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

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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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laboratories (Gilleland and Shore, 1970; Young and Wang, 1971). In addition, since the K_d's for binary and ternary complexes are similar for native and apo(alcohol dehydrogenases) (Iweibo and Weiner, 1972), and antibody prepared against native enzyme[1] interacts with essentially the same K_d with the native enzyme and apoenzyme (Coleman *et al.*, 1972), it seems logical to conclude that the gross structure of the apoenzyme is very similar to that of the native enzyme. However, it has been well established that the apoenzyme is a catalytically inactive species (Åkeson, 1964; Oppenheimer *et al.*, 1967; Drum *et al.*, 1967). In an attempt to explain this phenomenon, we present, in this paper, results that indicate that the nature of enzyme–coenzyme interaction differs for the two forms of alcohol dehydrogenase as determined by the techniques of fluorescence, fluorescence polarization, and equilibrium dialysis. Furthermore, using absorption, fluorescence, fluorescence polarization, and optical rotatory dispersion spectroscopies, ultracentrifugation, and denaturation of the two forms of the enzyme, we present results that clarify the role of zinc in the secondary, tertiary, and quaternary structures of the enzyme.

Experimental Section

Enzyme and Reagents. Preparation of native enzyme and apoenzyme, determination of enzyme activity and protein concentration, and fluorescence measurements were performed as described in the preceding paper (Iweibo and Weiner, 1972), except where otherwise indicated.

Fluorescein-labeled alcohol dehydrogenase was prepared as described by Coleman *et al.* (1972). Native enzyme was reacted with fluorescein isothiocyanate to yield a labeled enzyme containing approximately 1 mole of dye/mole of enzyme. The labeled enzyme possessed full enzymatic activity and was converted with the labeled apoenzyme by dialysis against 1 mM EDTA (Iweibo and Weiner, 1972).

Ultra Pure Gdn·HCl was obtained from Mann, fluorescein isothiocyanate from Calbiochemical and [^{3}H]NAD (adenine labeled) from New England Nuclear. The [^{3}H]NAD was diluted with unlabeled NAD and then purified by eluting from a DEAE column with pH 6.0 phosphate buffer. Sources of the other materials were those described in the preceding paper (Iweibo and Weiner, 1972).

Binding of Coenzymes to Native and Apo(Alcohol Dehydrogenase). NADH binding to apoenzyme as a function of pH was determined by polarization of fluorescence technique as previously described (Hoagstrom *et al.*, 1969). NAD binding was determined by equilibrium dialysis of the enzyme and [^{3}H]NAD in phosphate buffer, $\mu = 0.05$ at 4°. The dialysis was performed with 0.2-ml dialysis cells purchased from Interscience with membranes that were treated and stretched according to the method of England *et al.* (1969). Such treated membranes were stored in azide solution and rinsed thoroughly before use. Small glass beads were put in the dialysis cells to increase the rate of attainment of equilibrium which was reached in less than 2 hr. When equilibrium had been established, an aliquot from each half of the cell was withdrawn, placed in a scintillation vial containing Beckman's ambient temperature fluor (Beckman, 1967), and counted with a Beckman liquid scintillation counter, LS-100, to a 3% standard error.

Optical Rotatory Dispersion Studies. Optical rotatory dispersion spectra were obtained in a 1-cm path-length cuvet at 25° with a Cary 60 spectropolarimeter. The rotation of 1–5 μM enzyme in pH 7.0 phosphate buffer, $\mu = 0.1$, was measured between 500 and 220 nm. It was observed that the dynode voltage never exceeded 0.4 kV even in the presence of 7 M Gdn·HCl. To measure the effect of Gdn·HCl on the spectrum, aliquots of concentrated denaturant were added to the cuvet. To convert observed rotations, α, to reduced mean residue rotation, $[m']$, the following formula was used (Urnes and Doty, 1961)

$$[m'] = \frac{3}{(n^2 + 2)} \frac{100 \cdot \alpha \cdot MRW}{dc} \quad (1)$$

where $3/(n^2 + 2)$ in absence of Gdn·HCl was obtained from the literature (Sober, 1968). *MRW*, mean residue weight, was taken to be 104.8 (Theorell *et al.*, 1966), d was path length, 0.1 dm, and c, concentration in g/100 ml. The refractive index, n, in the presence of Gdn·HCl was calculated accordingly from the data of Hooker.[2]

Ultracentrifuge Analysis. A Beckman Model E ultracentrifuge equipped with schlieren optics was used to determine the sedimentation coefficients of native enzyme and apoenzyme. Samples of native enzyme and apoenzyme at any one concentration of Gdn·HCl were run simultaneously, as one of the cells was provided with a 2° positive wedge window so that the schlieren patterns from both samples appeared on the same photograph. All runs were performed at 60 μM protein in pH 7.0 phosphate buffer, $\mu = 0.1$, at 20°. Sedimentation coefficients were calculated as described by Schachman (1957).

Rate of Change of Fluorescence of Alcohol Dehydrogenase During Denaturation by Guanidine Hydrochloride. Rates of denaturation were determined by measuring changes of tryptophan fluorescence as a function of time in various concentrations of Gdn·HCl in pH 7.0 phosphate buffer, $\mu = 0.1$ at 25°. To initiate the denaturation, concentrated enzyme was added to a Gdn·HCl solution in an Aminco-Bowman spectrophotofluorometer, and the change in the emitted light at 350 nm with time was recorded with a Sargent recorder. Initial fluorescence was determined by measuring the fluorescence intensity of the same concentration of enzyme after dilution with an appropriate volume of Gdn·HCl-free buffer.

Influence of Temperature on Enzyme. The temperature of the enzyme solution in the cuvet was raised in 2–3° increments by appropriately adjusting a constant-temperature bath. Absorbance spectra were measured with a Cary 15 spectrophotometer and fluorescence spectra with an Aminco-Bowman spectrophotofluorometer connected to an Autograph X–Y recorder. Spectra were recorded only after both the temperature of the solution in the cuvet and the observed parameter had become constant (usually 10–15 min). The melting point was considered to be the temperature at which transition was half-completed.

Results

Interaction of Coenzyme with Alcohol Dehydrogenase. The effect of pH on binding of coenzyme to apo(alcohol dehydro-

[1] Abbreviations used are: native enzyme, horse liver alcohol dehydrogenase; apoenzyme, zinc-free alcohol dehydrogenase; Gdn·HCl, guanidine hydrochloride; ADP-Rib, adenosine diphosphoribose; K_d, dissociation constant of a ligand from its complex; T_m, melting temperature.

[2] Personal communication, T. M. Hooker, Jr., University of California, Santa Barbara, Calif.

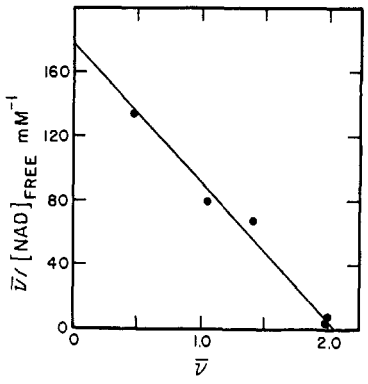

FIGURE 1: The interaction of NAD with apo(alcohol dehydrogenase) as measured by equilibrium dialysis and plotted according to the method of Scatchard (1949). The dialyses were performed at 4° in pH 7.5 phosphate buffer, μ = 0.05, as described in the text. [^{3}H]NAD was counted to an accuracy of ±3%.

genase) was investigated since it has been suggested that the pH profile for K_d with the native enzyme is governed by which of the species, H_2O or OH^{-1}, is liganded to zinc in the enzyme (Theorell and McKinley-McKee, 1961). Figure 1 presents a representative Scatchard plot from equilibrium dialysis experiments with apoenzyme and NAD. Data were gathered only between pH 6 and 8 as the apoenzyme was unstable to the 2-hr dialysis at pH 8.5 and above. NADH binding from pH 6 to 10, as determined by polarization of fluorescence, yielded biphasic Scatchard plots as previously reported (Hoagstrom *et al.*, 1969). In Figure 2 are presented the K_d's for NAD and the K_d's for the "tight" binding site for NADH from apoenzyme. For comparative purposes the K_d's for coenzyme with native enzyme (Theorell and McKinley-McKee, 1961) are also presented.

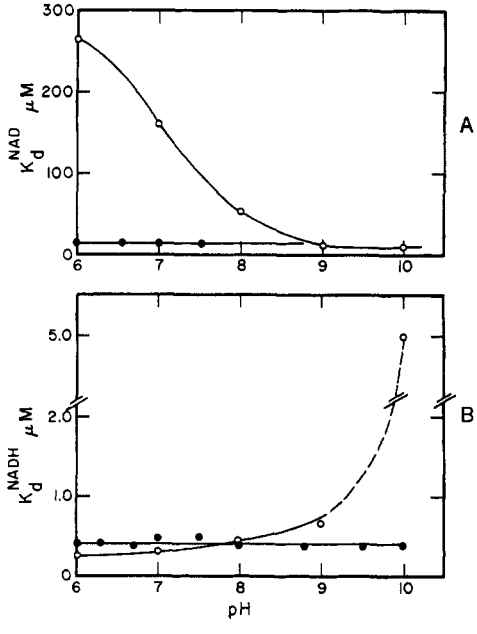

FIGURE 2: Dissociation constants of NAD (A) and NADH (B) from apo- (●) and native (○) enzymes as a function of pH. The K_d's for NAD from apoenzyme were obtained by equilibrium dialysis and for NADH by fluorescence polarization. The K_d's for NAD and NADH from the native enzyme were obtained from Theorell and McKinley-McKee (1961).

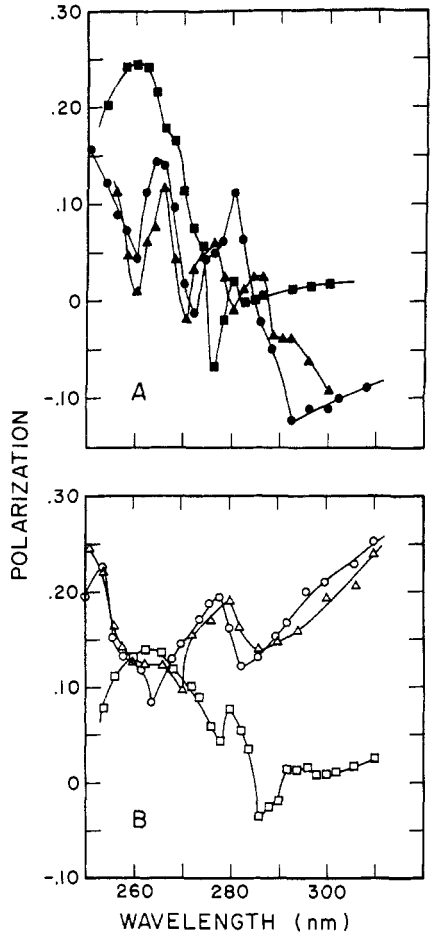

FIGURE 3: (A) Fluorescence polarization spectra at 16° of apoenzyme (●), apoenzyme–NAD complex (■), and apoenzyme–NAD–nitroethanol complex (▲). Concentrations were for apoenzyme (0.5 μM), NAD (100 μM), nitroethanol (2.5 mM) in pH 7.5 phosphate, μ = 0.05. (*B*) Fluorescence polarization spectra of native enzyme (○), native enzyme–NAD complex (□), and native enzyme–NAD–nitroethanol complex (△). Concentrations were for native enzyme (0.5 μM), NAD (300 μM), and nitroethanol (2.5 mM) in pH 7.5 phosphate buffer, μ = 0.05.

The observation that K_d is virtually independent of pH for the binding of either oxidized or reduced NAD to apoenzyme suggests structural differences may exist between native enzyme and apoenzyme. Various physical techniques were therefore employed in order to obtain data concerning the structures of the two forms of alcohol dehydrogenase and their corresponding binary and ternary complexes.

Fluorescence Polarization Spectra. The fluorescence polarization spectra in Figure 3 were obtained by varying the excitation wavelength and maintaining constant the emission wavelength at 350 nm. It can be seen that the fluorescence polarization spectra of the apoenzyme and the native enzyme and of their respective binary and ternary complexes differ not only in shape but also in absolute value at any wavelength. The difference, especially in the 280:295 nm ratio, has been used to indicate that conformational differences exist (Weber, 1960; Anderson and Weber, 1966).

Comparison of the Structure of Native Alcohol Dehydrogenase and Apo(Alcohol Dehydrogenase). SECONDARY STRUCTURE. Optical rotatory dispersion studies revealed that native enzyme and apoenzyme have essentially the same $[m']_{233nm}$ and $[m']_{220nm}$ values (−1250 and +500, respectively). A Moffitt plot (Urnes and Doty, 1961) gave a b_0 value of −90

TABLE I: Comparison of Spectroscopic Properties of Native and Apo(Alcohol Dehydrogenases) at pH 7.0.

Property	Apo:Native
E_{280nm}	1.0
Fluorescence (tryptophan)[a]	1.5
Polarization (tryptophan)[a]	0.85
Fluorescence (fluorescein)[b]	1.0
Polarization (fluorescein)[b]	1.4
$[m']_{233nm}$	1.0
$[m']_{220nm}$	1.0
Moffitt b_0 parameter	1.0

[a] Excitation 280 nm; emission 350 nm. [b] Excitation 480 nm; emission 530 nm.

for both enzymes, A b_0 of -100 has been reported for native enzyme (Rosenberg *et al.*, 1965). It can be concluded that, to the extent that optical rotatory dispersion (ORD) is a measure of secondary structure, the two forms of alcohol dehydrogenase possess the same overall secondary structure.

TERTIARY STRUCTURE. The results of various spectroscopic properties of native enzyme and apoenzyme are summarized in Table I. It has previously been shown that the extinction coefficient at 280 nm is essentially the same for both forms of the enzyme (Hoagstrom *et al.*, 1969). The intrinsic fluorescence intensities of the two forms differ, however, the apoenzyme being 32% more fluorescent in pH 7.0 phosphate buffer, $\mu = 0.05$. The effect of pH on the fluorescence intensities of the enzymes is presented in Figure 4. These data were replotted (Figure 5) in the Henderson–Hasselbach form (eq 2) as outlined by Edsall and Wyman (1958), in order to determine the pK_a of the ionizing species that is causing a change in the enzyme tryptophan's fluorescence.

$$pH = pK_a + \log \frac{\Delta F_m - \Delta F}{\Delta F} \qquad (2)$$

where the maximum fluorescence change $\Delta F_m = F_l - F_h$, F_l being a maximum fluorescence value of enzyme at the low pH of 6.0 and F_h a minimum fluorescence value at the high pH of 11.0. $\Delta F = F - F_h$, where F is the fluorescence at any point between these two limits of pH. $(\Delta F_m - \Delta F)/\Delta F$ represents the ratio of the ionized and protonated species.

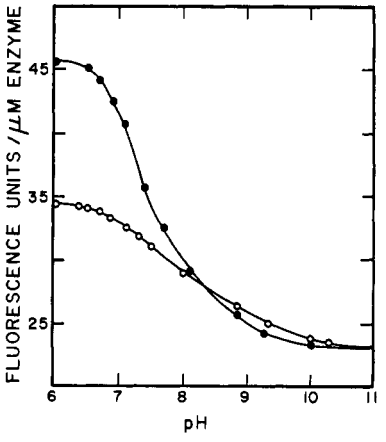

FIGURE 4: Comparison of the fluorescence of native enzyme (O) and apo(alcohol dehydrogenase) (●) as a function of pH. The relative fluorescence units are normalized for enzyme concentration in order to compare the two proteins to each other. Samples were excited at 280 nm and emission measured at 350 nm in sodium phosphate, $\mu = 0.05$ at 25°.

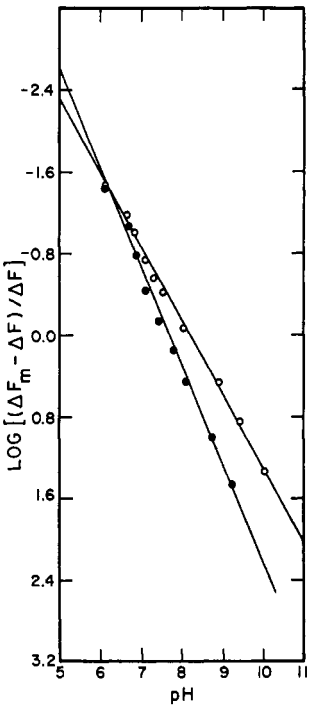

FIGURE 5: A Henderson–Hasselbach plot of the data from Figure 4. Symbols are described in eq 2 (see text). The slope for the apo-(alcohol dehydrogenase) (●) is 1.0 with $pK = 7.6$; those for native enzyme (O) are 0.74 and 8.2, respectively.

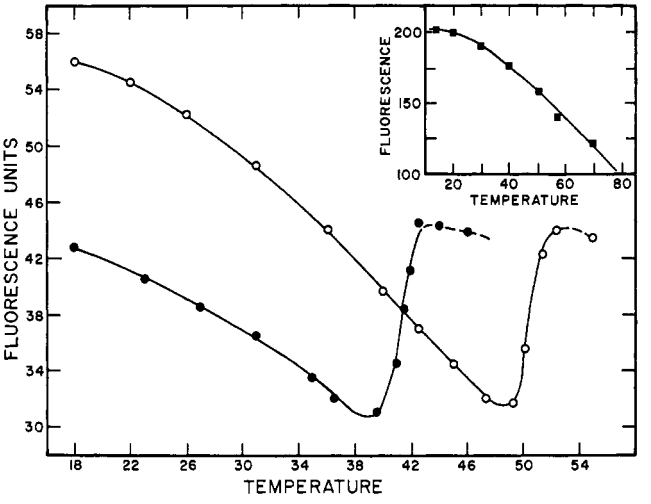

FIGURE 6: Examples of melting curves of apoenzyme (●) and native enzyme (O) at an enzyme concentration at 1.7 μM. In the insert is the fluorescence of *N*-acetyltryptophanamide (6.0 μM) as a function of temperature. At each temperature sufficient time (generally 10–15 min) was allowed for both the temperature in the cuvet and the fluorescence emission to equilibrate. The solution was excited at 280 nm and emission measured at 350 nm in pH 7.0 phosphate $\mu = 0.1$. Results at other enzyme concentrations mimicked those in this example with the exception that the temperature where fluorescence increases was displaced to either higher or lower temperatures. Dashed lines indicate that protein was aggregating and precipitating.

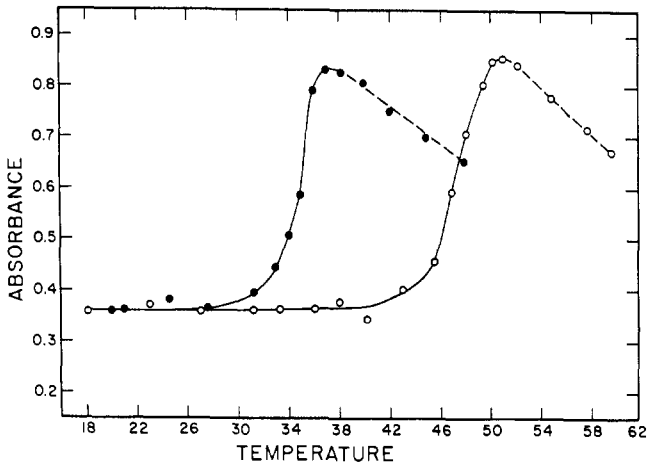

FIGURE 7: The absorbance of alcohol dehydrogenase at 280 nm as a function of temperature. The absorbance of a 10 μM solution of apo(alcohol dehydrogenase) (●) or native enzyme (○) in pH 7.0 phosphate, μ = 0.1, was measured after thermal equilibration (see Figure 6). There was no change in absorbance for *N*-acetyltryptophanamide with changing temperature (not shown). Dashed lines indicate that protein was aggregating and precipitating.

From Figure 5, pK_a's of 7.6 for the apoenzyme and 8.2 for the native enzyme were obtained. In addition, the slopes of the lines were 1.0 for apoenzyme and 0.74 for native enzyme.

The melting curves for the native enzyme and apoenzyme, determined by measuring changes in fluorescence and in absorption are presented in Figures 6 and 7. The steady decrease in fluorescence as temperature increased was the result of a temperature-dependent loss of fluorescence of tryptophan (see insert, Figure 6), not of a change in the protein's structure (Gally and Edelman, 1962). The melting phenomenon itself is associated with a concerted increase in both absorbance at 280 nm and fluorescence at 350 nm. By either technique the data show that the native enzyme is more stable than the apoenzyme toward thermal denaturation. It was also observed that at a few degrees above the melting temperature a large increase in turbidity occurred, suggesting that aggregation was occurring. As this aggregated protein precipitated the fluorescence or absorbance decreased (see dashed line Figures 6 and 7).[3] In addition, it was observed that the melting temperature, T_m, was inversely proportional to the enzyme concentration, a result presented in Figure 8. These data are compiled from both fluorescence and absorbance experiments similar to those presented in Figures 6 and 7 and reveal that the concentration effect on T_m, as measured by the slopes of the lines, is different for the two enzymes, −0.35 and −0.55 deg/μM for native enzyme and apoenzyme, respectively.

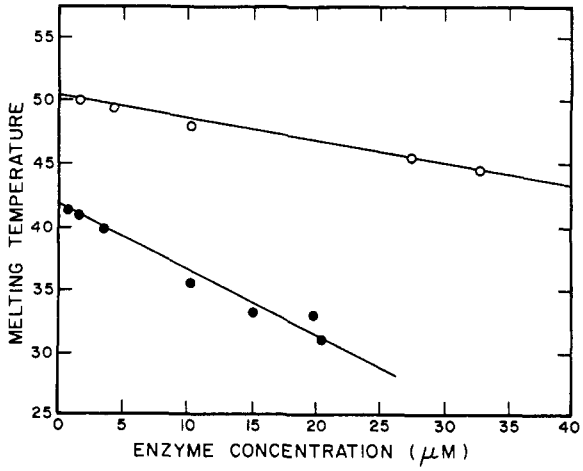

FIGURE 8: Effect of enzyme concentration on its melting temperature. Data for both fluorescence and absorbance experiments (Figures 6 and 7) at various concentrations are presented for apoenzyme (●) and native enzyme (○).

TABLE II: The Effect of pH on the Melting Temperature (T_m) of Native and Apo(Alcohol Dehydrogenase).[a]

pH	Melting Temp (T_m), °C[b]	
	Apo	Native
7.0	37	48
7.5		37
8.0	32	33
10.0		30

[a] The protein concentration was 10 μM. Buffers were phosphate, μ = 0.1, except at pH 10.0 where a 0.1 M glycine (NaOH) solution was used. [b] Temperature ±1°.

The results of experiments performed at other pH's at a constant enzyme concentration of 10 μM, appear in Table II. The general trends revealed are that both the absolute T_m and the difference between the values for native enzyme and apoenzyme decrease with increasing pH. This latter effect is similar to that observed in Figure 4 for fluorescence as a function of pH.

Effect of Guanidine Hydrochloride Concentration on the Maintenance of the Structure of Native Alcohol Dehydrogenase and Apo(Alcohol Dehydrogenase). SECONDARY STRUCTURE. Gdn·HCl did not change the magnitude of $[m']_{233nm}$ in either native enzyme or apoenzyme. Increasing Gdn·HCl concentration, however, did cause a significant decrease in the rotation of the apoenzyme and native protein at 220 nm from a value of +500 in the absence of Gdn·HCl to −1800 at 3.5 or 7 M Gdn·HCl. This effect, similar in both apoenzyme and native enzyme, is summarized in Table III. In general, Gdn·HCl produces quantitatively identical changes in the optical rotation spectra of both enzymes.

TERTIARY STRUCTURE. To determine whether zinc were stabilizing the tertiary structure, changes in tryptophan's emission and polarization in both native enzyme and apoenzyme as a function of Gdn·HCl concentration were measured. As revealed in Figure 9 for native enzyme, half of the total fluorescence change occurred at 1.6 M Gdn·HCl while 1.3 M was needed to produce a 50% change in polarization. The corresponding values for apoenzyme were 1.9 and 2.0 M Gdn·HCl, respectively. Not only was the amount of Gdn·HCl required to cause a 50% change with apoenzyme greater, but the shape of the curve differed. The transition as measured by fluorescence covered the range of 1–3 M Gdn·HCl for apoenzyme but only over a half-molar range for the native enzyme. The polarization results for the dye-conjugated pro-

[3] A value for ΔH was not calculated for the transitions since the proteins aggregated and precipitated, and, therefore, the transitions were not reversible. It was observed that the curves for the two forms of enzyme were superimposable, suggesting that their respective values for ΔH were the same.

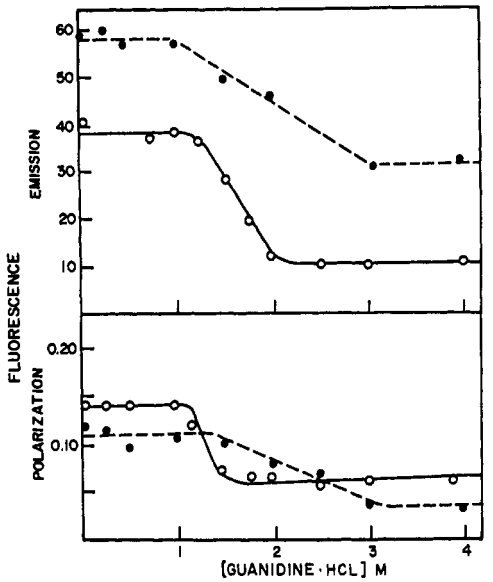

FIGURE 9: Comparison of fluorescence properties of native and apo(alcohol dehydrogenase) as a function of Gdn·HCl concentration. Solutions were excited at 280 nm and emission at 350 nm observed. Samples contained 0.75 μM enzyme in a pH 7.0 phosphate buffer, μ = 0.1 at 25°. Native enzyme (○) and apoenzyme (●).

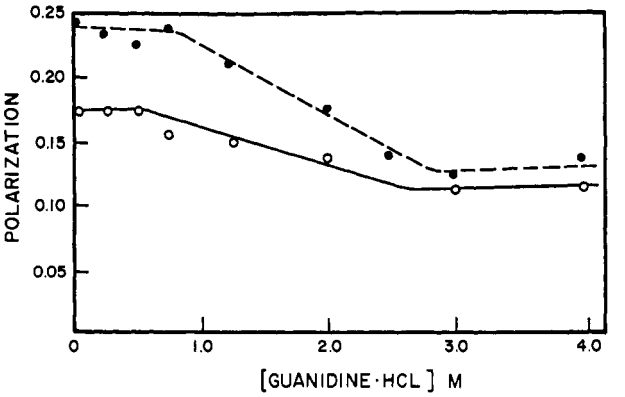

FIGURE 10: Comparison of the polarization of fluorescence of fluorescein-labeled native enzyme (○) and apo(alcohol dehydrogenase) (●). Conditions as described in Figure 9 except solutions were excited at 480 nm and emission of 530 nm observed.

tein are presented in Figure 10. The shapes of the two denaturation curves are basically the same for the two dye-labeled proteins in contrast to those presented in Figure 9, in which both the range of denaturation and the sharpness of the curve differ for the two enzyme forms. The fluorescence emission of the dye in both the native enzyme and apoenzyme did not change over the range of 0–7 M Gdn·HCl (not shown).

Possible differences in the structures of native enzyme and apoenzyme were also investigated by measuring the rate of denaturation of the two enzymes at a fixed Gdn·HCl concentration. A representative curve for the denaturation of native enzyme and apoenzyme, determined by measuring the changes in enzyme fluorescence with time, is shown in Figure 11A. Figure 11B is a plot of the log of the per cent fractional change in fluorescence *vs.* time according to eq 3; where F_0 is the initial emission; F_∞, the final emission,

$$\log \frac{F - F_\infty}{F_0 - F_\infty} = -kt \qquad (3)$$

and F, the emission at any time. As seen in this example and in the other experients summarized in Table IV, the data could best be fitted at two first-order processes. By the techniques available to us it was not possible to determine whether there are more rapid denaturation steps as was demonstrated with glutamate dehydrogenase (Fisher and Bard, 1969). However, Heitz and Brand (1971) have denatured horse liver alcohol dehydrogenase with acid and found no step more rapid than those reported here. The native enzyme and the apoenzyme, however, exhibited the same rate of denaturation at each Gdn·HCl concentration.

QUATERNARY STRUCTURE. The values of the sedimentation coefficients at various Gdn·HCl concentrations are presented

TABLE III: $[m']_{220nm}$ Data for Native and Apo(Alcohol Dehydrogenases) as a Function of Gdn·HCl.[a]

[Gdn·HCl], M	$[m']_{220nm}$ Native	Apo
0.0	500	400
0.5	500	100
1.0	200	−250
1.5	−400	−600
2.0	−1400	−1400
2.5	−1500	−1500
3.5	−1700	−1700
7.0	−1800	−1800

[a] The optical rotation curves for native enzyme and apoenzyme were identical with each other from 500 to 233 nm in all Gdn·HCl concentrations. Spectra were independent of Gdn·HCl concentration from 500 to 233 nm. Spectra were obtained in a 1-cm cuvet in pH 7 phosphate buffer, μ = 0.1 at 25°. Reproducibility in $[m']_{220nm}$ was ±5%.

TABLE IV: Rate Constants for the Denaturation of Native and Apo(Alcohol Dehydrogenases) at Various Concentrations of Gdn·HCl.[a]

[Gdn·HCl], M	$k_1 \times 10^2$ sec^{-1} Native	Apo	$k_2 \times 10^2$ sec^{-1} Native	Apo
1.40	4.0	4.7	2.0	1.5
1.75	6.8	7.1	2.6	2.5
2.10	8.8	11.5	2.5	2.5
2.45	35	18	11.7	6.7
2.80	30	25	12.7	14
3.15	95	71	40	43
3.50	143	128	62	63

[a] Decrease in fluorescence of the enzyme was observed as a function of time at 350 nm (excitation 280 nm). The two first-order rate constants, k_1 and k_2, were obtained from the slopes of a semilog plot of the fractional decrease in fluorescence *vs.* time. Denaturations were observed in pH 7.0 phosphate, μ = 0.1, at 25° with enzyme concentration of 0.75 μM.

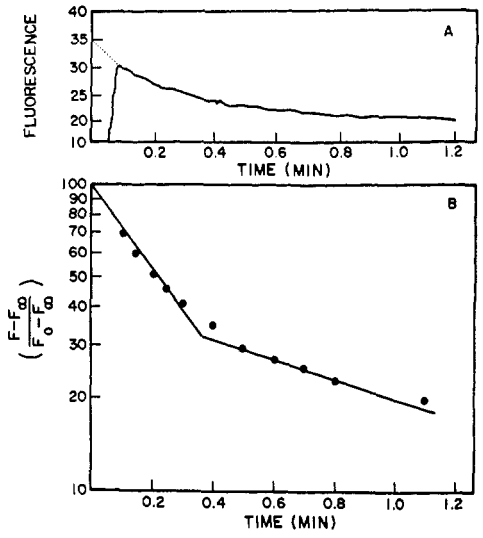

FIGURE 11: Rates of denaturation of alcohol dehydrogenase. The decrease in tryptophan fluorescence was taken as a measure of the rate of denaturation. Conditions as in Figure 9. (A) Representative recording of the change in fluorescence with time; (B) the conversion of the data into a first-order plot as described in eq 3 (see text). The particular curve shown is for native enzyme in 1.75 M Gdn·HCl. The rates constants for all experiments are tabulated in Table IV.

TABLE V: Effect of Gdn·HCl on the Sedimentation Coefficient of Native and Apo(Alcohol Dehydrogenases).[a]

	$s^{obsd}_{20,w}$	
[Gdn·HCl], M	Native	Apo
0.0	5.1	5.0
0.0	5.1[b]	
1.0	5.2[b]	
1.25	4.7	4.7
2.5	2.4	2.3
3.0	2.3[b]	

[a] $s^{obsd}_{20,w}$ values were calculated from sedimentation velocity runs in pH 7.0 phosphate, μ = 0.1 at 20° with an enzyme concentration of 5 mg/ml. [b] From Green and McKay (1969).

in Table V. The demonstration of an identical $s_{20,w}$ value for apoenzyme and native enzyme indicates that the removal of zinc does not lead to dissociation of the two subunits, in good agreement with previous reports (Green and McKay, 1969; Drum *et al.*, 1967). In addition, as Gdn·HCl concentration is increased the two forms of the protein have identical $s_{20,w}$ values, suggesting that their molecular weights in any Gdn·HCl concentration are virtually the same.

Discussion

Zinc in horse liver alcohol dehydrogenase could be involved in substrate or coenzyme binding, in the structural integrity of the enzyme or could act as a general acid.

Vallee's laboratory has presented much data that suggest that the two zinc atoms per subunit of horse liver alcohol dehydrogenase are in different environments and hence each may perform a different role in the enzyme (Drum *et al.*, 1967, 1969; Drum and Vallee, 1970). Since it has been established that zinc is not directly involved in substrate or coenzyme binding (Iweibo and Weiner, 1972), the catalytic inactivity of apo (alcohol dehydrogenase) suggests a structural role for the metal, although it may still be justifiably argued that zinc may be involved in catalysis as a general acid (Abeles *et al.*, 1957). The results reported in the previous paper (Iweibo and Weiner, 1972) are consistent with primarily a structural role for the metal.

The possible structural role for zinc in native alcohol dehydrogenase could be (1) to maintain the overall structure of the enzyme and (2) to allow proper change in conformation to occur upon coenzyme and/or substrate binding. The necessity of zinc to hold the subunits together can be eliminated by the equivalence of the sedimentation constants of the apoenzyme and native enzyme (Green and McKay, 1969; Drum *et al.*, 1967), a fact which was reverified in this study.

Fluorescence data presented in this and the preceeding paper (Iweibo and Weiner, 1972) reveal that indeed there is some difference between the overall structure of the two forms of the enzyme. The optical rotatory dispersion data in Table III imply that there are no measurable differences between the secondary structures of apoenzyme and native enzyme, while the identical $s_{20,w}$ values show that the quaternary structures are the same. Thus it can be concluded that any structural differences are in the tertiary structures. However, since apoenzyme binds substrates and coenzymes as well as does native enzyme (Iweibo and Weiner, 1972) the overall structures of the two forms of the enzyme must not be grossly different.

Differences in the tertiary structures of native enzyme and apoenzyme were assessed by the technique of fluorescence polarization. One experiment involved measuring the polarization of tryptophan, and another the polarization of fluorescein in dye-labeled native enzyme and apoenzyme. The relation of the polarization, P, to molecular properties has been defined by Perrin (1926): $1/P - 1/3 = (1/P_0 - 1/3)[1 + (3\tau/\rho_h)]$, where P_0 is the limiting polarization in a rigid medium; ρ_h is the harmonic mean of the relaxation times of the molecule, and τ is the fluorescence lifetime of the oscillator.

A change in P_0, ρ_h, or τ would therefore lead to a change in P. That the apoenzyme and native enzyme have the same sedimentation coefficients suggests that ρ_h for the molecule is similar for both enzyme forms (Weber and Daniel, 1966). The lower value of polarization of tryptophan fluorescence in the apoenzyme near neutral pH can be accounted for at least in part by the higher fluorescence yield and hence a higher τ value at pH 7. This argument is predicated on an assumption that the enzyme is behaving as a rigid body. If the portion of the polypeptide chain holding the two tryptophans (identified as residues 15 and 314 by Jornvall, 1970) has freedom of rotation independent of the rotating enzyme molecule, then an additional mode of depolarization is possible. Hence ρ_h for the individual tryptophan residues may be different. Similarly with the dye-labeled enzyme, the fluorescein may have freedom of motion independent of the protein. That the fluorescence yield from the dye is the same for native enzyme and apoenzyme suggests τ for fluorescein is the same with either form of the enzyme. The greater polarization found with native enzyme is related to either a change in P_0 or ρ_h. In either case it can be interpreted that the structures of the two forms of the enzyme are different in the region of the conjugated dye or of the tryptophans. In the light of the results of the optical rotation and ultracentrifuge studies it

can be concluded that the differences between the two forms of the enzyme are in the tertiary structure.

Supporting the notion that native enzyme and apoenzyme have different tertiary structures was the observation that the polarization of fluorescence spectra measured by varying the excitation wavelength differed for the two enzyme forms. In addition the *ca.* 10° difference in T_m at pH 7 and the dependency of T_m on concentration also suggest that the presence of zinc does influence the conformation of the enzyme

Not only do we conclude then that the tertiary structures of apoenzyme and native enzyme are different but the effect of pH on each of their respective structures is different in that there is: (1) a minimization of the fluorescence emission differences between native enzyme and apoenzyme at high *p*H; (2) a change in pK_a of the group responsible for the pH-mediated fluorescence change after zinc is removed from native enzyme; and (3) a decrease in the difference in T_m between native enzyme and apoenzyme as pH is increased.

The role of zinc in tertiary structure can be further subdivided: (1) orientation of tertiary structure; and (2) maintenance of that tertiary structure. The fact that the tertiary structure is different when zinc is removed suggests that point 1 is operative. The second possibility was not found to occur since in no case did the apoenzyme denature at a lower Gdn·HCl concentration than did native enzyme. The identical rates of change in fluorescence upon adding Gdn·HCl to either form of the enzyme supports the conclusion that zinc offers no stability to the structure, but only influences the structural orientation.

It has been suggested (Theorell and McKinley-McKee, 1961) that the lower K_d of NAD from native enzyme at high pH was due to the interaction of the positively charged nicotinamide ring with a hydroxyl group liganded to zinc in the enzyme ($Zn–OH^-$), while the poorer binding at low pH was due to the repulsion between the oxidized nicotinamide ring and the $Zn–OH_2$ complex, the prevalent form at neutral pH. The finding that K_d at all pH's for the apoenzyme–NAD complex is nearly as low as that for the native enzyme–NAD complex at pH 10 is interpreted to mean that some other process prevails. One possibility is that the coenzyme-induced conformational change in the presence of zinc is different from the change in the absence of zinc since evidence has been presented which shows that the basic difference in tertiary structures of the native enzyme and apoenzyme diminishes at high pH.

Other differences in the interaction of coenzyme with the two forms of the enzyme exist: (1) the apoenzyme binds only two cofactors as opposed to six to eight cofactors for the native enzyme as indicated with the spin-labeled analog of NAD by electron spin resonance technique (Weiner, 1969) and with the coenzymes by equilibrium dialysis experiments (Weiner *et al.*, 1972); (2) polarization of fluorescence experiments with the apoenzyme (Hoagstrom *et al.*, 1969) revealed that the sites interact in the binding of NADH while fluorescence enhancement revealed the sites are independent when NADH binds to the native enzyme (Theorell and Winer, 1959); and (3) although NADH has the same polarization when bound to either enzyme form, its fluorescence enhancement is different; yet the coenzyme is bound with approximately the same K_d by either enzyme form (Iweibo and Weiner, 1972). The differences in the polarization spectra of the apoenzyme and native enzyme and of their respective binary and ternary complexes indicate that conformational differences exist between both enzyme forms and that these enzymes are induced to different conformations in their binary and ternary complexes with coenzyme and substrate. This latter conclusion is also supported by the different degrees of quenching of the fluorescence of the enzymes observed when NADH, NAD, or ADP-Rib is bound (Iweibo and Weiner, 1972).

Thus the effect of zinc on the tertiary structure may be twofold: the first is in influencing the folding of the nascent polypeptide chain and the second is to allow the coenzyme (and possibly stubstrate) to induce a conformational change on the structure.

It has been shown that there are two classes of zinc in the enzyme (Drum *et al.*, 1967, 1969; Drum and Vallee, 1970). We suggest that both types of zinc are involved in the structure of the enzyme. It is also attractive to propose that one class may orient the polypeptide chains, perhaps by influencing the folding of the nascent chain into its active tertiary structure and that the other zinc is required in order that the enzyme be converted by substrate and coenzyme into its correct conformation for catalysis.

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Dihydrofolate Reductase from Amethopterin-Resistant *Lactobacillus casei*†

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ABSTRACT: Two forms (I and II) of dihydrofolate reductase from sonically disrupted cells of an amethopterin-resistant strain of *Lactobacillus casei* have been isolated using a procedure that involves fractionation with ammonium sulfate and chromatography on hydroxylapatite and CM-Sephadex. As shown previously (Dunlap *et al.*, *Biochem. Biophys. Res. Commun. 42*, 772 (1971)), these forms differ by the presence of an equimolar amount of noncovalently bound TPNH in II. Homogeneity of I was established by polyacrylamide electrophoresis and ultracentrifugation. Form II was also homogeneous with respect to extraneous proteins but some preparations contained traces of I arising from the loss of TPNH. Form I had a molecular weight of 14,900 as judged by gel filtration, electrophoresis on sodium dodecyl sulfate–polyacrylamide and ultracentrifugation. Amino acid analyses of I revealed the presence of three tryptophan residues per molecule and an absence of cysteine residues. The enzyme was not activated by mercurials and only slightly activated by urea. At pH 6.5 and 30°, the turnover number was 180 moles of dihydrofolate reduced per min per mole of enzyme. Polyacrylamide electrophoresis was used to demonstrate the following interrelationships between the two forms of the enzyme: (a) the TPNH-dependent conversion of I → II; (b) the dihydrofolate-dependent conversion of II → I; (c) the TPN- and tetrahydrofolate-dependent conversion of I → II; and (d) the AP-TPN- and tetrahydrofolate-dependent conversion of II → I.

Using the procedures comparable to those employed by Kisliuk and coworkers (Crusberg *et al.*, 1970), an amethopterin-resistant strain of *Lactobacillus casei* has been selected (Dunlap *et al.*, 1971b) in which the levels of thymidylate synthetase and dihydrofolate reductase (EC 1.5.1.3) are elevated several 100-fold over the corresponding levels in the wild-type organism. In a preliminary communication (Dunlap *et al.*, 1971a), some of the properties of the two principal forms (I and II) of the dihydrofolate reductase from amethopterin-resistant *L. casei* have been described. These forms, which can be separated chromatographically or electrophoretically, differ only by the presence of an equimolar amount of noncovalently bound TPNH in II. The present paper further characterizes this enzyme,[1] particularly with respect to the interconversion of forms I and II. Subsequent communications from the laboratory will be concerned with the nature of the substrate binding sites of this enzyme and with the mechanism by which it catalyzes both the TPNH-dependent reduction of dihydrofolate and a novel transhydrogenation reaction (Huennekens *et al.*, 1970) between TPNH and AP-TPN.[2]

Experimental Section

Materials. Commerical materials included: TPN, TPNH, and AP-TPN (Sigma); Sephadex G-50 and CM-Sephadex (Pharmacia); cellulose CC31 (Whatman); Celite (Johns Manville); and Bio-Gel P-150 and hydroxylapatite (Bio-Rad Lab-

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[1] A similar dihydrofolate reductase has been isolated recently from another amethopterin-resistant strain of *L. casei* (Newbold and Harding, 1971).

[2] Abbreviations used are: AP-TPN, 3-acetylpyridine-TPN; SDS, sodium dodecyl sulfate.