Electrostatic effects of bound NADH and NAD$^+$ on ionizing groups in liver alcohol dehydrogenase

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The affinity of liver alcohol dehydrogenase for its coenzyme NAD$^+$ increases drastically when the pH is raised from 7 to 10, whereas the affinity for NADH remains essentially constant up to pH 9 and then decreases [1]. Theorell and coworkers early suggested that this differential effect of pH on the binding of oxidized and reduced coenzyme derives from electrostatic interactions of the NAD$^+$ nicotinamide ring charge with an ionizing water molecule bound at the active-site zinc ion of the enzyme subunit [2]. That idea formed an essential ingredient of the mechanism of enzyme action proposed by Kvassman and Pettersson [3], according to which all main effects of pH on the catalytic reaction in the pH range 6–10 reflect analogous ionizations of either zinc-bound water or of the zinc-bound alcohol substrate. In subsequent developments of this proposal, the pH dependence of NADH binding was attributed to electrostatic effects of the coenzyme pyrophosphate group on the ionization of zinc-bound water, which were assumed to shift the pK$_a$ of the latter group from 9.2 in free enzyme to 11.2 in the enzyme · NAD$^+$ complex [4, 5]. The pH dependence of NAD$^+$ binding was envisaged to derive from the combined effects of the coenzyme pyrophosphate group and nicotinamide ring charges causing a net perturbation of the pK$_a$ of zinc-bound water to 7.6 in the enzyme · NAD$^+$ complex.

The role thus proposed for zinc-bound water in the coenzyme binding process has been questioned on the basis of results obtained with chemically modified enzyme [6], metal-substituted enzyme [7–10], or enzyme depleted of catalytic zinc [11–13]. Tyr-286, Lys-228, His-51, and His-67 have been mentioned as alternative groups which could account for the pH-dependent affinity of native enzyme for its coenzymes [10–17]. In the latter cases, also, the stability of the enzyme-coenzyme complexes must be assumed to show a pH dependence reflecting minimally the energetic contributions provided by electrostatic interactions of the ionizing groups with the charges present in the coenzyme molecules.

We have now estimated the magnitude of such electrostatic binding energy contributions for different ionizing groups from the available structural information on the liver alcohol dehydrogenase system by application of a point-charge model and reported semi-empirical relationships for the effective dielectric permittivity characterizing charge interactions in water-accessible regions of proteins [18]. The results confirm that the drastic differential effect of pH on NADH and NAD$^+$ binding derives from ionization of zinc-bound water.

THEORY

The binding of a charged ligand to an enzyme may show a pH dependence reflecting electrostatic interactions with ionizing enzymic groups. If the ligand is positively charged, it will be more tightly bound when an adjacent ionizing group is unprotonated. Conversely, the presence of the positively charged ligand will stabilize the unprotonated form of the ionizing group such that the group shows a lower pK$_a$ in the enzyme-ligand complex than in free enzyme. The magnitude of this pK$_a$ shift may be used as a measure of the strength of the electrostatic interaction.

The relationship between the perturbed (K$'_a$) and unperturbed (K$_a$) acid dissociation constant for the ionizing group is given by

$$\Delta \mu_a - \Delta \mu_0 = R T \ln (K'_a/K_a)$$  \hspace{1cm} (1)

where $\Delta \mu_a$ and $\Delta \mu_0$ are the standard chemical potential changes for the ionizing group in the presence and absence, respectively, of the charged ligand. Eqn (1) can be expressed as

$$N \cdot \Delta w = 2.303 R T \cdot \Delta p K_a$$  \hspace{1cm} (2)

where $N$ is Avogadro's number and $\Delta w$ denotes the change in electrostatic free energy caused by introduction of the ligand.
charge. Applying a point charge model for the electrostatic interaction, we have

\[ \Delta \omega = \frac{q_1 q_2}{\varepsilon r} \]

where \( r \) stands for the distance between the interacting charges \( q_1 \) and \( q_2 \).

The value of the effective dielectric permittivity \( \varepsilon \) is dependent on the environment of the charges and cannot be readily estimated by theoretical considerations. Semi-empirical relationships for \( \varepsilon \) as a function of distance are available, however, and the scaled dielectric permittivity function reported by Mehler and Eichele [18] has been shown to lead to reasonable agreements between calculated and experimentally observed electrostatic \( \Delta pK_a \) shifts for ionizing groups in water-accessible regions of proteins. Using their expression for \( \varepsilon \), the \( \Delta pK_a \) shift for an ionizing monobasic group contributed by an adjacent monovalently charged group at zero ionic strength can be described as a function of the distance \( r \) between the interacting charges. The relationship thus obtained from Eqns (2) and (3) is shown in Fig. 1 and was used for all \( \Delta pK_a \) shift calculations reported below.

RESULTS

Electrostatic effects of the coenzyme charges

Table 1 lists the ionizing enzymic groups which have been suggested as possible contributors to the pH dependence of coenzyme binding to liver alcohol dehydrogenase. Distances between the proton-donating atom in these ionizing groups and the nitrogen atom of the nicotinamide ring of bound coenzyme were calculated from crystallographic data reported for the enzyme \( \cdot \) NADH \( \cdot \) dimethylsulphoxide complex [20, 21]. Since essentially identical binding positions have been observed for oxidized and reduced coenzyme in complexes crystallizing in the closed conformational state [21], these distances were taken to be representative for the electrostatic effect of the NAD\(^+\) nicotinamide ring charge on deprotonation of the ionizing groups. The corresponding \( \Delta pK_a \) shift contribution was estimated for each ionizing group by application of the relationship in Fig. 1; the results are given in Table 1. The \( \Delta pK_a \) shifts contributed by the two negative charges of the pyrophosphate group of bound coenzyme were similarly calculated with the simplifying assumption that the latter charges are located at the phosphorus atoms of the coenzyme (Table 1).

According to molecular orbital data presented by Pullman and Pullman [22], the positive charge on NAD\(^+\) is delocalized over the nicotinamide ring as shown in Table 2. More precise estimates of the \( \Delta pK_a \) shifts induced by the NAD\(^+\) ring charge were calculated with consideration of the charge distribution indicated by these quantum mechanical data. The results are detailed in Table 2 for zinc-bound water; the refined estimates of \( \Delta pK_a \) obtained for other ionizing groups did not differ significantly from those reported in Table 1.

Electrostatic effects of the enzyme conformational change

Coenzyme binding induces a conformational change of the liver alcohol dehydrogenase subunit which brings the catalytic metal site closer to the coenzyme binding region and hence alters the position of zinc-bound water relative to that of charged groups in the protein. The \( \Delta pK_a \) shift for zinc-bound water contributed by these positional changes was estimated for aspartate, glutamate, lysine and arginine residues within a radius of 1.5 nm from the ionizing group, distances between the interacting charges being calculated from crystallographic data determined for, respectively, free enzyme (open conformation [21, 23]) and the enzyme \( \cdot \) NADH \( \cdot \) dimethylsulphoxide complex (closed conformation [20, 21]). The results in Table 3 indicate that the open-to-closed conformational change per se has no major electrostatic effect on the acidity

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**Table 1. Electrostatic effects of the charges of bound coenzyme on the acidity of ionizing groups in the liver alcohol dehydrogenase subunit**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nicotinamide ring N1</th>
<th>Pyrophosphate group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \Delta pK_a )</td>
<td>( \Delta pK_a )</td>
</tr>
<tr>
<td>( r )</td>
<td>( \Delta pK_a )</td>
<td>( \Delta pK_a )</td>
</tr>
<tr>
<td>( nm )</td>
<td>( nm )</td>
<td>( nm )</td>
</tr>
<tr>
<td>Zn-H(_2)O</td>
<td>0.46</td>
<td>-3.0</td>
</tr>
<tr>
<td>His-51 (N(_3))</td>
<td>0.80</td>
<td>-1.0</td>
</tr>
<tr>
<td>His-67 (N(_3))</td>
<td>0.89</td>
<td>-0.7</td>
</tr>
<tr>
<td>Lys-228</td>
<td>1.39</td>
<td>-0.3</td>
</tr>
<tr>
<td>Tyr-286</td>
<td>2.64</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

**Table 2. Shift in \( \Delta pK_a \) for zinc-bound water contributed by the NAD\(^+\) nicotinamide ring charge**

Calculations based on the charge distribution indicated by reported [21] molecular orbital data

<table>
<thead>
<tr>
<th>Ring atom</th>
<th>Charge</th>
<th>Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta pK_a )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( nm )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>+0.415</td>
<td>0.46</td>
</tr>
<tr>
<td>C2</td>
<td>+0.135</td>
<td>0.46</td>
</tr>
<tr>
<td>C3</td>
<td>+0.064</td>
<td>0.41</td>
</tr>
<tr>
<td>C4</td>
<td>+0.189</td>
<td>0.35</td>
</tr>
<tr>
<td>C5</td>
<td>+0.052</td>
<td>0.35</td>
</tr>
<tr>
<td>C6</td>
<td>+0.145</td>
<td>0.41</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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![Fig. 1. Distance dependence of the shift in \( \Delta pK_a \) contributed by the coulombic interaction of an ionizing group with an adjacent negative charge. Relationship calculated from Eqns (2) and (3) for 25°C using the scaled dielectric permittivity function reported by Mehler and Eichele [18]](image)
of zinc-bound water, and similar results were obtained for other ionizing groups in the neighbourhood of bound coenzyme.

To a first approximation, therefore, the $pK_a$ shifts induced by coenzyme binding may be attributed mainly to electrostatic effects of the coenzyme charges, i.e. can be estimated as the sum of the $pK_a$ shift contributions indicated for each ionizing group in Table 1. The net $pK_a$ perturbation estimates thus obtained for NADH and NAD$^+$ binding are given in Table 4.

**Effect of the coenzymes on ionizing groups in enzyme depleted of catalytic zinc**

Removal of catalytic zinc from liver alcohol dehydrogenase does not cause any substantial structural changes of the enzyme [24], but yields a selectively metal-depleted derivative in which ionization of the freed zinc ligands Cys-46, Cys-174, and His-67 may contribute electrostatically to the pH dependence of coenzyme binding. The expected magnitude of these contributions was estimated as described above for other ionizing groups, distances between the interacting charges being obtained from the crystallographically determined structure of the complex formed between NADH and enzyme depleted of catalytic zinc [25]. The coenzyme phosphorus atoms and nicotinamide nitrogen atom were taken as the origin of the NADH and NAD$^+$ point charges, and the coenzyme-induced electrostatic $pK_a$ shifts calculated for the freed zinc ligands with these assumptions are given in Table 5.

**DISCUSSION**

**Effect of the NAD$^+$ nicotinamide ring charge**

Crystallographic results have shown that NADH and NAD$^+$ are bound at one and the same site in the liver alcohol dehydrogenase subunit through bonding interactions which appear to be essentially identical except for those which may be dependent on the difference in charge of the coenzyme nicotinamide ring [21]. This means that all pronounced differences in pH dependence between the binding of oxidized and reduced coenzyme can be anticipated to derive from electrostatic interactions of ionizing enzymic groups with the positive ring charge of NAD$^+$. The deprotonation of any ionizing group which interacts significantly with this ring charge would be expected [5, 26] to affect the quotient between equilibrium constants for the dissociation from the enzyme of reduced ($KE_R$) and oxidized ($KE_O$) coenzyme according to the relationship

$$\frac{K_{E,R}}{K_{E,O}} = \frac{1 + 10^{pK_{a,O} - pK_{a,R}}}{1 + 10^{pK_{a,R}}.}$$

where ($K_{E,R}/K_{E,O}$)0 denotes the limiting value of the quotient at low pH, and where the difference between $pK_a$ values for the ionizing group in the presence of bound NADH ($pK_{a,R}$) and NAD$^+$ ($pK_{a,O}$) reflects the strength of the electrostatic interaction as indicated by Eqs (1-3). If several ionizing groups interact with the NAD$^+$ ring charge, their deprotonation invariably will add a stabilizing contribution to NAD$^+$ binding relatively to NADH binding [26], and the accumulated effects will conform to the relationship

$$\frac{K_{E,R}}{K_{E,O}} = \frac{1 + 10^{pK_{a,O} - pK_{a,R}}}{1 + 10^{pK_{a,R}}.}$$

Equilibrium data for coenzyme binding to liver alcohol dehydrogenase are available for the pH range 6 – 12 and indicate that Eqn (4) applies with $pK_{a,O} = 7.6$ and $pK_{a,R} = 11.2$ [26]. This observation and other lines of evidence [27, 28] support attribution of the pronounced differential effect of pH on NADH and NAD$^+$ binding to a single ionizing group which has its $pK_a$ perturbed by 3.6 through the electrostatic effect of the NAD$^+$ ring charge. Results in Table 1 seem to establish that ionizing groups such as Tyr-286, Lys-228, His-51, and His-67 are far too distant from the nicotinamide binding site to undergo such a drastic $pK_a$ perturbation by the positive charge on NAD$^+$. The estimated binding energy contribution provided by the interaction of the latter charge with the charged form of the closest one of these ionizing groups (His-51) is more than two orders of magnitude too low to account for the observed differential effect of pH on the binding of oxidized and reduced coenzyme.

The calculated strength of interaction of the NAD$^+$ ring charge with a zinc-bound hydroxide ion, however, corre-
responds to a \( pK_a \) shift of 3.6 (Table 2). This datum is in remarkably excellent agreement with the experimentally determined \( pK_a \) shift for the ionizing group which causes the difference in pH dependence of NADH and NAD\(^+\) binding (\( \Delta pK_a = 3.6 \) [26]), and agrees also closely with the \( pK \) shifts observed for cyanide (\( \Delta pK = 3.4 \)) and decanoate (\( \Delta pK = 3.3 \)) binding to catalytic zinc in the presence of oxidized versus reduced coenzyme [26, 27]. It has been previously concluded from the magnitude of the latter \( pK \) shifts that electrostatic interactions of the NAD\(^+\) ring charge with zinc-bound anions (including the hydroxide ion) are of such exceptional strength that it becomes chemically unreasonable to attribute the experimentally observed pH dependence of the quotient \( K_{ER}/K_{EO} \) to ionization of a group distinct from zinc-bound water [26]. The present theoretical calculations corroborate that conclusion by confirming the experimental estimates of the electrostatic \( pK_a \) shift for zinc-bound water, and by showing that the \( pK_a \) shifts for other ionizing groups proposed to account for the differential effect of pH on NADH and NAD\(^+\) binding are of insufficient magnitude to explain the effect observed.

Effect of the coenzyme pyrophosphate group

Results discussed above leave little doubt that the ionizing group showing a \( pK_a \) of 7.6 in the enzyme \( \cdot \) NAD\(^+\) complex and 11.2 in the enzyme \( \cdot \) NAD complex has been correctly identified as zinc-bound water. The kinetic evidence indicating that the corresponding group exhibits a \( pK_a \) of 9.2 in the absence of bound coenzyme [4, 17, 29] is strongly supported by data in Tables 1 and 4, which establish that NADH binding must be assumed to cause a most significant decrease in acidity of zinc-bound water due to the electrostatic effect of the negatively charged pyrophosphate group of the coenzyme. The theoretically estimated magnitude (1.5) of this \( pK_a \) shift is in satisfactory agreement with the \( pK_a \) shift of 2.0 deduced from pH-dependence data for NADH binding [5], as well as with the \( pK \) shifts of 1.7 reported for the NADH-induced destabilization of cyanide [26] and decanoate [27] binding to catalytic zinc.

It may be concluded also from data in Table 4 that zinc-bound water is the only ionizing group among those considered which would be expected to show a decreased \( pK_a \) following NAD\(^+\) binding. All other groups appear to interact at least as strongly with the coenzyme pyrophosphate group as with the nicotinamide ring charge and, therefore, can be anticipated to exhibit an unchanged or increased \( pK_a \) in the enzyme \( \cdot \) NAD\(^+\) complex. The electrostatic effect of the coenzyme pyrophosphate group should be of particular significance in the case of Lys-228. Deprotonation of the latter group would be expected to destabilize drastically the binding of both NADH and NAD\(^+\) (Table 4), and the pH dependence thus contributed to the affinity of the enzyme for its coenzymes should show an onset at the \( pK_a \) for ionization of Lys-228 in free enzyme and extend in value by more than 2 towards higher pH. Such a destabilizing proton-dependent interaction affecting the binding of both oxidized and reduced coenzyme has been experimentally detected in the pH range 10–12 and was found to correspond to a \( pK_a \) of 10.4 for the unperturbed form of the ionizing group [5]. Since the latter datum agrees well with the expected \( pK_a \) for an unperturbed \( \epsilon \)-amino group, it seems reasonable to conclude that the observed \( pK_a \) of 10.4 dependence of NADH and NAD\(^+\) binding most likely derives from ionization of Lys-228.

According to Table 4, ionization of His-51 (first deprotonation step) and His-67 (second deprotonation step [10]) should affect mainly the binding of reduced coenzyme. While the latter ionization is unlikely to show a \( pK_a \) below 12 in free enzyme [30], the former could occur with a \( pK_a \) of 6–7 and might hence contribute detectably to the pH dependence of coenzyme binding within the experimentally examined pH range 6–12. Indeed, reported equilibrium data for NADH binding indicate the presence of minor pH-dependence contributions below pH 7 [1], in addition to the major effects deriving from the ionizations with \( pK_a \) 9.2 and 10.4. These minor effects of pH are qualitatively consistent with those expected for ionization of His-51 with a \( pK_a \) slightly above 6 in free enzyme, but are much less pronounced than indicated by the data in Table 4. This quantitative disagreement could be attributable to attenuation of the electrostatic interactions by solvent ions in the buffer solutions of 0.1 M ionic strength used for the experimental measurements; corrections for ionic strength (not included in Tables 1–5) may be of most significant magnitude for a surface group such as His-51 [18, 31].

Coenzyme binding to zinc-depleted enzyme

The possibility that ionization of zinc-bound water accounts for the major effects of pH on coenzyme binding to liver alcohol dehydrogenase in the pH range 7–10 has been questioned on basis of the observation of similar pH dependencies for coenzyme binding to enzyme selectively depleted of catalytic zinc [11–13]. As indicated by the results in Table 5, however, coenzyme binding to the metal-depleted enzyme should be strongly electrostatically affected by ionization of the freed protein ligands of catalytic zinc. The pH dependence of coenzyme binding thus contributed by ionization of Cys-174 would be expected to be similar to that contributed by zinc-bound water in native enzyme due to the similarity in strength of the electrostatic effects (Table 1 and 5) and in intrinsic acidity of the ionizing groups. Data in Table 5 should be interpreted with caution, however, since they refer to spatially neighbouring groups which cannot be assumed to ionize independently of each other. Furthermore, the mobility of the side-chains of the freed zinc ligands introduces some uncertainty as to their position when ionized in the presence of NAD\(^+\); the \( pK_a \) shifts for Cys-46 and Cys-174 contributed by the NAD\(^+\) ring charge could be considerably greater than indicated by the results in Table 5. On the other hand, there seems to be little doubt that the pH dependencies reported for coenzyme binding to zinc-depleted enzyme reflect electrostatic effects of the ionization of one (at least) of the freed zinc ligands. Observations made with zinc-depleted enzyme, therefore, would seem to provide confirmatory evidence for the energetic significance of charge interactions of the coenzymes with ionizing groups in the catalytic metal-site region of the enzyme, i.e., can be taken to strongly support, rather than bring into question, the role proposed for zinc-bound water in regulation of the pH dependence of coenzyme binding to native enzyme.

Concluding remarks

The present investigation establishes beyond reasonable doubt that the much-discussed major effects of pH on coenzyme binding to liver alcohol dehydrogenase derive from ionization of zinc-bound water and consequent electrostatic interactions with the charged groups on the coenzymes. The agreement between the calculated strength of these interac-
tions and that deduced from experimentally observed effects on coenzyme binding of pH [5] or ligation of anions to catalytic zinc [26, 27] is surprisingly good, considering the crudity of the method used to obtain the theoretical pK_a shift estimates. The point-charge model now applied accounts only for the coulombic interactions of the coenzymes with the charged state of the ionizing group, and does not explicitly consider the multitude of additional factors (e.g. dispersion, polarization, hydrogen bonding, enzyme conformational readjustments, and cooperative interactions of the ionizing groups) that might provide protonation-state-dependent contributions to the energetics of coenzyme binding. It would appear that such additional contributions either are of minor significance in the liver alcohol dehydrogenase system or implicitly accounted for by the semi-empirical dielectric permittivity function determined by Mehler and Eichele [18]. However that may be, data now reported lend credence to the latter authors' supposition that their approach for calculation of electrostatic pK_a shifts in proteins should lead to roughly correct results for enzyme systems in general.

With regard to the catalytic action of liver alcohol dehydrogenase, the present investigation provides the important inference that zinc-bound alcohols should be subjected to coenzyme-induced electrostatic pK_a shifts similar to those determined for zinc-bound water. This is consistent with the conclusions drawn from kinetic studies of alcohol binding and catalytic oxidation [29, 32] and strongly supports the mechanism of enzyme action proposed by Kvassman and Pettersson [3]. According to that proposal, ionization of the substrate in productive enzyme - NAD^+ - alcohol complexes represents a catalytic key step which exhibits a pK_a shifted to a value below 7 in order to ensure that the substrate is predominantly bound as an alkoxide ion at physiological pH. Data in Table 2 confirm the supposition that the positive nicotinamide ring charge on NAD^+ provides a most significant contribution to this pK_a shift, i.e. to stabilization of the alkoxide ion which may be considered as a transition-state intermediate in the catalytic process of hydride transfer from the alcohol substrate to NAD^+. Such coenzyme-dependent electrostatic transition-state stabilization may be a main energetic factor facilitating hydride transfer from substrate to NAD^+ in all reactions catalyzed by NAD- or NADP-dependent dehydrogenases.

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