Nevertheless, the ability to enrich for active DNA segments, independently of the normal RNA–DNA hybridization procedure, adds to the repertoire of approaches to the mechanism of gene regulation in eucaryotes.

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Role of Zinc in Horse Liver Alcohol Dehydrogenase. Coenzyme and Substrate Binding†

Idowu Iweibo and Henry Weiner*

ABSTRACT: Formation of binary and ternary complexes of horse liver alcohol dehydrogenase (EC 1.1.1.1) and the zinc-free (apo) form of the enzyme has been investigated by fluorescence techniques. Both the native enzyme and the catalytically inactive apoenzyme bind NAD, NADH, and ADP-Rib, all of which quench the tryptophan's fluorescence emissions of the enzymes. The dissociation constants for the coenzymes are not significantly different for the two forms of the enzyme. In contrast to the native enzyme, the two sites in the apoenzyme do not appear to bind NADH independently. The sites are independent, however, in the binding of NAD in both apo- and native enzymes. The apoenzyme forms ternary complexes of coenzyme and substrate competitive inhibitors, such as isobutyramide and nitroethanol, with dissociation constants comparable to those of the native enzyme. The apoenzyme also forms an enzyme–NAD–butanol complex. The demonstration that the apoenzyme binds coenzymes and forms enzyme–coenzyme–substrate (or inhibitor) complexes with essentially the same dissociation constants as the native enzyme's rules out a role for zinc in binding coenzyme or substrate.

Zinc is known to be a constituent of many metalloproteins. Among the best-studied zinc enzymes are perhaps carboxypeptidase and carbonic anhydrase. It has been found that zinc in these two enzymes directly interacts with the substrates for the enzymes and presumably participates in catalysis (Lipscomb *et al.*, 1968; Wang and Riepe, 1968). Alcohol dehydrogenase (EC 1.1.1.1) is one of the few pyridine nucleotide dependent oxidoreductases that is a zinc-containing

† From the Department of Biochemistry, Purdue University, Lafayette, Indiana 47907. *Received October 22, 1971.* Supported in part by National Science Foundation Grant GB 18489 and a Purdue University Cancer Grant from the Indiana Elks Club. Journal Paper No. 4579 from the Purdue University Agriculture Research Station. Paper II of the series, where paper I is by Hoagstrom *et al.* (1969).

metalloenzyme. The two-subunit horse liver alcohol dehydrogenase, LADH,[1] contains two zinc atoms per subunit (Åkeson, 1964; Oppenhiemer *et al.*, 1967; Drum *et al.*, 1969), while the four-subunit yeast alcohol dehydrogenase possesses one zinc atom per subunit (Vallee and Hoch, 1955). The actual role of zinc in either enzyme is not known, but both are inactive if zinc is chelated or removed (Kagi and Vallee, 1960; Drum *et al.*, 1967). It has been suggested that zinc in the liver enzyme might play a role in the maintenance of structure (Drum *et al.*, 1967), in binding coenzymes and/or substrate (Theorell and McKinley-McKee, 1961) or in functioning catalytically as a general acid (Abeles *et al.*, 1957). Evidence has been presented which suggests that the two zinc atoms in each subunit of liver alcohol dehydrogenase perform different functions (Drum *et al.*, 1967; Drum and Vallee, 1970). Also, preliminary X-ray data suggest that the two zinc atoms in the subunit are actually in different environments (Branden *et al.*, 1969).

We have previously shown that a zinc-free (apo) liver alcohol dehydrogenase binds NADH with essentially the same K_d as the native enzyme (Hoagstrom *et al.*, 1969), and also, that a spin-labeled analog of NAD binds nearly as well to the apoenzyme as to the native enzyme (Weiner, 1969). Thus, zinc in horse liver alcohol dehydrogenase is not essential for binding coenzymes.

Quenching of the intrinsic fluorescence of tryptophan when coenzyme binds to some dehydrogenases has been reported (Velick, 1958; McKay and Kaplan, 1964; Geraci and Gibson, 1967). Recently, elaborate fluorescence quenching studies have been made on LADH to determine coenzyme binding (Theorell and Tatemoto, 1971; Luisi and Favilla, 1970). We have extended the observed quenching by coenzyme of the fluorescence of tryptophan of LADH to determine whether coenzymes and substrates can bind to the apoenzyme. In this paper we discuss the necessity of zinc in substrate binding and in the accompanying paper (Coleman *et al.*, 1972) we shall discuss the role of zinc in the maintenance of structure.

Experimental Section

Reagents. Sodium phosphate buffers were used except where Tris buffers are indicated. Metal-free buffers were prepared by extracting a concentrated buffer solution with dithizone (100 mg/l. of carbon tetrachloride) according to the procedure of Vallee and Gibson (1948). Water used to prepare the buffer was doubly deionized and then distilled. Coenzymes were obtained from Boehringer-Mannhein Corp. and adenosine diphosphoribose from Sigma and were used without further purification. L-Tryptophan, *N*-acetyl-L-tryptophan, and *N*-acetyl-L-tryptophanamide were Sigma products. Nitroethanol was purchased from Aldrich Chemical Co., Inc. Butanol was purchased from Baker and redistilled before use. Fresh solutions of coenzymes and substrates were prepared daily. Coenzyme and tryptophan concentrations were determined spectroscopically with a Gilford spectrophotometer. Bromoacetaldehyde diethyl acetal, purchased from Aldrich Chemical Co., Inc., was hydrolyzed to bromoacetaldehyde with Dowex 50 (H^+) resin as described by Ballou and Fischer (1956). The pH of the solution was adjusted to 6.7 before use. Bromoacetaldehyde concentration was determined by oxidation to bromoacetic acid with an excess of NAD and a horse liver aldehyde dehydrogenase isolated in this laboratory (Feldman and Weiner, 1972). The absorbance at 340 nm of the NADH produced when the reaction is complete corresponds to bromoacetaldehyde concentration.

Enzymes. Horse liver alcohol dehydrogenase was purchased from Worthington Biochemicals and exhaustively dialyzed against phosphate buffers before use. The enzyme was homogeneous on DEAE- and CM-cellulose columns. Titration of catalytic sites with isobutyramide (Winer and Theorell, 1960) and the specific activities, determined by the method of Dalziel (1957), indicated a purity greater than 95%. Apo-(alcohol dehydrogenase) was prepared as described previously (Hoagstrom *et al.*, 1969) except that dialysis to remove the zinc was against 1.0 mM EDTA in phosphate buffer, μ = 0.05, for 18 hr at 4° instead of against 0.1 mM EDTA for 72 hr, as was originally described.

Enzyme concentration was determined by the absorbance at 280 nm, and the number of titratable sulfhydryl groups was determined by reacting with Ellman's reagent as previously described (Hoagstrom *et al.*, 1969). The apoenzyme prepared in 1.0 mM EDTA contained 26 sulfhydryl groups/molecule as does the native enzyme (Jornvall, 1970), which suggests that the partial loss of sulfhydryl groups in our previous preparation (Hoagstrom *et al.*, 1969) was due to oxidation during the longer hours of dialysis.

Kinetics. The initial velocities of the oxidation of ethanol were determined in the presence and absence of nitroethanol at constant NAD concentration and various ethanol concentrations in phosphate buffer, pH 7.5, μ = 0.05, by monitoring the appearance of NADH on an Aminco fluoromicrophotometer. The latter was equipped with excitation filter 4-7113 (Corning 760) and an emission filter 4-7116 (Wratten 2A). The rate of bromoacetaldehyde reduction was determined by following the disappearance of NADH fluorescence.

Fluorescence Experiments. An Aminco-Bowman spectrophotofluorometer was used in all experiments with a 1 × 1 cm quartz cuvet. To minimize light scattering, glan prisms supplied by the company were mounted on the cuvet holders to replace slits 2 and 5 on the excitation and emission paths, respectively (Chen, 1966). The emission intensities at 90° to the excitation beam were recorded on a Sargent recorder while emission spectra were recorded on an Autograph X–Y recorder.

The cell and slit positions in the instrument were altered according to the method described by Chen and Hayes (1965) to diminish the distance between the cuvet surface and the optical path in the excitation pathway and the distance through which the emitted light passed before reaching the cuvet surface. Samples were allowed to equilibrate at 20° with the thermostatic cuvet holders. Binary complexes were formed by adding small aliquots of a concentrated solution of coenzyme or ligand to the solution of enzyme in 2.0 ml of buffer. Ternary complexes were formed by adding small aliquots of the concentrated solution of substrates or analogs to a buffered solution of enzyme containing an excess of cofactor in order to ensure saturating all the binding sites. Only substrate analogs, which act as substrate competitive inhibitors, could be used for forming ternary complexes with coenzyme-bound native enzyme. In the case of the catalytically inactive apoenzyme both inhibitors and real substrates were employed to study the formation of ternary complexes.

Equation 1, derived by Parker and Barnes (1957), was ap-

[1] Abbreviations used in this paper are: LADH or E, horse liver alcohol dehydrogenase; apo, zinc-free alcohol dehydrogenase; native, zinc-containing alcohol dehydrogenase; ADP-Rib, adenosine diphosphoribose; IBA, isobutyramide; K_d, dissociation constant of a ligand from its complex; K_i, kinetic inhibitor constant.

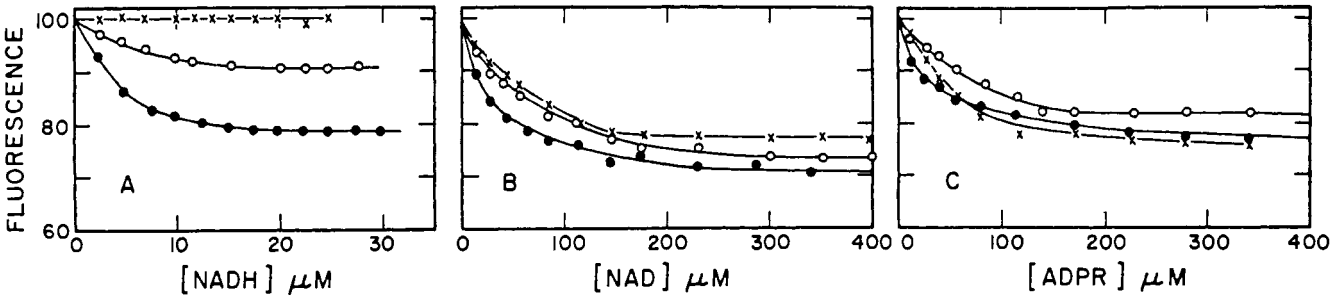

FIGURE 1: Quenching of indole fluorescence. 2.82 μM *N*-acetyltryptophanamide (×), 2.55 μM *N*-acetyltryptophan (○), and 2.67 μM tryptophan (●) by cofactors (A) NADH, (B) NAD, (C), ADP-Rib. The above relative fluorescence data have been corrected for quenching by absorbing molecules. The experiment was performed in pH 7.5 phosphate buffer, $\mu = 0.05$ at 20°. Excitation was at 280 nm and emission at 350 nm.

plied to correct for the overall concentration quenching of all absorbing molecules

$$\frac{F_0}{F} = \frac{2.3D(d_2 - d_1)}{10^{-Dd_1} - 10^{-Dd_2}} \quad (1)$$

where F is the measured fluorescence intensity, F_0 is the fluorescence intensity that would have been observed in the absence of inner filter effect, D is the optical density of the solution, d_2 is the optical path length, and d_1 the distance between the inner surface of the cuvet and the surface of the optical volume. The modification of cell and slit positions such that $d_1 = 0$ allowed corrections to be applied with certainty even to solutions in a millimolar range of cofactor concentrations.

The concentration of bound ligand was obtained from eq 2,

$$[L]_{bound} = \frac{nE_T\left(\frac{F}{F_i} - 1\right)}{\left(\frac{F_f}{F_i} - 1\right)} \quad (2)$$

where $[L]_{bound}$ is the molar concentration of bound ligand, E_T is the total molarity of enzyme, n is the number of binding sites and equals 2 for LADH, F_i is the initial fluorescence of the enzyme in the absence of ligand, F is the fluorescence at any point in the titration, and F_f is the final fluorescence when all the enzyme sites are saturated with ligand. F_f/F_i is the enhancement or quenching ratio. The assumption incorporated in eq 2 is that every ligand bound not only causes a change in the fluoresence of the enzyme but does so to the same degree.

Results

Fluorescence of Tryptophan Derivatives in the Presence of NAD, NADH, ADP-Rib, and Ethanol. It has been well established that nicotinamide derivatives form molecular complexes with tryptophan (Shifrin, 1967). The data presented in Figure 1 indicate that the degree of quenching of indole fluorescence is a function not only of the form of the coenzyme, but also of the derivative of tryptophan. To assess the role of the adenine portion in the quenching process, ADP-Rib was also investigated. From the curves in Figure 1C compared to those in 1A and 1B, it can be readily observed that the adenine portion of the coenzymes also quenches the fluorescence of trytophan. The data from Figure 1 were plotted according to the equation of Shinitzky *et al.* (1966) to obtain a quenching constant K_Q.

$$\frac{F_i}{F} = 1 + K_Q[Q] \quad (3)$$

where F_i and F are the fluorescence intensities in the absence and presence of the quencher Q. $K_Q = K_a + k\tau$, where K_a is the association constant for the formation of a complex, k is a diffusion-controlled collision constant, and τ is the fluorescence lifetime. The calculated values for K_Q are tabulated in Table I. The quenching by cofactors, except that of tryptophan by ADP-Rib gave linear plots as predicted by eq 3.

By assuming that tryptophan or one of its derivatives has a fluorescence life $\tau = 4$ nsec (Spencer and Weber, 1969) and that the diffusion-controlled collision rate constant $k = 10^{11}$ M^{-1} sec^{-1}, a maximum value for $k\tau = 0.4$ mM^{-1}. Since the data in Table I show that K_Q was between 2 and 30 mM^{-1}, the quenching was mainly due to complex formation.

The fluorescence spectrum of *N*-acetyltryptophanamide was measured in 95% and in 20% aqueous ethanol (v/v), the excitation wavelength being 280 nm. Compared to the fluorescence emission at 370 nm in pH 7.0 phosphate buffer, the fluorescence yields in 95 and 20% ethanol were 160 and 135%, respectively.

Fluorescence of Liver Alcohol Dehydrogenase in the Presence of Various Ligands. Representative fluorescence spectra of native enzyme and its binary and ternary complexes are presented in Figure 2. Data such as these made possible measurement of the fluorescence change at a particular wavelength as coenzyme and substrate were added to the enzyme. Figure 3 shows the fluorescence quenching titration curves by cofactors. The ratio of moles of cofactor bound to a mole of enzyme at any point in a curve was calculated by the use of eq 2. The Scatchard plot (Scatchard, 1949), presented in Figure 4, for the binding of NADH to native enzyme is essentially linear with a n value of 2.3± 0.1 and K_d of 1.2 ± 0.04 μM. The Scatchard plot for the apoenzyme is, however, biphasic. For the site that binds more tightly $n_1 = 1.0 \pm 0.15$ and $K_d = 0.82 \pm 0.06$ μM. Thus the fluorescence quenching technique and fluorescence polarization technique (Hoag-

TABLE I: Quenching Constants for the Interaction of NAD, NADH, and ADP-Rib with Tryptophan and Two Derivatives.

	Quenching Constant (K_Q) (mM^{-1})		
Tryptophan Derivative	NADH	NAD	ADP-Rib
N-Acetyltryptophanamide		2.4	2.8
N-Acetyltryptophan	8.8	3.4	1.9
Tryptophan	27.4	5	

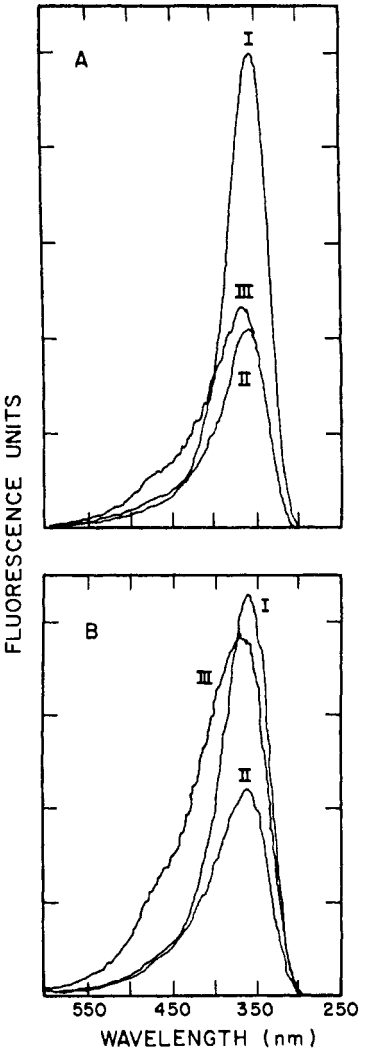

FIGURE 2: (A) Emission spectra (excitation at 280 nm) of native enzyme (I), native enzyme–NAD (II), and native enzyme–NAD–nitroethanol (III). Conditions were 0.47 μM enzyme, 300 μM NAD, and 0.3 mM nitroethanol in P_i, pH 7.5, μ = 0.05 at 20°. The spectra are uncorrected for quenching due to absorbance of the molecules. (B) Emission spectra (excitation at 280 nm) of apoenzyme (I), apoenzyme–NAD (II), and apoenzyme–NAD–nitroethanol (III). Conditions were 0.5 μM enzyme, 100 μM NAD and 1.2 mM nitroethanol in pH 7.5 phosphate buffer, μ = 0.05 at 20°. The spectra are uncorrected for quenching due to absorbance of the molecules.

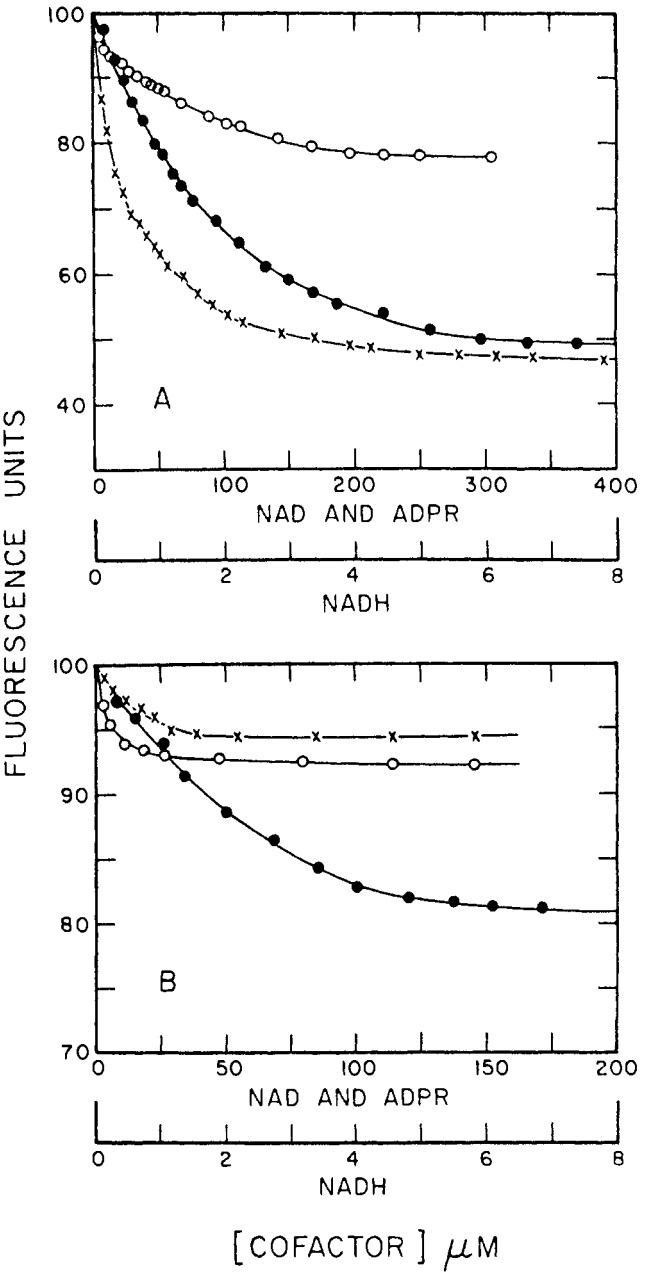

FIGURE 3: Fluorescence quenching titration curves of native (A) and apo- (B) enzyme with ligands: NADH (●), NAD (×), ADP-Rib (○). The experiments were performed at 20° in phosphate buffer pH 7.5, μ = 0.05. Native, enzyme concentration was 0.47 μM and the apoenzyme's 0.5 μM. The solution was excited at 280 nm and emission intensity was recorded at 350 nm. The experimental points were corrected for inner filter effect (see text).

strom *et al.*, 1969) give qualitatively the same results for NADH binding to apoenzyme. The interaction of NAD and ADP-Rib with apoenzyme appear to fit well with the assumption made in eq 1 as indicated by the linearity of the Scatchard plots in Figures 5 and 6. The Scatchard plot for ADP-Rib interaction with the native enzyme is, however, biphasic. In Table II are tabulated the K_d's for NADH, NAD, and ADP-Rib interacting with native and apo(alcohol dehydrogenases).

Double-reciprocal plots of the initial velocities *vs.* concentration of ethanol in the absence and presence of a known concentration of nitroethanol showed that nitroethanol is a competitive inhibitor of ethanol, suggesting that nitroethanol binds in the same site as ethanol in the ternary complexes. The K_i calculated from the data is 0.8 mM. The spectra of binary and ternary complexes in Figure 2 reveal that addition of nitroethanol (and butanol in the case of the apoenzyme) causes a gross alteration of the emission spectrum of the binary complex. Not only is the enzyme's fluorescence emission at 370 nm less quenched in the ternary than in the binary complex but a general broadening of the emission spectrum above 400 nm occurs with the ternary complex formation. The observed changes in fluorescence at 450 nm as nitroethanol was titrated against enzyme–NAD complex were related to the amount of nitroethanol bound by eq 2. The Scatchard plots of the results of such an analysis, as presented in Figure 7, reveal that between 2 and 3 moles of nitroethanol bind per mole of either native or apoenzyme with K_d's of 0.4 and 1.4 mM, respectively.

As the apoenzyme is catalytically inactive, substrates were also used to form the ternary complexes. 1-Butanol mimicked

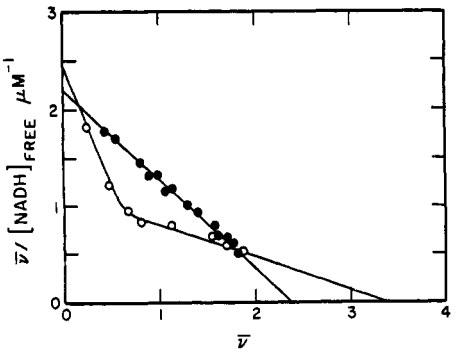

FIGURE 4: Scatchard plots for the binding of NADH to native (●) and apo- (○) enzymes. $\bar{\nu}$ is the ratio of molar concentration of bound ligand to molar concentration of enzyme. Conditions were as described in Figure 3.

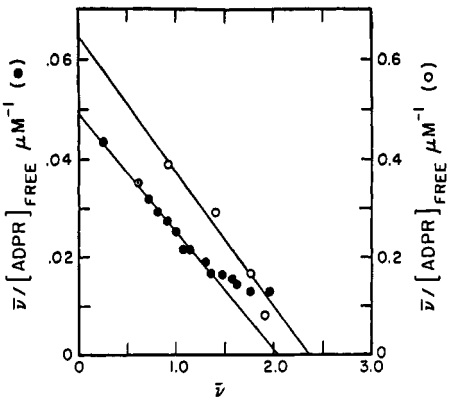

FIGURE 6: Scatchard plots for the binding of ADP-Rib to native (●) and apo- (○) enzymes. $\bar{\nu}$ is the ratio of molar concentration of bound ligand to molar concentration of enzyme. Conditions were as described in Figure 3.

the results found for the apo–NAD–nitroethanol complex and hence the data are not presented. Again, essentially two moles of butanol bind per mole of enzyme. The ADP-Rib ternary complexes of native enzyme and apoenzyme with both 1-butanol and nitroethanol were obtained in a similar manner. The results of titrating the ADP-Rib binary complexes with substrate or inhibitor yielded the K_d's presented in Table III.

Isobutyramide forms a ternary complex with native enzyme–NADH and produces a large increase in the fluorescence of bound NADH (Winer and Theorell, 1960). The formation of the ternary complex could also be followed by measuring the change in quenching of the tryptophan emission. The two titrations, involving either enhancement of bound NADH's fluorescence or a change in the quenching of enzyme fluorescence, yielded the same stoichiometry for the reaction: $E\text{-}(NADH)_2(IBA)_2$.

At the excitation and emission wavelengths of 340 and 430 nm, respectively, it was found for the apoenzyme that the fluorescence of bound NADH is enhanced only 5% over free NADH's compared to the 500% observed in the presence of native enzyme. In our previous paper (Hoagstrom *et al.*, 1969) this 5% enhancement of NADH fluorescence by the apoenzyme was not observed because we did not use as sensitive a setting on the fluorometer. In the isobutryamide ternary complex the fluorescence of apoenzyme-bound NADH was less than 10% of that of the native enzyme ternary complex. A sensitive setting on the fluorometer, however, allowed the formation of ternary complexes to be followed as isobutryamide was added to preformed apoenzyme–NADH complex. Analysis of data by eq 2 and then by the method of Scatchard (Figure 8) revealed that essentially 2 moles of aldehyde inhibitor bind to either native or apoenzyme with approximately the same K_d. The dissociation constants for the various ternary complexes are summarized in Table III.

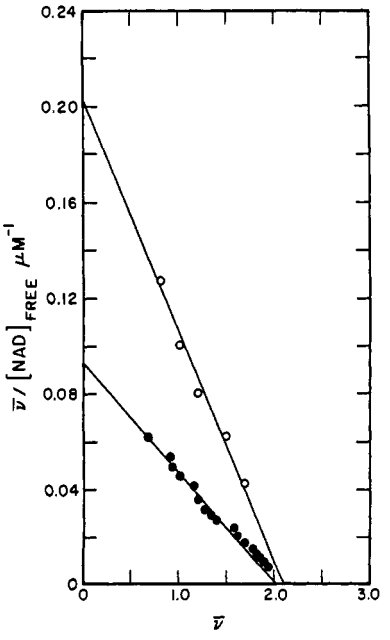

FIGURE 5: Scatchard plots for the binding of NAD to native (●) and apo- (○) enzymes. $\bar{\nu}$ is the ratio of molar concentration of bound ligand to molar concentration of enzyme. Conditions were as described in Figure 3.

Discussion

The fluorescence data presented in this study clearly show that apo(alcohol dehydrogenase) is capable of forming binary complexes with NADH, NAD, and ADP-Rib. This is in excellent agreement with what we have previously shown with NADH by fluorescence polarization (Hoagstrom *et al.*, 1969) and with a spin-labeled analog of NAD by electron spin resonance and proton relaxation rates techniques (Weiner, 1969;

TABLE II: Interaction of NAD, NADH, and ADP-Rib with Native and ApoLADH at pH 7.5, 20°.

Binary Complex	K_d (μM)
Apo–NADH	0.82 ± 0.06[a]
Apo–NAD	10.4 ± 0.5
Apo–ADP-Rib	3.5 ± 0.3
Native–NADH	1.2 ± 0.04
Native–NAD	21.7 ± 0.5
Native–ADP-Rib	39.5 ± 0.4[b]

[a] By polarization of fluorescence a K_d of 0.27 was obtained (Hoagstrom *et al.*, 1969). [b] K_d for ADP-Rib was calculated from the initial slope of Figure 4. The calculated errors are standard deviations.

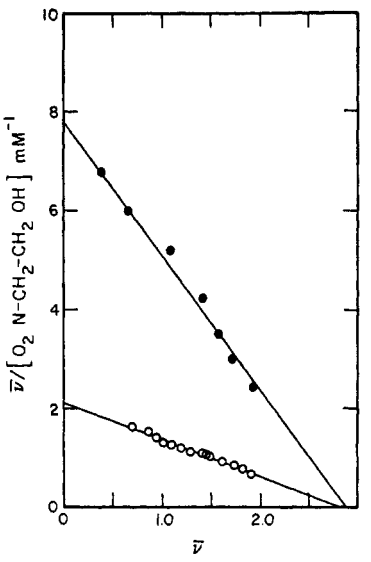

FIGURE 7: Scatchard plots for the binding of nitroethanol to preformed NAD binary complexes of native (●) and apo- (○) enzymes. $\bar{\nu}$ is the ratio of molar concentration of bound ligand to molar concentration of enzyme. Conditions were for the native enzyme 0.47 μM enzyme and 300 μM NAD, and for the apoenzyme 0.5 μM enzyme and 100 μM NAD, in pH 7.5 phosphate buffer, μ = 0.05 at 20°. Excitation was at 280 nm and emission at 350 nm.

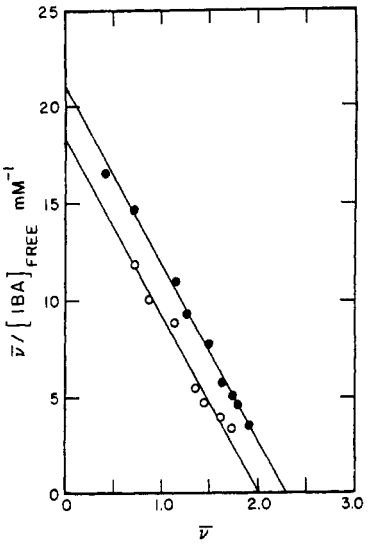

FIGURE 8: Scatchard plots for the binding of isobutyramide to performed NADH binary complexes of native (●) and apo- (○) enzymes as measured by fluorescence enhancement. Conditions were, for native enzyme, 0.47 μM enzyme and 7.0 μM NADH, and for the apoenzyme, 0.5 μM enzyme and 6.0 μM NADH. Buffer was pH 7.5 phosphate, μ = 0.05 at 20°. Excitation and emission wavelengths were 340 and 430 nm, respectively.

Mildvan and Weiner, 1969a). Using fluorescence enhancement and quenching, we now further show that the apoenzyme can form ternary complexes with both inhibitors and substrates. Thus, zinc is not required by the enzyme to form complexes with alcohol and aldehydes or with inhibitors such as isobutryamide and nitroethanol. Not only could apo(alcohol dehydrogenase) form binary and ternary complexes, but the dissociation constants for the formation of such complexes were found to be approximately equal in magnitude to those of the native enzyme. It can be concluded that in the catalytic process zinc is not the binding site for coenzyme or substrate. Hence, the only step where zinc could still possibly be essential is the hydride-transfer step. The inability of apo(alcohol dehydrogenase) to execute this step may be due to structural alterations resulting from the removal of zinc or to the absence of zinc as a general acid that is absolutely required for the reactions.

Besides the above findings, there exists much evidence against invoking zinc as the binding site for coenzyme or substrate in LADH. Lack of binding of azide, a substrate–competitive inhibitor, to cobalt in cobalt–alcohol dehydrogenase (Young and Wang, 1971), the finding that the rate-determining step in the formation of ternary complex between enzyme–NAD and pyrazole is not the binding of pyrazole but some step after that (Gilleland and Shore, 1970), the fact that Branden's (1969) X-ray crystallographic data indicate that zinc is *ca.* 25 Å from the coenzyme[2] in the binary complex while proton relaxation experiments with the spin-labeled analog of NAD indicate that substrate binds less than 5 Å from the nicotinamide ring (Mildvan and Weiner, 1969b) all suggest that zinc may not be necessary in binding substrate. Since, however, the enzyme has different conformations in the binary and ternary complexes (Zeppezauer *et al.*, 1967), reorientation when substrate binds to enzyme–coenzyme complex could bring the zinc close enough to the carbonyl group and thus allow the metal to act as a general acid without a substantial contribution to the binding free energy of substrate.

In an attempt to determine if zinc were necessary to act as a general acid, bromoacetaldehyde was investigated as a possible substrate for the apoenzyme, the rationale being that the electron-withdrawing bromo group would make the carbonyl–carbon more electron deficient, and thus mimic the possible general acid property of zinc interacting with the carbonyl–oxygen. However, this compound, which is a substrate for the native enzyme, was found not to be reduced by the apoenzyme. This negative finding cannot be used to prove that zinc is not acting as a general acid, but is consistent with the data from various laboratories which do suggest that zinc may not play any catalytic role. To definitely prove that no interaction occurs between metal and substrate it appears that continuous wave nuclear magnetic resonance with a paramagnetic metal substituted for zinc in the enzyme will have to be employed (Mildvan, 1970; Mildvan and Cohn, 1970).

It is not clear why the K_d for the interaction of NADH with native alcohol dehydrogenase would be dependent on which

TABLE III: Dissociation Constants of Substrates and Inhibitors from LADH–Ternary Complexes at pH 7.5, 20°.

Ternary Complex	K_d (mM)
Apo–NADH–isobutyramide	0.11 ± 0.03[a]
Apo–NAD–nitroethanol	1.39 ± 0.05
Apo–ADP-Rib–nitroethanol	4.20 ± 0.17
Apo–NAD–butanol	0.80 ± 0.30
Native–NADH–isobutyramide	0.11 ± 0.02
Native–NAD–nitroethanol	0.40 ± 0.02
Native–ADP-Rib–nitroethanol	4.40 ± 0.47

[a] The calculated errors are standard deviations.

[2] C. I. Branden reported in his paper that, owing to low resolution, he was not able to locate the position of all four zinc atoms.

of the fluorescent species in the complex was chosen for investigation. For example in this study a K_d = 1.2 μM was obtained by measuring quenching of the enzyme's fluorescence while a value of 0.30 μM was obtained by the enhancement of NADH's fluorescence at 430 nm, with excitation at 340 nm (data not presented). The latter K_d value is in good agreement with the results in the literature (Theorell and McKinley-McKee, 1961; Anderson and Weber, 1965). The observed difference in K_d, which corresponds to a free-energy difference of approximately 0.6 kcal/mole, may not be critically important, however, and in no way alters the conclusions of the experiments in this study. The calculated K_d values for the interaction of ADP-Rib and NAD are in good agreement with the respective K_i values of Yonetani (1963) for ADP-Rib and of Taniguchi *et al.* (1967) for NAD.

Kinetic experiments with simple substrates such as ethanol and acetaldehyde show no subunit interaction (Theorell and McKinely-McKee, 1961; Shore and Gutfreund, 1970) in LADH. Using aromatic substrates, Bernhard *et al.* (1970) have, however, presented data which suggest that the two subunits are intereacting with each other. In addition, the recent fluorescence data of Theorell and Tatemoto (1971) suggest that there might be some type of subunit interaction, as do the chloride ion binding studies by nuclear magnetic resonance (Lindman *et al.*, 1972). Experiments with the spin-label analog of NAD (Mildvan and Weiner, 1969a) showed that the enhancement of the proton relaxation rates of water differs as the occupany of the sites by the bound label increased from 0 to 2. These independent lines of evidence indicate that there is some form of site–site interaction in LADH. However, the monophasic nature of the Scatchard plots for the formation of the native enzyme–coenzyme binary complexes presented in this study suggests that the two sites are independent of each other. The interaction between the sites is thus not observed by measuring changes in the intrinsic fluorescence of the native enzyme or coenzyme as the binary complex was formed nor by following difference absorption spectra (Taniguchi *et al.*, 1967). The Scatchard plot for the binding of NADH to the apoenzyme, however, is biphasic. A similar biphasic curve obtained by the polarization technique (Hoagstrom *et al.*, 1969) was interpreted to mean two independent types of sites. In the present circumstances the observance of a biphasic Scatchard plot in the binding of NADH and a monophasic Scatchard plot in the binding of NAD to the apoenzyme indicates that, although the two coenzyme sites are identical, the binding of NADH causes interaction of these sites. This effect of NADH is therefore observed only when zinc is removed from the enzyme.

The mechanism of quenching of an enzyme's tryptophan fluorescence when coenzyme binds to the enzyme is not known. Three mechanisms have been suggested (Velick, 1958): (1) Forster transfer (Forster, 1959) from tryptophan to the reduced nicotinamide ring, (2) molecular complex between tryptophan and the coenzyme, and (3) alteration of the environment of tryptophan, and hence its fluorescence emission, produced by binding coenzyme. The data presented do not prove which of the above mechanisms best explains the quenching. However, since it was observed in these studies that NAD and ADP-Rib quench the emission of free tryptophan and derivatives of the amino acid (Figure 1 and Table I) as efficiently as, or better than, NADH, it does not appear that quenching by NADH is exclusively due to Forster energy transfer. The data in Figure 1 can best be explained by assuming that a complex between the indole moiety and some portion of the coenzyme contributes significantly to the overall quenching.

The observation that *N*-acetyltryptophanamide fluorescence increases in ethanol shows that solvent effect on quantum yield is important as has been demonstrated for indole (Van Duuren, 1960) and *N*-acetyltryptophanamide (Steiner *et al.*, 1964). Binding of ligands to the enzyme could alter the microenvironment of the tryptophans and may thus result in an alteration of the fluorescence yield. As the degree of quenching by NAD, NADH, or ADP-Rib is different for the native enzyme and the apoenzyme and there is no parallel between the enzymes' degree of quenching and that observed with an amino acid derivative, it is more attractive to postulate that the quenching is largely due to conformational alteration in the enzyme, rather than to molecular complex between ligand and tryptophan of the enzyme.

Low-resolution X-ray data for LADH have revealed that conformational changes do occur as binary and ternary complexes of the enzyme are formed (Zeppezauer *et al.*, 1967). Similarly, optical rotatory dispersion data suggest a change in the tertiary structure occurs when coenzyme binds to the native enzyme (Rosenberg *et al.*, 1965). Also pertinent to the hypothesis that the quenching of fluoresence in alcohol dehydrogenase is due to a conformational change rather than to a direct interaction of coenzyme and tryptophan is the observation of Adams *et al.* (1971) with another dehydrogenase, dogfish lactate dehydrogenase, in which tryptophan was found to be remote from the coenzyme in the binary complex, yet the intrinsic fluorescence of this enzyme is quenched when an NADH binary complex is formed.[3]

Irrespective of the mechanism of quenching that is operating, the fact that coenzyme partially quenches the tryptophan fluorescence emission of the enzyme and this emission is altered by adding substrates or inhibitors which form ternary complexes allows us to conclude that zinc is not necessary for binding coenzyme or substrate to liver alcohol dehydrogenase.

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Role of Zinc in Horse Liver Alcohol Dehydrogenase. Influence on Structure and Conformational Changes†

Patrick L. Coleman, Idowu Iweibo, Henry Weiner*

ABSTRACT: Coenzyme binding and structural properties of zinc-free (apo) and native horse liver alcohol dehydrogenase (EC 1.1.1.1) have been investigated in order to further elucidate the role of zinc in the enzyme. Differences in the fluorescence polarization spectra for apoenzyme and native enzyme and in their respective binary and ternary complexes indicate that not only are the structures of the two enzyme forms different but that they are induced to different conformations by the coenzymes. Difference in the interaction of coenzymes with apoenzyme and native enzyme is also indicated by the difference in the pH profile of their dissociation constants. Identical sedimentation velocity constant and optical rotatory dispersion properties for the apoenzyme and the native enzyme eliminate a role for zinc in the quaternary and secondary structures. The differences observed by fluorescence and fluorescence polarization techniques and melting temperatures are therefore ascribed to a fundamental difference in the tertiary structures of the two enzymes, such that the apoenzyme can no longer catalyze the oxidation–reduction reaction. In addition the presence of zinc does not appear to stabilize the enzyme against denaturation by guanidine hydrochloride.

In previous papers (Weiner, 1969; Mildvan and Weiner, 1969; Hoagstrom *et al.*, 1969; Iweibo and Weiner, 1972) we have shown that zinc is not involved in the formation of binary and ternary complexes of coenzymes and substrates with horse liver alcohol dehydrogenase (EC 1.1.1.1). Evidence corroborating the above finding has been reported by other

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