**Coenzyme Interaction with Horse Liver Alcohol Dehydrogenase**

**EVIDENCE FOR ALLOSTERIC COENZYME BINDING SITES FROM THERMODYNAMIC EQUILIBRIUM STUDIES***

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**SUMMARY**

The techniques of fluorescence enhancement, fluorescence quenching, fluorescence polarization, and equilibrium dialysis are utilized to study the binding properties of coenzyme to horse liver alcohol dehydrogenase. Polarization of fluorescence and equilibrium dialysis show that NADH binds to alcohol dehydrogenase with a stoichiometry of 6 mol per mol of enzyme, in contrast to the value of 2 determined from fluorescence enhancement measurements. NAD+ also binds with a stoichiometry of six as was determined by equilibrium dialysis. The two NADH sites which bind coenzyme more tightly and which are revealed by fluorescence enhancement measurements are designated the catalytic sites. Binding of coenzyme to the four ancillary sites does not alter the quantum yield of NADH but results in a 20% contribution to quenching of enzyme's tryptophan fluorescence. From the emission anisotropy of bound NADH of 24.0% for the additional sites and 28.1% for the catalytic sites and their relative fluorescence lifetimes at the same wavelengths of excitation and emission, we conclude that the nicotinamide ring of NADH bound to the additional sites exhibits a freedom of motion independent of the macromolecule, while that bound to the catalytic sites is more rigidly held. Polarization of fluorescence yields negative intrinsic free energies of 9.2 and 7.5 Cal m−1 for NADH interaction with the catalytic and additional sites, respectively. Although these values are 1.3 to 2.0 Cal higher than those determined by fluorescence anisotropy and equilibrium dialysis, the mean Hill coefficient of 1.76 ± 0.06, the titration span of 2.4 logarithmic units and couping free energies (in magnitude and sign) are the same for all these techniques. The above difference in the intrinsic free energies are attributed largely to the different modes of interaction of excited and unexcited NADH molecules with alcohol dehydrogenase.

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‡ This work is a part of the thesis submitted by I. I. to Purdue University in 1972 in partial fulfillment of the requirement for the Ph.D. degree. Present address, Department of Chemistry, University of Ibadan, Ibadan, Nigeria.

Horse liver alcohol dehydrogenase (EC 1.1.1.1) is a two-subunit, nicotinamide adenine dinucleotide-dependent enzyme that catalyzes the interconversion of alcohols and aldehydes. Coenzyme interaction properties of this enzyme have been investigated previously by various authors (1-3). By the technique of enhancement of fluorescence of bound NADH it was shown (1) that the stoichiometry of binding is two, as might be anticipated for a protein with two identical subunits (4). In the course of comparative studies of the zinc-free alcohol dehydrogenase and native enzyme, it was observed that the latter possesses additional coenzyme binding sites (5-7).

In this paper, we report coenzyme binding properties of alcohol dehydrogenase by the techniques of fluorescence enhancement, fluorescence quenching, fluorescence polarization, and equilibrium dialysis. Our results indicate differences in and the complementary nature of these techniques. Kinetic evidence shows that the binding of coenzyme to the allosteric sites inhibits catalysis (5).

**EXPERIMENTAL PROCEDURES**

Reagents—Sodium phosphate buffer was employed in all experiments except where it is otherwise stated. Water used to prepare the buffer was doubly distilled. The last distillation step was carried out in the presence of KMnO₄ to remove traces of alcohols and aldehydes (2). Isobutyramide was the product of Eastman Organic Chemicals and was further purified by recrystallization from ethanol. Traces of ethanol were removed from the crystals in a dessicator containing Drierite (W. A. Hammond Drierite Co., Xenia, O.) and Parafilm (Marathon Products).

Both oxidized and reduced coenzymes were purchased from Boehringer-Mannheim Corp. and were more than 95% reducible or oxidizable with the liver enzyme. New England Nuclear was the source of [³H]NAD⁺ labeled in the C-4 position of the nicotinamide ring.

Preparation and Purification of ³H Labeled Coenzyme—[³H]NAD⁺ was diluted with unlabeled NAD⁺ and purified on a DEAE-cellulose column as was described previously (8). [³H]NAD⁺ was synthesized in batches by the reduction of purified [³H]NAD⁺ with an excess of ethanol in 0.1 M glycine buffer, pH 9.0, in the presence of high concentrations of yeast alcohol dehydrogenase as a catalyst. [³H]NAD⁺ was precipitated from this solution by the method of Lehninger (9).

Prior to use, a solution of the precipitated [³H]NAD⁺ was further purified on a DEAE-cellulose column equilibrated with 0.05 M bicarbonate buffer, pH 8.0. The purified [³H]NAD⁺ and [³H]NAD⁺ were finally desalted by passage through Sephadex G-15 columns (3 X 5 cm) equilibrated with pH 7.5 phosphate buffer, μ = 0.05. The concentrations of the coenzyme solutions were determined spectrophotometrically with a Gilford spectrophotometer.
Molecular Weight Determinations—The molecular weight of horse liver alcohol dehydrogenase was determined in the absence and presence of low and high concentrations of coenzyme by means of gel permeation chromatography on a calibrated Sephadex G-100 column. The eluate fractions were tested for alcohol dehydrogenase activity and for absorbance at 280 nm.

Purification and Determination of Concentration of Enzyme—Horse liver alcohol dehydrogenase was purchased from Worthington Biochemical as an ammonium sulfate suspension. Such suspensions were dissolved in a minimal volume of pH 7.5 phosphate buffer, \( \mu = 0.05 \). The resulting solution was dialyzed extensively against solutions of the same buffer. The enzyme was finally crystallized by dialysis for 24 hours at 4° against 8 to 10% (v/v) ethanol in phosphate buffer as has been described (10). The resulting solution was dialyzed extensively against 

\[ \text{NADH} \text{Solutions} \]

The elution was followed through a carboxymethylcellulose column with 

\[ \text{pH 7.5 phosphate buffer, } \mu = 0.05 \]

The eluate was removed with a microliter syringe after attainment of equilibrium. Dialysis experiments were performed to check the possibility of exchange of the solvent or enzyme was occurring.

Equilibrium Dialysis—Equilibrium dialysis and radioactivity counts of samples were performed as have been described previously (8). In each set of experimental determinations controls were run concurrently at the lowest and highest coenzyme concentrations to ensure attainment of equilibrium. Dialysis experiments were also performed to check the possibility of exchange of the solvent or enzyme was occurring. In the latter experiments, aliquots from the same buffer were removed with a microliter syringe after attainment of equilibrium and pipetted into an activated charcoal-pulp column (12) equilibrated with pH 7.5 phosphate buffer, \( \mu = 0.05 \). The eluate was collected in 1.0-ml fractions. Fractions showing ultraviolet absorption were tested both for enzyme activity and for radioactivity. All other fractions were also tested for radioactivity. No radioactivity was found in the eluate nor in the peak showing enzyme activity. This indicates no exchange of tritium with the solvent or enzyme was occurring.

Measurement of Fluorescence of NADH—Fluorescence intensity was measured at 90° to the excitation beam in an Aminco-Bowman Spectrofluorimeter with cell and slit positions modified as have been described (12). In the titration of binding sites, microliter amounts of a concentrated solution of NADH were added to a Hamilton microsyringe to a fixed concentration of enzyme in 2.0 ml of buffer. The concentrations of both enzyme and NADH were corrected for dilution.

Equation 1, derived by Laurence (13), was used to relate the fraction, \( \alpha \), of coenzyme bound to fluorescence intensity:

\[ \frac{A - \alpha}{\alpha} = \frac{(A - \alpha)}{(A - \alpha)} \]

where \( A \) is the measured fluorescence intensity of NADH in the presence of the enzyme, \( A_0 \) is the fluorescence intensity of the same concentration of NADH in the absence of the enzyme. \( \lambda \) is the enhancement factor and represents the ratio of the fluorescence intensity of bound NADH to that of the free NADH of the same concentration.

For the solution of the number of sites and the dissociation constant, \( \lambda \) was determined by the method of Laurence in which a fixed amount of coenzyme was titrated with an excess of enzyme until no further enhancement of the fluorescence intensity of NADH occurred. For routine determinations, in which the intrinsic dissociation constants under different conditions were to be compared, each \( \lambda \) was determined from the corresponding titration curve of a fixed concentration of enzyme with a variable concentration of NADH by use of the following equation:

\[ F = (\lambda - 1)F_0\left[ R_0 \right] + F_0 [R_0] \]

where \( F \) is the fluorescence intensity at any point in the titration, \( F_0 \) is the molar emission of the coenzyme, \([R_0] \) is the molar concentration of bound coenzyme, and \([R] \) is the sum of the concentration of the bound and free coenzymes. When all the sites that enhance coenzyme fluorescence are saturated \( [R] = n[E] \) where \( n \) equals the number of "fluorescent sites" and \( E \) the molar concentration of enzyme. Under this condition, a plot of \( F \) against \([R] \) should yield a straight line with slope \( = F_0 \) and intercept \( = (\lambda - 1)F_0\left[ R_0 \right] \) and \( \lambda = (\text{intercept/slope} \times n E) + 1 \) can be evaluated.

Fluorescence Quenching—The procedure and the instrument used for observing the quenching by coenzyme of enzyme’s tryptophan fluorescence have been described previously (11).

Polarization of Fluorescence of NADH—Polarization of fluorescence, \( P \), was measured with the same modified instrument described above, Glan prisms being mounted to replace Slits 2 and 5 on the excitation and emission pathways, respectively. In the titration of NADH against a fixed concentration of enzyme and vice versa, fluorescence intensities were measured at different settings of the polarizers and \( P \) was calculated as was previously described (6). Polarization was measured with a precision of \( \pm 0.005 \) and is related to emission anisotropy, \( A \), by the formula \( A = 2P/(3 - P) \).

The fraction, \( \alpha \), of coenzyme bound was related to emission anisotropy by Equation 3, derived (see Ref. 5 and “Appendix”) by use of the additivity of emission anisotropy principle of Weber (14) and the procedure of Laurence (13):

\[ A = 1 - \left( \frac{A_0 - A}{A_0 - A} \right) \]

where \( \lambda \) is the measured emission anisotropy at any point in the titration. \( A_0 \) is the emission anisotropy of bound NADH and was determined to be 28.17% from the asymptote of the titration curve of a variable enzyme concentration with a small concentration of NADH; \( F_0 \) and \( F \) are the measured fluorescence intensities of NADH in the presence and absence of enzyme, respectively.

The data at high NADH concentrations where more than an average of 2 mol of coenzyme were bound per mol of enzyme were then refined with Equation 4 (see “Appendix”).

\[ A + 1 = \frac{(A'_0 - A)}{(A_0 - A)} = 1 - \frac{(A - A)}{(A_0 - A)} \]

The second term of the left-hand side of Equation 4 arises from the additional stipulated conditions that there are two types of bound coenzyme in alcohol dehydrogenase, one with an emission anisotropy, \( A' \), and an enhancement factor \( \lambda' > 1 \), and the other with an emission anisotropy, \( A' \), and an \( \lambda' = 1 \). \( \alpha \) and \( \alpha' \) are the fractions of coenzyme bound to the first and second types of sites, respectively, and the sum of these two parameters is the total fraction of coenzyme bound. All the other terms have the meanings described in Equation 3. The practical utility of Equation 4 can be realized by multiplying through by \( [R] \) and setting \( A_0 [R] - n[E] \).

The only unknown, \( \alpha'[R] = [R] \), and the total amount of coenzyme bound, \( \alpha + \alpha'[R] \) can then be obtained.

RESULTS

NADH Interaction with Alcohol Dehydrogenase by Fluorescence Emission—In Fig. 1 is shown the fluorescence titration curve of NADH against a fixed amount of enzyme. At the titration point beyond which enhancement cannot be observed, the curve becomes a straight line parallel to that of the free coenzyme. The enhancement factor of 4.5, determined from this region of the curve under the condition that \([R] = n[E] \) in Equation 2, is in excellent agreement with those determined either by titration of enzyme against a fixed concentration of coenzyme or by dilution of a solution of enzyme-NADH complex with a NADH solution of concentration equal to that of the unbound coenzyme in the original solution (3).

The amount of coenzyme bound at each point in the titration of NADH against a fixed concentration of enzyme was determined by use of Equation 1. A representative Bjerrum plot (15) of the data is presented in Fig. 2. It shows a stoichiometry of two and
FIG. 1. Fluorescence titration curve at 20° of a variable amount of NADH against 0.5 μM enzyme in pH 7.5 phosphate buffer, μ = 0.05. The straight line represents that of titration of NADH against pH 7.5 phosphate buffer alone. Excitation was at 340 nm and emission was at 430 nm.

an intrinsic dissociation constant of 0.32 μM from the midpoint of the titration. Although most of the data in Fig. 2A are confined to a region where V > 1, it can be assumed that the formation curve is symmetrical about the midpoint. A span of 1.9 logarithmic units between V = 0.2 and 1.8 and the order of binding of 1.0 from the Hill plot (Fig. 2B) indicate a simple type of binding and that the sites are identical in agreement with previous findings (2, 3). The synthesized [3H]NADH, which contains only 0.1% radioactive coenzyme, is bound with approximately the same dissociation constant as the commercial NADH.

Coenzyme Interaction with Alcohol Dehydrogenase by Polarization of Fluorescence and Equilibrium Dialysis—Fig. 3A shows the fluorescence polarization curve as concentrated NADH solution was added to a fixed concentration of alcohol dehydrogenase and Fig. 3B is a replot of the data with the ordinate in logarithmic form. The solid curves in both plots represent the theoretical computer-generated titration curves with the assumption of two identical and equivalent sites for NADH, an intrinsic dissociation constant of 0.19 μM and an emission anisotropy of 28.1% for bound enzyme. The logarithmic plot of the experimental data clearly indicates that there are two types of sites exhibiting different Aₙ values for NADH bound to alcohol dehydrogenase.

The values of the emission anisotropy of bound coenzymes can be calculated from the data presented in Fig. 3. From the upper ordinate intercept of the logarithmic plot, the fluorescence emission anisotropy of coenzyme bound to the sites that bind coenzyme more tightly can be calculated to be 28.1% in excellent agreement with that determined by titrating an excess of enzyme against a fixed concentration of NADH at the same wavelengths of excitation (340 nm) and emission (430 nm). From the lower ordinate intercept (broken line), (A₀ - A_f) can be computed and hence A₂. The latter represents the mean of contributions from A₀ and A_f weighted according to their fractional sites. Equation 5 describes this relationship.

\[
\frac{\Delta_{b}}{\Delta} = \frac{\Delta_{b}}{\Delta_{L}} + \frac{\Delta_{b}}{\Delta_{S}}
\]

1 This approach will be published in detail elsewhere by I. I.
Table 1

Parameters for binding of coenzyme to alcohol dehydrogenase

<table>
<thead>
<tr>
<th>Technique</th>
<th>K_d</th>
<th>K_M</th>
<th>$\Delta F^0$</th>
<th>$\Delta F^0$</th>
<th>$\Delta F^0$</th>
<th>$j$</th>
<th>Log [coenzyme] span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence Enhancement (NADH)</td>
<td>0.32</td>
<td>-8.9</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescence Polarization (NADH)</td>
<td>0.19</td>
<td>3.0</td>
<td>-9.2 -7.5</td>
<td>-1.7</td>
<td>1.78</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Equilibrium Dialysis (NADH)</td>
<td>1.2</td>
<td>25</td>
<td>-8.2 -6.3</td>
<td>-1.9</td>
<td>1.65</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Equilibrium Dialysis (NAD+)</td>
<td>15</td>
<td>140</td>
<td>-6.6 -5.3</td>
<td>-1.3</td>
<td>1.74</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

Equation 8 predicts that in a plot of $F_i/F$ against $Q$, a straight line with an intercept = 1 and a slope = $K_Q = (K_{AQ} + k_{q} \tau_0)$ should be obtained. Fig. 5 gives the result of quenching by coenzyme of enzyme's tryptophan fluorescence. For either NADH or NAD+ as a quencher the curve is biphasic. The concentration of NADH at the point at which linearity breaks down is that required to saturate all the sites that enhance the fluorescence of bound NADH (Fig. 1). The second phase of each curve is therefore due to binding of coenzyme to the additional sites. From the breaking points in the curves 20% of the total quenching is calculated to be due to the binding of coenzyme to the

molecules. When $Q$ is small, as was the case in our experiments, the squared $[Q]$ term in Equation 7 drops out and

$$F_i/F = 1 + (K_{AQ} + k_{q} \tau_0)[Q]$$

Equation 8. In the analysis of the data for each titration curve, we gave $K_{AQ}$ its appropriate value and set $i = 1.0$, the Hill coefficient indicated by the technique of enhancement of NADH fluorescence.

Quenching of Enzyme's Tryptophan Fluorescence by Coenzymes—
The general equation which describes the quenching of a fluorophore, $A$, by a quencher $Q$, is given by Equation 7 (17-19).

$$F_i/F = (1 + K_{AQ}[Q])(1 + k_{q} \tau_0 + [Q])$$

In the analysis of the data for each titration curve, we gave $K_{AQ}$ its appropriate value and set $i = 1.0$, the Hill coefficient indicated by the technique of enhancement of NADH fluorescence.

FIG. 4. Bjerrum formation curves for the binding of coenzyme to alcohol dehydrogenase. a, binding of NADH by polarization of fluorescence as was described in Fig. 3. The branch curve (broken line) is that for the uncorrected data computed with Equation 3. b, [PH][NADH] interaction with 2.0 $\mu$M enzyme by equilibrium dialysis at $4^\circ$ in pH 7.5 phosphate buffer, $\mu = 0.05$. c, [PH] NAD+ interaction with 2.0 $\mu$M enzyme by equilibrium dialysis at $4^\circ$ in pH 7.5 phosphate buffer, $\mu = 0.05$.

FIG. 5. Quenching of enzyme's tryptophan fluorescence by coenzyme in pH 7.5 phosphate buffer, $\mu = 0.05$. The concentration of enzyme is 0.5 $\mu$M. Curve a is quenching by NADH and Curve b by NADH. The plots are constructed according to Equation 8.
The use of absorption and fluorescence spectroscopies in determining the stoichiometric number of ligand binding sites in macromolecules is based on the implicit assumption that every ligand bound has its absorption, or fluorescence spectrum or intensity, or both, perturbed. The reliability of the fluorometric technique in determining stoichiometry for dehydrogenases has been questioned formerly (27), since all the binding sites in some pyridine nucleotide-dependent dehydrogenases may not perturb the absorption or fluorescence spectrum of the coenzyme (to the same extent). The earlier discrepancy as to whether n-glyceraldehyde 3-phosphate dehydrogenase possesses three or four NAD+ binding sites, as was determined by the perturbation of the absorption spectrum of the coenzyme, has been ascribed to the inability of the fourth subunit to alter the spectrum of the bound coenzyme (28). The limitation of the perturbation technique in determining the total number of binding sites for a ligand is also exemplified in the binding of a drug, a pyrazolidinedione analog, to human serum albumin. This protein has been shown by fluorescence technique to have one binding site for a pyrazolidinedione analog, while equilibrium dialysis indicated two strongly binding and four weakly binding sites (29). A similar disparity is exhibited in the binding of dicoumarol to human serum albumin (30). The revelation of additional coenzyme binding sites in alcohol dehydrogenase by polarization of fluorescence and by equilibrium dialysis, techniques which do not necessarily impair the requirement that the chromophoric group in a ligand be perturbed, further underscores this limitation. Yet the results yielded by the fluorescence technique have been utilized to complement those of fluorescence polarization to allow simple but meaningful interpretations to be made. It is in this perspective that we emphasize the usefulness of these techniques.

The Bjerrum formation curves (Fig. 4) can be given two alternative interpretations: (a) there exist two independent types of sites with overlapping titration curves; (b) the binding of coenzyme to the second type of site is conditional upon binding to the first type of site, the conditional or coupling of free energies ($\Delta F^0$) being $-1.7$ and $-1.3$ Cal $\text{m}^{-1}$ for NADH and NAD+, respectively. The sign of the free energies indicate cooperativity of the first and second types of sites in their saturation by coenzyme. No distinctive choice can as yet be made between these two interpretations, which by themselves are oversimplified as each type of site contains more than one coenzyme binding site. A more complex system could be envisaged if binding is considered in terms of statistical rather than intrinsic free energies. Approaches to the theoretical estimates of the statistical free energies for relatively simple systems and the compatibility of such estimates with experimental data have been described (31-33).

The constancy of the molecular weight at high and low coenzyme concentrations excludes the possibility that the observed cooperativity, as is indicated by the Hill coefficient of 1.7 for the second type of sites, is due to dissociation of subunits and subsequent creation of new sites, a condition that would have amounted to the phenomenon called relaxation effect. The conclusion is therefore apparent that the generalization that in dehydrogenases one coenzyme is bound per subunit (34) cannot be extended to liver alcohol dehydrogenase.

It has been well established that alcohol dehydrogenase has two catalytic sites for the oxidation of ethanol (35). The two coenzyme binding sites, as was determined by fluorescence enhancement, therefore, have been equated logically with the catalytic sites. The finding that horse liver alcohol dehydrogenase possesses additional coenzyme binding sites poses the
problem of which really are the catalytic sites. We have shown previously that in the inactive zinc-free alcohol dehydrogenase only two coenzyme binding sites are detectable by the technique of fluorescence polarization and equilibrium dialysis (6, 8). These sites were shown to be equivalent in the binding of NAD+ and have the same intrinsic dissociation constant as the two "tight" binding sites in the native enzyme. In addition a mole of the zinc-free enzyme-coenzyme complex binds either 2 mol of substrates or substrate analogs to form ternary complexes. The above two facts suggest that the two sites in the zinc-free enzyme-coenzyme complex binds either 2 mol of NAD + and have the same intrinsic dissociation constant as the two sites previously that in the inactive zinc-free alcohol dehydrogenase of fluorescence polarization and equilibrium dialysis (6, 8).

The equivalence of the quantum yield of the free coenzyme and that bound to the additional sites and lack of perturbation of the absorption spectrum of such bound coenzyme lead to the conclusion that the fluorescence lifetimes and the environment of the fluorophore are the same, and that possibly the nicotinamide ring is hanging out from the macromolecule into solution. A theoretical estimate of the emission anisotropy of such bound coenzyme can be made from the ratio of the emission anisotropies of the bound and free coenzymes.

\[ A_1 = A_2 \left( \frac{1 + \frac{3n_2}{n_1}}{1 + \frac{3n_1}{n_2}} \right) \]  

Equations 14 and 15 can be written to relate the fraction of coenzyme bound to the two types of sites, respectively, at any point in the titration and F = F f (1 - α - α') + dF f (16) becomes Equations 16 and 17, respectively.

\[ F = F f (1 - \alpha - \alpha') + \omega F_f + \alpha F_f \] (14)

\[ F = F f (1 - \alpha - \alpha') + \frac{\omega F_f}{(1 - \alpha - \alpha') + \omega} \] (15)

where \( \alpha \) and \( \alpha' \) are the fractions of coenzyme bound to the two types of sites, respectively, at any point in the titration and \( F \) and \( F_f \) are the fluorescence intensities of the coenzyme in the presence and absence of enzyme, respectively. \( A_f \) is the emission anisotropy of free NADH, and \( A \) is the measured emission anisotropy representing the average of the emission anisotropies of all bound and free forms weighted according to their fractional intensities. For any species, \( i \), in solution, the fluorescence intensity \( F_i = F_{fi} + 2F_{fi} \).

When \( \lambda' = 1 \), as is the case for the additional sites in alcohol dehydrogenase (see Fig. 1), Equations 14 and 15 become Equations 16 and 17, respectively.

\[ F = F f (1 - \alpha - \alpha') + \omega F_f \] (16)

\[ F = F f (1 - \alpha - \alpha') + \frac{\omega F_f}{(1 - \alpha - \alpha') + \omega} \] (17)

By substituting \( \lambda \) from Equation 16 into Equation 17 and rearranging, we obtain Equation 4 in the text. Setting \( \alpha' = 0 \) in Equation 4 yields Equation 3 for the simple system.

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