasing the rates of breakdown of the enzyme- NAD^+ complex (Sogin & Plapp, 1975; Dworschack *et al.*, 1975). In order to attempt to identify the group involved in the fluorescence quenching and to assess its role in conformational changes, we chose to study chemically modified enzymes. Coenzyme-induced conformational changes have now been characterized crystallographically (Brändén, 1977) and a pressure-induced conformational relaxation has been described (Coates *et al.*, 1977).

Carboxymethylation of the sulphur atom of cysteine-46, which is ligated to the zinc in the active site (Eklund *et al.*, 1976), partially inactivates the enzyme (Reynolds & McKinley-McKee, 1975), but does not change the structure of the protein as observed by X-ray crystallography at a resolution of 0.45 nm (Zeppezauer *et al.*, 1975). However, for-

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mation of the neutral thioether ligand should increase the net positive charge on the zinc atom (Zeppezauer *et al.*, 1975) and decrease the pK_a of the water bound to the zinc (Woolley, 1975). Perhaps lower basicity of zinc-bound hydroxide ion provides an explanation for the 700-fold lower rate of hydrogen transfer in the carboxymethylated enzyme (Hardman, 1976). Similarly, acetimidylation of amino groups raises the pK_a by about 2 pH units (Hunter & Ludwig, 1962). We have titrated the protein fluorescence of the modified enzymes as a function of pH and examined the effects of the formation of binary and ternary complexes.

Materials and Methods

Alcohol dehydrogenase was prepared from horse livers by a modification of the method of Theorell *et al.* (1966). NAD^+ (grade II) was from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.; for rapid-titration experiments NAD^+ (Boehringer Corp. (London), Ealing, London W5 2TZ, U.K.) was purified by the method of Stinson & Holbrook (1973). Trifluoroethanol and ethyl acetimidate were purchased from Aldrich Chemical Co., Gillingham, Dorset SP8 4BR, U.K., and fluorimetric-grade imidazole was from Sigma. Iodoacetic acid (BDH Chemicals, Poole, Dorset, U.K.) was recrystallized from heptane.

Carboxymethylation of alcohol dehydrogenase was carried out at pH 7.9 as described previously (Reynolds & McKinley-McKee, 1975; Hardman, 1976). Enzyme was acetimidylated as described previously (Dubied *et al.*, 1977), except that four additions of ethyl acetimidate were used and the modified enzyme was freed of ethanol and by-product by gel filtration and dialysis overnight.

The rapid titration of the protein fluorescence of the modified enzyme as a function of pH was carried out on the apparatus described by Shore *et al.* (1975a). The protein fluorescence was excited at 297 nm and emission was measured through a Kodak no. 18A filter (about 340 nm). The enzyme ($1 \mu M$ in active sites) was initially in 5 mM-sodium phosphate buffer containing 45 mM- Na_2SO_4 , pH 7.0, and the fluorescence of the apoenzyme form of either the native or chemically modified alcohol dehydrogenase was set to 100% for any given series of experiments. Although we did not make direct comparisons of the intrinsic fluorescence of the different chemically modified forms of the enzyme we did not notice major differences between them. The pH was changed by addition of 2M-NaOH or 2M-formic acid, and the protein fluorescence and pH were monitored simultaneously at 25°C (Wolfe *et al.*, 1977). Each titration was completed in 200 s. The record of each experiment was averaged from 2048 10-bit bytes to 256 8-bit bytes and stored on a digital recorder. The

results of a series of experiments could then be superimposed on a storage oscilloscope by using an Altair 8800a computer. The NADH-recycling system of Shore *et al.* (1977) was used when NAD^+ was present.

The binding of NAD^+ at pH 7 to the carboxymethylated enzyme ($2.5 \mu M$) was detected by observing the effect of NAD^+ (0, 0.9 mM, 1.8 mM) on the apparent dissociation constant for NADH binding ($2.5 \mu M$, $10 \mu M$, $16.5 \mu M$) and the results were analysed as described by Stinson & Holbrook (1973). $[NADH]$, varied from 0 to $50 \mu M$ depending on $[NAD^+]$.

Measurements of rapid changes in protein fluorescence on mixing the carboxymethylated enzyme with NAD^+ , or with NAD^+ and ethanol, were carried out on a stopped-flow fluorimeter (Bagshaw *et al.*, 1972). The excitation monochromator was set at 288 nm and the fluorescence was observed through Schott WG345 and UG11 filters. The photomultiplier signals were stored by using a Data Laboratories DL 905 transient recorder, transferred to magnetic tape and subsequently analysed by means of a FOCAL program on a PDP-8e computer (Hardman *et al.*, 1978). Buffers for stopped-flow experiments were sodium phosphate (pH 7.0 or 8.0) or sodium glycinate (pH 9.8), 1.0 l.

Results

pH titration of carboxymethylated enzyme

The pH of a solution of carboxymethylated alcohol dehydrogenase ($1 \mu M$ in enzyme active sites) was increased from 7.0 to 10.8 and the protein fluorescence was recorded as a function of pH (Fig. 1a). The trace is almost identical with one previously obtained for native enzyme (Fig. 1b). The apparent pK_a of the transition monitored by protein fluorescence quenching is about 9.6 in the carboxymethylated enzyme, as in the native enzyme. On the basis of the rationale presented in the introduction, these results imply that the group with pK_a of about 9.8 is not the zinc-bound water molecule. (The estimated pK_a values of the titrations in this work are ± 0.2 .)

This conclusion was further substantiated by the observation that the pK_a of native enzyme was not shifted by the presence of 30 mM-imidazole (well above the 1 mM dissociation constant; Theorell & McKinley-McKee, 1961), which displaces the water molecule on the zinc without altering the structure of the protein (Boiwe & Brändén, 1977).

With the native enzyme, NAD^+ binding lowers the apparent pK_a from 9.8 to 7.6 (Wolfe *et al.*, 1977). In contrast, even at an NAD^+ concentration of 0.9 mM, the pK_a of the carboxymethylated enzyme shifts by less than 0.2 unit (Fig. 2). Nevertheless, the protein fluorescence of a mixture of carboxymethylated

Fig. 1. pH-dependence of the intrinsic fluorescence of carboxymethylated and native liver alcohol dehydrogenases (a) Carboxymethylated enzyme; (b) native enzyme. For experimental conditions, see the text.

Fig. 2. pH-dependence of protein fluorescence of binary and ternary complexes of carboxymethylated enzyme —, Modified enzyme alone; ----, including 0.9 mM-NAD⁺; ····, including 90 μM-NAD⁺ and 13 mM-trifluoroethanol. Values are percentages of the values with enzyme alone at pH 7.

enzyme with NAD⁺ and trifluoroethanol is quenched by about 35% over the pH range 7–9 (Fig. 2). We conclude that the ternary complex with trifluoroethanol and NAD⁺ is in the alkaline form at pH 7, just as is the ternary complex with unmodified enzyme (Wolfe *et al.*, 1977). The slight additional quenching between pH 9 and 10.5 after the enzyme is in the alkaline form is very similar to that observed with the

Fig. 3. Time course of protein-fluorescence quenching on mixing carboxymethylated alcohol dehydrogenase with NAD⁺ and ethanol

Syringe 1 contains 4.6 μM-modified enzyme and syringe 2 2 mM-NAD⁺ and 330 mM-ethanol. Reaction was pH 7.0 in phosphate buffer (10.1). The curve shown is the average of two transients.

native enzyme (Wolfe *et al.*, 1977) and is due to tyrosine ionization.

Rates and strength of complex-formation with carboxymethylated enzyme

When carboxymethylated alcohol dehydrogenase was mixed with NAD⁺ and ethanol in the stopped-flow fluorimeter, a rapid quenching of protein fluorescence was observed (Fig. 3), followed by a slow additional quenching (not shown) due to the steady-state production of enzyme-bound NADH. Only two phases were observed, compared with three for the native enzyme (Shore *et al.*, 1975b), because hydrogen transfer is rate-limiting for the modified enzyme (Reynolds & McKinley-McKee, 1975; Hardman, 1976). The rate constant for the rapid phase is $270 \pm 70 \text{ s}^{-1}$ for the carboxymethylated enzyme at pH 7.0, and this value is effectively the same as that for the native enzyme ($300\text{--}500 \text{ s}^{-1}$; Shore *et al.*, 1975b). A biphasic process was also observed at pH 8.0 and pH 9.8; the rate constant for the rapid phase is lower at higher pH ($50 \pm 10 \text{ s}^{-1}$ at pH 9.8).

However, when the modified enzyme was mixed with NAD⁺ (2 mM or 10 mM after mixing) in the absence of ethanol, there was little or no quenching of fluorescence (less than 10% of the change observed in the presence of ethanol). This result is consistent with the absence of an effect of NAD⁺ in the titration experiment.

A possible explanation for our failure to observe any effect of NAD⁺ alone is that the binding of NAD⁺

to the modified enzyme is so weak that a negligible proportion of enzyme sites contained NAD^+ under the conditions of our experiments. This explanation has been excluded. Firstly, the kinetically determined dissociation constant for the carboxymethylated enzyme in the binary complex with NAD^+ is $53\mu\text{M}$ at pH 7.4 (Reynolds & McKinley-McKee, 1975). Secondly, we showed that NAD^+ could bind to the modified enzyme by observing competition between NAD^+ and NADH . The dissociation constant for NAD^+ from the binary complex, obtained by the method of Stinson & Holbrook (1973), was $0.2 \pm 0.1\text{ mM}$ at pH 7.0; for the native enzyme at the same pH it is 0.13 mM (Taniguchi *et al.*, 1967). Thus the NAD^+ concentrations used in our rapid-titration and stopped-flow experiments were at least 3 times the dissociation constant of the modified enzyme- NAD^+ binary complex, and therefore most of the enzyme was in the form of binary complex with NAD^+ yet no quenching of protein fluorescence was observed.

pH titration of acetimidylated enzyme

The protein fluorescence of free, acetimidylated enzyme showed an apparent pK_a of 9.8, the same as native enzyme (Fig. 4). This result implies that the amino groups of lysine-228, and any of the other 25

Fig. 4. pH-dependence of fluorescence of acetimidylated alcohol dehydrogenase and its complexes

These five experiments were performed (from right to left): (i) without additions; (ii) in the presence of 1 mM-NAD^+ ; (iii) with $0.13\text{ mM-trifluoroethanol}$ and 1 mM-NAD^+ ; (iv) with $1.3\text{ mM-trifluoroethanol}$ and 1 mM-NAD^+ ; (v) with $130\text{ mM-trifluoroethanol}$ and 1 mM-NAD^+ . (The curves appear as dotted lines because the results were stored in digital form and later displayed at 8-bit precision as a composite on an oscilloscope.) The decrease in fluorescence above pH 10.2 in (i) is due to time-dependent denaturation. The decrease in (ii) above pH 9.1 is due to production of NADH from ambient ethanol.

of 30 lysine residues that are acetimidylated, are not directly involved in the fluorescence quenching.

When NAD^+ was added to the enzyme solution, the apparent pK_a was shifted to lower values. But even at 1 mM-NAD^+ the pK_a was 9.0 ± 0.2 (Fig. 4), substantially higher than the value of 8.1 observed with native enzyme in $100\mu\text{M-NAD}^+$ (Wolfe *et al.*, 1977). Since the dissociation constant of the acetimidylated enzyme- NAD^+ complex is similar to that for native enzyme in the pH range 7–8.75, and less than $100\mu\text{M}$ in the pH range 8–8.75 (Zoltobrocki *et al.*, 1947; Dworschack, 1976), the acetimidylated enzyme should have been nearly saturated with NAD^+ at 1 mM .

When NAD^+ and trifluoroethanol were both added to the acetimidylated enzyme, the apparent pK_a values decreased in the manner predicted for a simple coupled system, with a change of 1 pH unit for a change of 10-fold in the concentration of trifluoroethanol (Fig. 5). At saturating concentrations of trifluoroethanol, a limiting pK_a value of about 5.0 was obtained, which is apparently the pK_a value for the enzyme- NAD^+ -trifluoroethanol complex. Since the binding of NAD^+ and trifluoroethanol are coupled equilibria, NAD^+ could not have dissociated from the complex in the pH 5 region, unless the binding of NAD^+ showed a higher-power dependence on H^+ concentration than the binding of trifluoroethanol does.

Fluorescence of native enzyme complexes at low pH

Since the inhibition constant for trifluoroethanol decreases logarithmically as a function of pH and

Fig. 5. Dependence of the apparent pK_a of acetimidylated enzyme on the concentration of trifluoroethanol

The protein fluorescence of acetimidylated enzyme ($1\mu\text{M}$ in sites) in the presence of 1 mM-NAD^+ and varied concentrations of trifluoroethanol was determined as a function of pH. The apparent pK_a values were estimated from the titration curves (such as those in Fig. 4) and are plotted as a function of the concentration of trifluoroethanol.

does not reach a limiting value as low as pH 5.5 (Shore *et al.*, 1974), we may conclude either that the pK_a is below 4.5 or that formation of enzyme-NAD⁺-trifluoroethanol displaces the ionizable group. [If the group were water bound to zinc, binding of the ethanol could displace the water, as in one mechanism proposed for catalysis (Brändén *et al.*, 1975).] To confirm this conclusion with native enzyme and to compare the behaviour of native and acetimidylated enzymes, native enzyme was titrated to low pH in the presence of NAD⁺ and trifluoroethanol.

Fluorescence titration of native enzyme in the presence of 1 mM-NAD⁺ and increasing concentrations of trifluoroethanol decreased the apparent pK_a value, until at pH 4 the trifluoroethanol-dependent change in protein fluorescence was obscured by an increase in fluorescence that was independent of added trifluoroethanol. These experimental difficulties precluded studies at lower pH. Thus the observed change in tryptophan fluorescence appears to be greater than 35%. Irreversible denaturation of the enzyme did not occur at a significant rate until pH 3.9 within 10 s. Thus with native enzyme there was no evidence of a protonated high-fluorescence form of enzyme-NAD⁺-trifluoroethanol complex at or above pH 4, where the enzyme is still reasonably stable.

Discussion

We may draw three major conclusions from the results of these studies. First, it appears that the group controlling protein fluorescence with a pK_a of about 9.8 is not the water molecule ligated to the zinc in the active site or the sulphur of cysteine-46 or the amino group of lysine-228. Furthermore, it is unlikely that the group is one of the other ligands to the zinc, the sulphur of cysteine-174 or the imidazole of histidine-67, since the introduction of the negatively charged carboxymethyl group should perturb the pK_a values of nearby groups by at least 0.5 unit, which could be detected by our titration methods. Since the apparent pK_a for protein fluorescence is still about 9.8 when imidazole has displaced the zinc-bound water, it definitely appears that the water does not have a pK_a of 9.8 and furthermore is not coupled to the group(s) controlling fluorescence.

It should be noted, however, that these results do not exclude a role for the zinc-bound hydroxide ion as the basic group involved in the removal of the hydroxy-group proton of the substrate in synchrony with hydride transfer (Blackwell & Hardman, 1975; Dworschack & Plapp, 1977). Brooks *et al.* (1972) have shown that a group with pK_a 6.4 must be unprotonated for maximum rates of hydrogen transfer catalysed by native enzyme, and Dworschack

& Plapp (1977) found a corresponding pK_a of 8.4 with an activated enzyme with modified amino groups. Since the pK_a determined from protein fluorescence titrations and proton-release experiments is less than 4.5, the basic group involved in catalysis may be different from the one controlling the fluorescence quenching.

The second major conclusion is that both protonated and unprotonated forms of the acetimidylated enzyme-NAD⁺-trifluoroethanol complex exist. Most importantly, this means that the binding of trifluoroethanol does not displace from the enzyme the ionizable group controlling protein fluorescence. It also means that fluorescence quenching is ultimately controlled by an ionizable group, not by the binding of trifluoroethanol. Although the limiting pK_a of 5.0 was observed with the acetimidylated enzyme, we think that the native enzyme must have the corresponding pK_a with a somewhat lower value. The modified enzyme is, after all, a quite respectable catalyst for alcohol dehydrogenation, being 2.6 times more active than the native enzyme at pH 8 (Zoltobrocki *et al.*, 1974). For some reason, the modification has made the pK_a experimentally accessible.

The equilibria shown in Scheme 1 are coupled, that is the binding constants of NAD⁺ and trifluoroethanol are pH-dependent and the apparent pK_a values for fluorescence quenching depend on the ligand concentrations. We assume that only one ionizing group is involved, and its pK_a can be perturbed by more than 5 units. (Although more complicated mechanisms involving two ionizable groups can also explain the data, we have chosen the simplest mechanism consistent with the available data.)

Two forms of enzyme, with different intrinsic fluorescence, are shown, and we should like to propose what these two forms may be. Since native free enzyme crystallizes at pH 8.4 in an orthorhombic space group (C222₁), and most ternary complexes, and particularly the enzyme-NAD⁺-trifluoroethanol complex (B. V. Plapp, H. Eklund & C.-I. Brändén, unpublished work), crystallize in another conformation in the triclinic P1 space group (Zeppezauer *et al.*, 1967), it is reasonable and most simple to interpret the scheme as showing conformational equilibria. Then the high-fluorescence form would correspond to the orthorhombic conformation and the low-fluorescence form to the triclinic conformation. (Just why the tryptophan fluorescence should differ in the two conformations requires high-resolution structural studies.) It would follow that deprotonation of the enzyme is coupled to the conformational change, as was proposed previously (Wolfe *et al.*, 1977). (Our interpretation that the fluorescence quenching in this case involves conformational changes does not mean that we think quenching

Scheme 1. *Unification of observations on the protein fluorescence titrations*

Abbreviations used: Nat, native enzyme; CM, carboxymethylated enzyme; AI, acetimidylated enzyme.

by other ligands, such as NADH or *o*-phenanthroline, must also involve conformational changes. Different agents can quench fluorescence by different mechanisms.)

The third major conclusion follows from this discussion. It appears that NAD⁺ alone or trifluoroethanol (in the presence of NAD⁺) can affect the fluorescence quenching and hence the conformational transition. The conformation of carboxymethylated enzyme, at pH 8 for instance, does not seem to be changed by the binding of NAD⁺, but the binding of both NAD⁺ and trifluoroethanol does change the conformation. In comparison, native enzyme would be largely changed by the binding of NAD⁺. This conclusion is consistent with the observation by Reynolds & McKinley-McKee (1975) that NAD⁺ binds no more tightly to carboxymethylated enzyme at pH 9.9 than at pH 7.4, whereas NAD⁺ binds much more tightly to native enzyme at high pH (Theorell & McKinley-McKee, 1961; Dalziel, 1963; Taniguchi *et al.*, 1967). Moreover, the carboxymethylated enzyme is catalytically active, from which we may infer that binding of ethanol leads to the triclinic conformation, which is the active form. The acetimidylated enzyme represents an intermediate situation. Thus it appears that the coupling of the binding of NAD⁺ and the conformational transition is impaired in the carboxymethylated and acetimidylated enzymes. Nevertheless, both enzymes can be driven into the other conformation with sufficient concentrations of alcohol. The mechanism of the linkage between ionization of the group with pK_a 9.8, the binding of ligands and the conformational change remains a subject for further investigation.

We thank Dr. J. F. Eccleston for assistance with the stopped-flow experiments. M. J. H. thanks Professor H. Gutfreund for his hospitality and for support from his Scientific Research Council grant. B. V. P. thanks the U.S. National Institute on Alcohol Abuse and Alcoholism for the support of Research Scientist

Development Award K02 AA00010. J. J. H. thanks The Royal Society and the Science Research Council for funds to construct the optical equipment of the rapid-titration apparatus. J. D. S. is grateful for support from the U.S. National Science Foundation (grant 74-04112 A02) and NATO grant 766.

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