An Unproductive Binary Complex of Liver Alcohol Dehydrogenase and a Chromophoric Aldehyde†

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ABSTRACT: Liver alcohol dehydrogenase forms a binary complex with 4-(2′-imidazolylazo)benzaldehyde (azoaldehyde) with a difference spectrum absorption maximum at 460 nm; the apparent binding constant for this complex is $K_{app} = 2 \times 10^{-5}$ M. Zn^{2+} forms a complex with azoaldehyde which has a very similar spectrum with a difference spectrum absorption maximum at 453 nm. This suggests that the binding site for the binary complex with enzyme involves a zinc atom on the enzyme surface. Substrate competitive inhibitors (isobutyramide, pyrazole) and a coenzyme competitive inhibitor (4-biphenylylcarboxylate) displace azoaldehyde from the binary complex indicating that the binding site for azoaldehyde overlaps the site for binding of coenzyme and substrate in the active ternary complex. Transient kinetic experiments indicate that the azoaldehyde–enzyme binary complex is unproductive since substrate must be removed from this complex before reaction can occur. This is consistent with other studies which suggest an ordered mechanism in which aldehyde combines only with the enzyme–coenzyme complex to form a reactive ternary complex. This is the first example of formation of an unproductive complex whose binding constant is of approximately the same order of magnitude as that for the productive complex (the apparent binding constant for the hydride transfer step is $K_{app} = 2 \times 10^{-5}$ M).

The role of zinc in the catalytic and binding processes of substrates with liver alcohol dehydrogenase (equine) has been under vigorous discussion recently. Early studies indicated that at least two zinc atoms per molecule of enzyme [there appear to be a total of between three and four zinc atoms per molecule (Drum *et al.*, 1967)] are involved in binding coenzymes at the active site. This conclusion was based upon the fact that *o*-phenanthroline competes with coenzymes for the active site (Vallee *et al.*, 1959) and furthermore that spectral properties of the enzyme–*o*-phenanthroline complex are similar to those of the Zn^{2+}–*o*-phenanthroline complex (Vallee *et al.*, 1958; Vallee and Combs, 1959). Later studies indicated that 2,2′-bipyridine also forms a binary complex with enzyme and that substrate, coenzyme, and substrate and coenzyme inhibitors displace bipyridine from the enzyme. The Zn^{2+} complex of 2,2′-bipyridine shows similar spectral properties to the enzyme–2,2′-bipyridine complex (Sigman, 1967). Nuclear magnetic resonance (nmr) experiments using ^{35}Cl as a probe also indicate that coenzyme binds near zinc on the enzyme surface (Ward and Happe, 1971). Stopped-flow results on the rate of binding of pyrazole and bipyridine have also been interpreted as indicating these inhibitors bind directly to the zinc at the active site (Gileland and Shore, 1970).

On the other hand several experiments concerning the apozinc enzyme indicate that coenzyme binds to the modified enzyme as tightly as to native enzyme although the apozinc enzyme is not active. This has been interpreted as evidence that zinc is not involved in binding coenzyme (Iweibo and Weiner, 1972). Recent preliminary X-ray investigation of the three-dimensional structure of the enzyme indicates that one zinc atom is located approximately 25 Å from the binding cleft in agreement with the view that zinc may not be involved in binding substrate and coenzyme or in catalysis (Branden *et al.*, 1969).

We wish to report the formation of an unproductive binary complex between liver alcohol dehydrogenase and 4-(2′-imidazolylazo)benzaldehyde (azoaldehyde) (Bernhard *et al.*, 1970); these data have bearing on the location of zinc with respect to the active site.

Experimental Section

The enzyme preparation has been described previously (Bernhard *et al.*, 1970; McFarland and Bernhard, 1972). Azoaldehyde was prepared as described previously (Bernhard *et al.*, 1970). Azoaldehyde was checked for purity by thin-layer chromatography on silica gel using 10% ethanol–ether and pure ether as solvents. In both solvents only one soluble product was detected. Spectral properties of the azoaldehyde were comparable to those reported previously. Coenzymes were obtained as follows: NADH was P-L Biochemical's Chromatopure and NAD^+ was Boehringer's highest quality. Isobutyramide was obtained from Aldrich Chemical and recrystallized from a mixture of water and acetonitrile (2:1). Pyrazole was obtained from Aldrich Chemical and used without further purification. 4-Biphenylylcarboxylate was obtained from Aldrich Chemical and recrystallized from acetonitrile. Ethyl alcohol was obtained from A.S. Industrial Chemicals (U.S.P.–N.F.) (reagent) and used without further purification. Zinc nitrate was obtained from Baker Chemical Co. and was used without further purification.

Difference Titration of Enzyme with Azoaldehyde. Difference spectra were obtained in pH 8.75, 0.05 M pyrophosphate buffer by adding azoaldehyde to both reference and sample cuvets where the sample cuvet contained $1–2 \times 10^{-5}$ N enzyme. Difference spectra were obtained on a Beckman Acta V double beam spectrophotometer and single wavelength titration data were obtained on a Cary 16 double beam spectrophotometer at 460 nm. Equilibrium constants were calculated using standard iterative computer techniques to calculate binding constants, extinction coefficients, and the number of independent binding sites from data taken under conditions where [enzyme] = $1–2 \times 10^{-5}$ N and [azoaldehyde] = 1×10^{-6} to $5 \times$

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TABLE I: Dissociation Constants of Inhibitor and Substrate Complexes with Enzyme.

[Inhibitor] or [Substrate] (M)	K_{eq}[a] (M) Azoaldehyde	K_{eq}[b] (M) 2,2'-Bipyridine	K_{ES} (M)
[Pyrazole] = 5.6×10^{-2}	4.8×10^{-2}	1.4×10^{-2}	1.6×10^{-4} [c]
[Pyrazole] = 2.4×10^{-1}	1.1×10^{-2}		
[Ethanol] = 7.0×10^{-1}	1.8×10^{-1}	2.7×10^{-1}	1.0×10^{-1} [d]
[Ethanol] = 9.1×10^{-1}	7.8×10^{-1}		
[Isobutyramide] = 2.5×10^{-1}	3.8×10^{-1}	7.6×10^{-2}	9.3×10^{-3} [d]
[Isobutyramide] = 3.7×10^{-1}	2.8×10^{-1}		
[Ph_2COOH] = 3.66×10^{-5}	4.5×10^{-5}	6×10^{-5}	
[Ph_2COOH] = 7.14×10^{-5}	4.8×10^{-5}		

[a] pH 8.75. [b] Sigman, 1967; pH 7.0. [c] Theorell and Yonetani, 1962. [d] Theorell and McKinley-McKee, 1961.

10^{-5} M. These concentrations are in the range in which $K_{app} \simeq [E] \simeq$ [azoaldehyde].

Model System Titrations. Azoaldehyde (1.6×10^{-5} M) was titrated with zinc nitrate in pH 6.0 Tris buffer (0.1 M).[1] Difference spectra were obtained by adding azoaldehyde to both reference and sample cells and adding zinc nitrate to the sample cell.

Displacement of azoaldehyde from the binary complex was studied by adding inhibitor to both reference and sample cells in the experiment described above. In all cases the total difference optical density (OD) at 460 nm could be abolished at high inhibitor concentrations. Dissociation constants for binding of inhibitor and substrate were determined by titrating enzyme–inhibitor complexes with azoaldehyde at varying concentrations of inhibitor reported in Table I.

Dissociation constants were calculated from the following equations. K_{eq} values shown in Table I were calculated using a

$$E + \text{aldehyde} \overset{K_{bin}}{\rightleftharpoons} E\text{-aldehyde}$$

$$E + I \overset{K_{eq}}{\rightleftharpoons} E\text{-}I$$

$$E\text{-}I + \text{aldehyde} \overset{K_{obsd}}{\rightleftharpoons} E\text{-aldehyde} + I$$

$$K_{eq} = K_{bin}/K_{obsd}$$

computer fit of the data to determine K_{obsd} and the known binding constant of the enzyme–azoaldehyde binary complex.

Stopped-Flow Experiments. RATE OF DISSOCIATION OF AZOALDEHYDE FROM THE BINARY COMPLEX. The formation of the NAD^+–pyrazole ternary complex with enzyme completely displaces azoaldehyde from its binary complex with enzyme. The rate of displacement of azoaldehyde from its binary complex was measured by mixing azoaldehyde and enzyme with NAD^+ and pyrazole in the stopped-flow spectrophotometer. The NAD^+–pyrazole concentration was such that formation of the enzyme–NAD^+–pyrazole ternary complex occurs with a rate constant $k_1 \geq 30$ sec^{-1}. The experiment was performed under two sets of conditions to ensure that the rate of dissociation is not dependent on the NAD^+ and azoaldehyde concentrations; optical density changes were recorded at 460 nm, the maximum in the absorption spectrum of the binary complex. The optical density changes observed as a function of time were single exponential changes. The dissociation rate constant is $k = 0.67$ sec^{-1} with [enzyme] = 5.6×10^{-6} N, [azoaldehyde] = 4.6×10^{-5} M, [NAD^+] = 4.5×10^{-4} M, and [pyrazole] = 0.02 M; the rate constant is $k = 0.59$ sec^{-1} with [enzyme] = 4.0×10^{-6} N, [azoaldehyde] = 3×10^{-5} M, [NAD^+] = 1×10^{-3} M, and [pyrazole] = 0.02 M.

Association Rate. The rate of association of azoaldehyde and enzyme was measured by observing formation of the binary complex at 460 nm. The pseudo-first-order rate constant is 1.5 sec^{-1} where [azoaldehyde] = 3×10^{-5} M and [liver alcohol dehydrogenase] = 1×10^{-5} M. This is equivalent to a bimolecular rate constant of 5×10^4 l. M^{-1} sec^{-1}.

Preincubation Experiment. Stopped-flow experiments were carried out under two configurations. In the first experiment a normal "suicide" transient kinetic experiment was carried out in which azoaldehyde–pyrazole in one syringe was mixed with NADH–enzyme in the other. In a second preincubation experiment, azoaldehyde–enzyme was mixed with NADH–pyrazole. These experiments were carried out at the following concentrations: first experiment (see Figure 3), [azoaldehyde] = 3.9×10^{-5} M, [pyrazole] = 0.02 M, [enzyme] = 4.1×10^{-6} N, [NADH] = 3.1×10^{-5} M; second experiment, [azoaldehyde] = 2.0×10^{-5} M, [pyrazole] = 0.02 M, [enzyme] = 1.7×10^{-5} N, [NADH] = 9.7×10^{-5} M. The optical density changes at 410 nm result almost exclusively from reduction of the azoaldehyde to azo alcohol since the isosbestic point for the binary complex of aldehyde and enzyme is near 410 nm. All stopped-flow experiments were performed on a Durrum stopped-flow spectrophotometer. The tungsten source was used during all determinations; flow was initiated with a gas-activated pushing device.

Results

Azoaldehyde forms a binary complex with liver alcohol dehydrogenase as shown in Figure 1. The λ_{max} for the azoaldehyde is only slightly shifted by complex formation; however, a shoulder appears on the enzyme–azoaldehyde absorption curve at 460 nm resulting in the observed difference spectrum. The difference spectrum of the binary complex shows a large red shift in the visible spectrum from λ_{max} 380 nm for free azoaldehyde to λ_{max} 460 nm for the complex with enzyme. The apparent binding constant is $K_{app} = 2.0 \times 10^{-5}$ M, the number of independent binding sites per molecule of enzyme is two, and the difference extinction coefficient for the complex is $\epsilon_{460} = 4.2 \times 10^3$ l. M^{-1} cm^{-1}. Figure 2 shows spectra for a complex between Zn^{2+} and azoaldehyde; $K_{app} = 2.9 \times 10^{-3}$ M (assuming that the number of independent binding sites is one) and the difference extinction coefficient is $\epsilon = 1.4 \times 10^3$ l. M^{-1} cm^{-1} for the Zn^{2+} complex. An isosbestic point is obtained over a limited range of zinc concentrations; however,

[1] The spectrum of azoaldehyde is pH independent between pH 6.0 and 9.0.

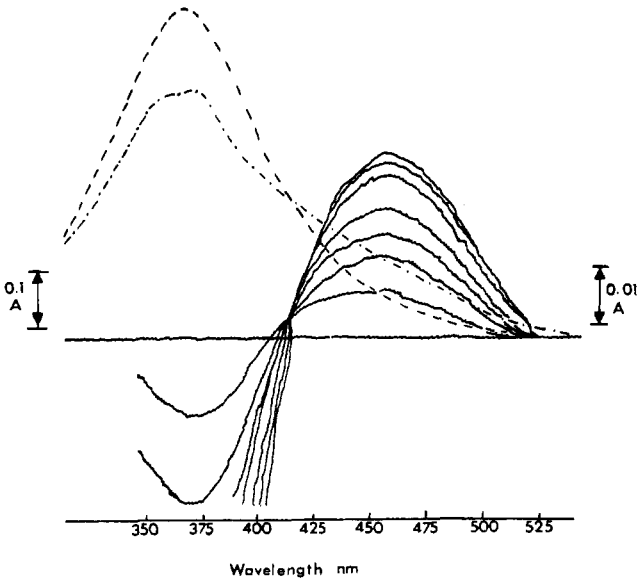

FIGURE 1: (- - -) Spectrum of azoaldehyde; [azoaldehyde] = 2.0×10^{-5} M; left hand absorbance scale. (- · -) Spectrum of the azoaldehyde–enzyme complex; [enzyme] = 1.1×10^{-4} N; [azoaldehyde] = 2.0×10^{-5} M; left-hand absorbance scale. (–) Difference spectrum of the azoaldehyde–enzyme complex; [enzyme] = 2.1×10^{-5} N; [azoaldehyde] = (1) 0.0 M, (2) 2.8×10^{-6} M, (3) 5.4×10^{-6} M, (4) 7.9×10^{-6} M, (5) 1.0×10^{-5} M, (6) 1.5×10^{-5} M, (7) 2.3×10^{-5} M; right-hand absorbance scale.

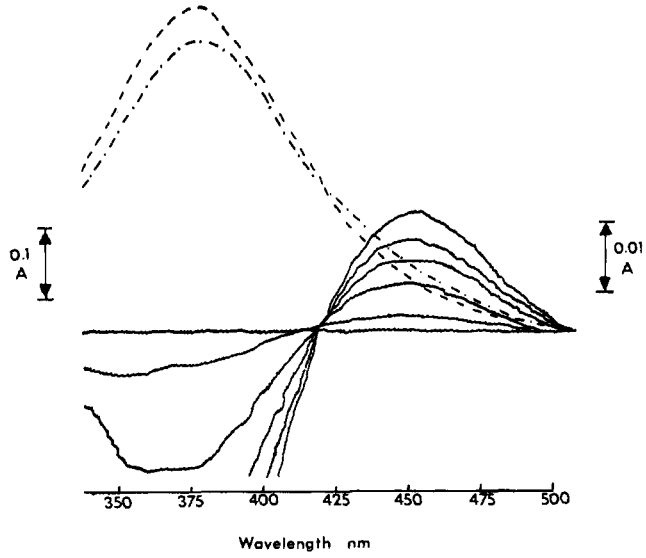

FIGURE 2: (- - -) Spectrum of azoaldehyde; [azoaldehyde] = 1.6×10^{-5} M; left-hand absorbance scale. (- · -) Spectrum of the azoaldehyde–zinc complex; [azoaldehyde] = 1.6×10^{-5} M; [zinc] = 4.1×10^{-2} M; left-hand absorbance scale. (–) Difference spectrum of the azoaldehyde–zinc complex; [azoaldehyde] = 2.2×10^{-5} M; [zinc] = (1) 0.0 M, (2) 2.7×10^{-4} M, (3) 9.3×10^{-4} M, (4) 1.6×10^{-3} M, (5) 2.2×10^{-3} M, (6) 4.0×10^{-3} M; right-hand absorbance scale.

there is considerable deviation from this behavior at lower zinc concentrations. This may be due to the formation of a 1:1 zinc complex at low zinc concentrations and a 2:1 complex over the remaining range of zinc concentrations. The λ_{max} (453 nm) for the difference spectrum of this zinc complex is quite similar to that (460 nm) for the difference spectrum of the enzyme complex.

A series of substrate and coenzyme inhibitors displaces azoaldehyde from the binary complex as evidenced by a decrease in the difference spectrum at 460 nm with added inhibitor; experiments were carried out in which azoaldehyde was forced to compete with inhibitor and substrate molecules for binding to the enzyme. Table I shows the dissociation constants calculated from these competition experiments as well as dissociation constants reported in the literature.

Our observed dissociation constants are similar to those observed by Sigman during displacement of 2,2′-bipyridine for an enzyme–bipyridine binary complex, but do not always show similarity to other values obtained by different experiments. The values for ethanol and isobutyramide were obtained from fluorescence titration of formation of a ternary ERI complex (Theorell and McKinley-McKee, 1961); the value for pyrazole was determined from the absorbance titration of the ternary enzyme–NAD^+–pyrazole complex (Theorell and Yonetani, 1963). Our data at pH 8.75 correlate reasonably with Sigman's data at pH 7.0 but are not similar to titration data for pyrazole and isobutyramide obtained by Theorell and coworkers, indicating that the mode of binding of small molecules listed in Table I is not the same in our competition experiments as in experiments carried out by direct titration.

Several experiments were performed using the stopped-flow spectrophotometer. In the first, azoaldehyde and liver alcohol dehydrogenase were preincubated in one syringe and rapidly mixed with NADH–pyrazole in the other; this will be referred to as a preincubation experiment. In a second experiment, azoaldehyde–pyrazole in the first syringe was mixed with NADH–liver alcohol dehydrogenase in the second. This configuration is the normal "suicide" single turnover experiment described previously (McFarland and Bernhard, 1972). The results of these experiments are shown in Figure 3. There is a striking difference in the number of enzyme sites undergoing reaction at the slower of the two rates in the two experiments. The normal experiment shows about one-half of the optical density change in each of the two kinetic steps while preincubation greatly increases the optical density change in the slow step. The normal suicide transient experiment consists of adding a large excess of pyrazole with aldehyde to a mixture of coenzyme and enzyme. Reaction of this mixture produces oxidized coenzyme, NAD^+, to which pyrazole quickly adds with a rate constant of 104 sec^{-1}. This fast addition serves to remove enzyme from the reactive pathway, stopping reaction after a single turnover. Under these conditions two kinetic steps are observed. In the case of the preincubation experiment part of the enzyme is complexed in an abortive binary substrate complex. This complex cannot be converted into productive complex by the addition of NADH. Aldehyde must first be removed from the enzyme and then added to the NADH–enzyme complex before reaction can occur. That part of the enzyme still reacting in a fast hydride transfer step is "free" enzyme not involved in the binary complex. The amount of "free" enzyme available is calculated from the equilibrium binding constant for this binary complex; the calculated concentration of aldehyde undergoing rapid hydride transfer corresponds to the amount of "free" enzyme. With the large excess of aldehyde used in this experiment it is possible that some impurity is responsible for the binary complex formation rather than azoaldehyde itself. In order to examine this possibility, the preincubation experiment was carried out at nearly equivalent aldehyde and enzyme concentrations; [azoaldehyde] = 2.0×10^{-5} M and [enzyme] = 1.7×10^{-5} N. The results are the same as those shown in Figure 3; the total absorbance change is the same in both the preincubation and normal single turnover experi-

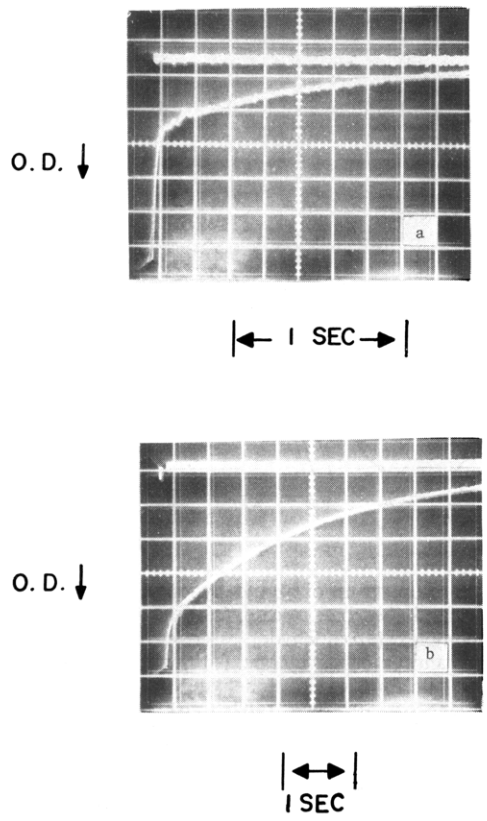

FIGURE 3: Stopped-flow experiment with different mixing of reagents: (a) normal mixing; [azoaldehyde] = 3.9×10^{-5} M; [pyrazole] = 0.02 M; [enzyme] = 4.1×10^{-6} N; [NADH] = 3.1×10^{-5} M; (b) preincubation: [azoaldehyde] = 3.9×10^{-5} M; [enzyme] = 4.1×10^{-6} N; [NADH] = 3.1×10^{-5} M; [pyrazole] = 0.02 M.

ments (one enzyme equivalent of reaction) and the ratio of absorbance changes in each kinetic step is the same as shown in Figure 3. If a minor impurity were responsible for the binary complex under conditions of aldehyde and enzyme equivalence, very little of the enzyme would be present as the abortive complex; therefore, the ratio of absorbance changes would be equal in the preincubation and normal single turnover experiments. On the other hand a major unreactive impurity in the azoaldehyde would result in less than one enzyme equivalent of reaction in the transient kinetic experiment. These results clearly show that the compound forming the abortive complex is azoaldehyde.

The enzyme–aldehyde complex dissociation rate was measured by rapidly mixing NAD^+–pyrazole solutions with enzyme–aldehyde at concentrations where the NAD^+–pyrazole association with enzyme is greater than 30 sec^{-1}. In this manner NAD^+–pyrazole complex is formed at a rate equal to dissociation of the enzyme–aldehyde complex; this measured rate is 0.63 ± 0.04 sec^{-1}. The association rate of azoaldehyde and enzyme is 5.0×10^4 l. M^{-1} sec^{-1}. This is consistent with a single step binding mechanism in which the equilibrium constant is $K_{eq} = k_{off}/k_{on} = 0.63/5.0 \times 10^{-4} = 1.3 \times 10^{-5}$ M/l.; this compares well with the measured equilibrium constant, $K_{eq} = 2 \times 10^{-5}$ M/l.

Discussion

The magnitude of the red shift in the visible spectrum produced upon binding azoaldehyde to liver alcohol dehydrogenase (Figure 1) is not characteristic of spectral changes usually encountered on binding an aromatic molecule to a hydrophobic site of an enzyme. For example, proflavine, upon binding to α-chymotrypsin, shows a spectral shift from 444 to 458 nm (Bernhard *et al.*, 1966). It is more reminiscent of the large red shifts found on formation of zinc complexes of organic nitrogen compounds (Sigman, 1967). Furthermore, the zinc complex of azoaldehyde shows a spectral shift similar to that in the enzyme–azoaldehyde complex (Figures 1 and 2). This suggests that the binding of azoaldehyde to the enzyme takes place at a zinc atom on the enzyme.

That the binding site for azoaldehyde overlaps the binding site for coenzyme and substrate is indicated by the inhibition data shown in Table I. Both pyrazole and isobutyramide, competitive inhibitors of substrate binding, and ethanol displace azoaldehyde from its binding site; a coenzyme competitive inhibitor, 4-biphenylylcarboxylate, and the NAD^+–pyrazole complex also displace azoaldehyde from its binary complex with enzyme. This strongly indicates that the binding site for azoaldehyde bound to a zinc on the enzyme is also proximate to the substrate and coenzyme binding sites.

Normal single turnover kinetics with the enzyme exhibit two steps. During preincubation experiments in which a binary complex of enzyme and azoaldehyde is formed and then allowed to react with NADH–pyrazole, the second of these two steps increases greatly in amplitude (Figure 3). This indicates that the binary complex is inactive and that azoaldehyde must dissociate from this complex before the enzyme can undergo reaction (*i.e.*, NADH cannot be added to the binary complex with subsequent reaction). This is totally in keeping with the ordered mechanism for this reaction postulated previously (Theorell and Chance, 1951; Wratten and Cleland, 1963); such a mechanism suggests that productive binding of substrate must be preceded by binding of coenzyme. The slower rate for the second step under preincubation conditions, 0.49 sec^{-1} *vs.* 0.89 sec^{-1} for the normal single turnover experiment, is consistent with the slower dissociation rate from the azoaldehyde–liver alcohol dehydrogenase binary complex, 0.63 sec^{-1}, compared to the dissociation rate from the ternary complex (normal second step rate = 1.0 sec^{-1}).

Several unexpected observations can be made from these studies. This is the first case in which an unproductive binary complex of a substrate is formed with enzyme with approximately the same binding constant and dissociation rate as the productive ternary complex ($K_{app} = 2 \times 10^{-5}$ M for hydride transfer step with the substrate). The apparent binding constant for the hydride transfer step is used as a better measure of the actual binding constant because the rate-limiting step for reaction is the desorption of products; therefore, the Michaelis binding constant ($K_m = 4 \times 10^{-6}$ M) (Bernhard *et al.*, 1970) would be expected to be tighter than the actual binding constant. It is interesting that this is the only aldehyde substrate we have found which will form such a tight binary complex. Preincubation experiments with other aromatic aldehydes (benzaldehyde, *p*-chlorobenzaldehyde, and naphthaldehyde) all give the same results whether or not enzyme and substrate are premixed. Presumably the ability of the imidazolyl azo nitrogen system to complex zinc leads to the formation of the unproductive complex with an otherwise normal aromatic substrate of the enzyme. However, in the presence of coenzyme the site for this unproductive binary complex is presumably not available for binding azoaldehyde. This is evidenced by the similarity in behavior during normal single turnover kinetics studies of azoaldehyde and other aromatic aldehydes which do not form unproductive binary complexes. Forma-

tion of some unproductive azoaldehyde–enzyme complex would reduce the amount of reaction in the hydride transfer step for this aldehyde with respect to other aromatic aldehydes in our normal transient kinetic experiment in which aldehyde is mixed with enzyme–NADH binary complex; this does not seem to be the case. An interesting point then is that the combination of binding forces of zinc complexation and hydrophobic interactions leads to approximately the same free energy of binding for the unproductive complex bound to zinc and the productive complex which most likely does not derive the same stabilization from binding zinc (evidenced by the fact that we find no kinetic anomalies for azoaldehyde reaction even though it does show the ability to complex Zn^{2+} in solution and on the enzyme which normal aldehydes do not). This may indicate that it is dangerous to imply that zinc is not involved in binding coenzyme (Iweibo and Weiner, 1972) because apozinc enzyme binds coenzyme tightly. The effect of binding zinc may only supply part of the overall binding free energy and the loss of this may be made up in hydrophobic interactions as seems to be the case in the productive ternary complex with azoaldehyde.

The dissociation constants for binding azoaldehyde in the binary and ternary complexes are nearly equal. Similarly the rate of substrate dissociation from the ternary complex is nearly equal to the rate of azoaldehyde dissociation from the binary complex. However, that this is coincidence rather than similarity in mechanism can be seen by the following argument. The formation and dissociation rates for the binary complex are consistent with a single binding step with a very slow bimolecular association rate. However, the association rate of azoaldehyde with enzyme–coenzyme binary complex cannot be this slow; transient kinetics show hydride transfer rates of 200 sec^{-1} with [azoaldehyde] = 2×10^{-5} M and [enzyme] = 1×10^{-5} N. Therefore, the association rate of azoaldehyde with the enzyme–coenzyme complex must be much faster than 200 sec^{-1}. Furthermore, even at very low aldehyde concentrations (5×10^{-6} M) the isotope effect on the transient kinetics remains unchanged indicating that no association rate is mixed with hydride transfer (McFarland and Bernhard, 1972). A lower limit of 1.0×10^{7} l. M^{-1} sec^{-1} can be assigned to the association rate in the active ternary complex; that is, the rate is at least 100 times greater than that for association of aldehyde in the binary complex. It follows that binding of aldehyde to the enzyme–NADH complex is probably not a simple single step process and that alcohol dissociation from the ternary complex is slow because it is not simple physical desorption, but is a much slower chemical step. More will be reported on this in future communications.

In conclusion, our chromophoric substrate forms an unproductive binary complex with liver alcohol dehydrogenase; this complex seems to involve direct complexation to zinc at the active site and includes the substrate and coenzyme binding site.

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References

Bernhard, S. A., Dunn, M. F., Luisi, P. L., and Schack, P. (1970), *Biochemistry 9*, 185.

Bernhard, S. A., Lee, B. F., and Tashjian, Z. H. (1966), *J. Mol. Biol. 18*, 405.

Branden, C. I., Zeppezauer, E., Boiwe, T., Soderlund, G., Soderberg, B. O., and Nordstrom, B. (1969), *in* Pyridine Nucleotide Dependent Dehydrogenase, Sund, H., Ed., New York, N. Y., Springer-Verlag, p 129.

Drum, D. E., Harrison, J. H., Li, T. K., Bethune, J. L., and Vallee, B. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1434.

Gileland, M. J., and Shore, J. D. (1970), *Biochem. Biophys. Res. Commun. 40*, 230.

Iweibo, I., and Weiner, H. (1972), *Biochemistry 11*, 1003.

McFarland, J. T., and Bernhard, S. A. (1972), *Biochemistry 8*, 1486.

Sigman, D. S. (1967), *J. Biol. Chem. 242*, 3815.

Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 15*, 11811.

Theorell, H., and McKinley-McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1811.

Theorell, H., and Yonetani, T. (1963), *Biochem. Z. 338*, 537.

Vallee, B. L., and Coombs, T. L. (1959), *J. Biol. Chem. 234*, 2616.

Vallee, B. L., Coombs, T. L., and Williams, R. J. P. (1958), *J. Amer. Chem. Soc. 80*, 397.

Vallee, B. L., Williams, R. J. P., and Hoch, F. L. (1959), *J. Biol. Chem. 234*, 2621.

Ward, R. L., and Happe, J. A. (1971), *Biochem. Biophys. Res. Commun. 45*, 1444.

Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.