Kinetics of the Reaction of Cyclopropanone Hydrate with Yeast Aldehyde Dehydrogenase: A Model for Enzyme–Substrate Interaction†

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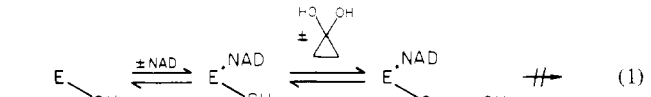
ABSTRACT: Cyclopropanone hydrate forms a thiohemiketal with an –SH group of yeast aldehyde dehydrogenase, analogous to the thiohemiacetal formed by aldehyde substrates [Wiseman, J. S., & Abeles, R. H. (1979) *Biochemistry 18*, 427–435], but cyclopropanone hydrate cannot undergo oxidation and, therefore, acts as an inhibitor of the catalytic process. We have investigated the kinetics of the reaction of cyclopropanone hydrate with yeast aldehyde dehydrogenase since we believe this reaction serves as a model for the interaction of the enzyme with the normal substrate. The reaction is ordered; nicotinamide adenine dinucleotide (NAD) binds first. Striking results are observed in both the thermodynamics and kinetics of binding. Equilibrium constants for binding of both NAD and cyclopropanone hydrate in the ternary complex are on the order of 10^{-9} M. The sources of such high binding energy for cyclopropanone hydrate are not clear. In both cases, the binding in the ternary complex is nearly 10^5 tighter than in the binary complexes. The ternary complex is also kinetically stable. NAD dissociates from the ternary complex with $t_{1/2} \approx 6$ days, i.e., 10^8-fold slower than from the binary complex. It is argued that a conformational change is rate limiting for this dissociation. The effect of cyclopropanone hydrate on the binding and dissociation rate of NAD is a unique property of that molecule. Alkylation of the –SH group which reacts with cyclopropanone hydrate by iodoacetamide does not effect the reaction of NAD with enzyme. Nicotinamide mononucleotide (NMN), which is also a cofactor for aldehyde dehydrogenase, does not form a kinetically stable complex with cyclopropanone hydrate inactivated enzyme, nor does it affect the binding of cyclopropanone hydrate to the enzyme. It is concluded that binding of NAD and cyclopropanone hydrate is cooperative. Binding of one results in a conformational change that enhances binding of the other. The adenosine 5′-monophosphate (AMP) portion of NAD is important in bringing about this conformational change. Results obtained for the catalytic reaction confirm that the reaction with cyclopropanone hydrate is an applicable model for the reaction with the normal substrate. In the presence of benzaldehyde, the reaction is ordered; NAD binds first. NAD enhances the binding of benzaldehyde at least 500-fold and dissociates slowly from the ternary complex as demonstrated by pulse–chase experiments [Rose, I. A., O'Connell, E. L., Litwin, S., & Bar-Tana, J. (1974) *J. Biol. Chem. 249*, 5163–5168] while NMN dissociates rapidly for the ternary complex (pulse–chase experiments). In the presence of NMN, K_m for benzaldehyde is 200-fold higher than in the presence of NAD.

The inhibition of aldehyde dehydrogenase (yeast) by cyclopropanone hydrate has recently been described (Wiseman & Abeles, 1979). The mechanism proposed for the inhibition is shown in eq 1.



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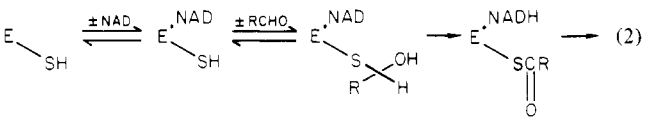

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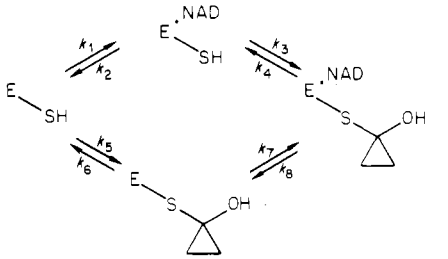
Cyclopropanone hydrate is thought to form a stable thiohemiketal with the active-site thiol, analogous to the thiohemiacetal formed with aldehyde substrates (eq 2), but the thiohemiketal is not oxidized by nicotinamide adenine dinucleotide (NAD). The inhibition reaction, therefore, provides a model for the binding steps in the oxidation of aldehydes (eq 2). This system has the advantage that the covalent enzyme–cyclopropanone complexes are kinetically quite stable. We, therefore, have chosen to study this reaction further in order to evaluate how cyclopropanone hydrate influences the interaction between NAD and aldehyde dehydrogenase. In particular, we evaluated all the rate constants of eq 3. The



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results which we obtained are a dramatic example of ordered binding in a two-substrate reaction. The rate of binding of NAD to the enzyme–cyclopropanone complex, k_7, is 3 orders of magnitude slower than the rate for free enzyme, k_1.

We have also investigated some aspects of the normal oxidation reaction in order to verify extrapolations which could be made from the model reaction to the normal catalytic process. The results for the model reaction predict that the normal order of binding for this enzyme is cofactor first, followed by aldehyde. There has been some controversy concerning the order of binding for the oxidation reaction, however. The original characterization of the enzyme (Bradbury & Jakoby, 1971) indicated that aldehyde binds first, while more recent data (Bostian & Betts, 1978a,b) indicate that NAD binds first. In addition to the studies of inhibitor

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binding, therefore, we have further investigated the enzyme-catalyzed oxidation of benzaldehyde. All of our results corroborate and extend the conclusions of the latter investigators: the binding is ordered with NAD binding first.

Materials and Methods

Chemicals. NAD, iodoacetamide, and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co., benzaldehyde was from Aldrich Chemical Co., and 2-mercaptoethanol was from Eastman Organic Chemicals.

[^{14}C]Iodoacetamide was purchased from New England Nuclear. Cyclopropanone hydrate and [^{3}H]cyclopropanone hydrate were prepared as described previously (Wiseman & Abeles, 1979). [^{3}H]NAD was the generous gift of A. Cheung.

Buffers. Buffer A is 0.1 M potassium phosphate and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0. Buffer B is 0.1 M Tris-HCl, 0.2 M KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 30% glycerol, pH 8.0. Buffer C is 0.1 M potassium phosphate, 1 mM EDTA, 50 mM 2-mercaptoethanol, and 0.01% phenylmethanesulfonyl fluoride, pH 7.0. Buffer D is 50 mM Tris-HCl, 100 mM potassium phosphate, 1 mM EDTA, 50 mM 2-mercaptoethanol, and 30% glycerol, pH 7.92. Buffer E contains, per liter, 100 mL of buffer D, 220 mL of glycerol, 1 g of KCl, and 3 mL of 2-mercaptoethanol.

Yeast aldehyde dehydrogenase is most stable in buffers containing polyhydric alcohols such as glycerol (Bradbury & Jakoby, 1972). Glycerol, at the high concentrations normally used, 30%, however, contains ~0.3 mM aldehydes which are subject to aldehyde dehydrogenase catalyzed oxidation by NAD. Such impurities would cause obvious difficulties in the interpretation of binding data for NAD and cyclopropanone hydrate as has been discussed previously (Bradbury & Jakoby, 1971; Bostian & Betts, 1978a,b). Buffer A was found to have no such impurities (<0.5 μM oxidized in 16 h by 2 IU of aldehyde dehydrogenase).

Aldehyde Dehydrogenase. Aldehyde dehydrogenase, ~2 × 10^{-3} IU/mL, was assayed in buffer B containing 0.5 mM NAD and 0.6 mM benzaldehyde by observing the change in absorbance at 340 nm. Protein was determined by the method of Bradford (1976).

The enzyme was purified from baker's yeast by a modification of a published procedure. An ammonium sulfate pellet (659 IU, 5600 mg) prepared according to Bradbury et al. (1975) was dissolved in and dialyzed against buffer C. The dialyzed enzyme solution, 465 mL, was treated successively with 121, 37, 8.4, 16.7, and 8.4 g of ammonium sulfate and centrifuged at 19000*g* for 20 min after each addition. The enzyme precipitated in the last two treatments. These last two pellets were combined and washed with 10 mL each of buffer D containing first 0.42 g and then 0.38 g of ammonium sulfate/mL. The supernatants from these two washes were combined and dialyzed against buffer E. This enzyme (470 IU, 550 mg, 35 mL) was chromatographed on a 3.6 × 12.5 cm column of Whatman DE-52-cellulose in buffer E. The column was washed with 50 mL of buffer E and then with 250 mL of buffer E plus 1.5 g/mL KCl. The enzyme was eluted with a linear 1-L gradient of buffer E containing 1.5 g/mL KCl at the start and 5 g/mL at the end. Aldehyde dehydrogenase containing fractions, which were of constant specific activity, were assayed by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis. A single minor impurity of 48 000 daltons was detected. Fractions which appeared 95% pure or better with Coomassie blue R staining were pooled. This enzyme was precipitated by dialysis against saturated ammonium sulfate in 25 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 75 mM 2-mercaptoethanol. The precipitate, 168 IU, was stored at −78 °C. A portion, 80 IU, was dissolved in 0.5 mL of buffer A plus 15 mM 2-mercaptoethanol and desalted on Sephadex G-25 (medium, 1 × 28 cm) in the same buffer. The peak fractions contained 30 IU/mL. The specific activity of this enzyme was 3.58 IU/mg.

Iodoacetamide was used to determine the number of enzyme active sites per milligram of protein. Aldehyde dehydrogenase, 0.19 IU, was incubated with 1.3 mM [^{14}C]iodoacetamide, 10.8 Ci/mol, in 0.11 mL of buffer A at 25 °C. Aliquots, 1 μL, were removed and assayed for remaining enzyme activity. After 30 min, 98% inactivation, this material was chromatographed on Sephadex G-50 (fine, 0.5 × 20 cm) in buffer A. The protein-containing fractions were pooled and found to contain 16.7 nmol of iodoacetamide/mg of protein. This value corresponds to a molecular weight of 60 000/enzyme active site and agrees closely with subunit molecular weights determined by $NaDodSO_4$–polyacrylamide gel electrophoresis: 56 000 (this preparation), 56 000 (Clark & Jakoby, 1970), and 62 500 (Bostian & Betts, 1978a,b). The values 60 000/active site, 240 000/tetramer, and 0.86 IU/nmol of tetramer were used in subsequent calculations of binding stoichiometries.

It has been reported that aldehyde dehydrogenase from yeast contains three cysteine residues/subunit (Clark & Jakoby, 1970). At least one more of these reacts with iodoacetamide after longer incubation times. For example, after a 180-min incubation under the above conditions, 22.6 nmol of iodoacetamide/mg of enzyme was found, 35% more than after 30 min. Care was taken, therefore, to avoid long reaction times and to determine stoichiometries of reactions in experiments for which covalent adducts of enzyme with iodoacetamide or cyclopropanone hydrate were to be isolated.

Pulse–Chase Experiments. Experiments were carried out at 25 °C. Enzyme and 330 μM [^{3}H]NAD (15.6 Ci/mol) were preincubated for 10 min in 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA and 5 mM 2-mercaptoethanol. An aliquot of the preincubation mixture (0.1 mL) was added with a mechanical pipet to 0.9 mL of a vigorously stirred solution containing 0.73 mM NAD and benzaldehyde in the above buffer. Two seconds later 0.2 mL of a 150 mM *N*-ethylmaleimide solution (made just prior to use) was added by mechanical pipet to stop the reaction. NADH carrier (0.25 mL, generally ~2.5 μmol, the exact concentration was determined spectrophotometrically at 340 nm) was then added, and the mixture was diluted to 3 mL with H_2O. The entire solution was then applied to a DEAE-cellulose column (6 × 1 cm diameter) equilibrated with 10 mM ammonium bicarbonate. The column was first eluted with 20 mL of 10 mM NH_4HCO_3 to remove all [^{3}H]NAD and then with 10 mL of 500 mM NH_4HCO_3 to remove NADH. Fractions (1 mL) were collected. A 0.5-mL aliquot was used for the determination of radioactivity and 0.25 mL was used for the spectrophotometric determination of total NADH; the amount of [^{3}H]NADH formed in the experiment was calculated. Control experiments were performed identically expect that the [^{3}H]NAD was already present in the chase solution (not preincubated with enzyme). The amount of [^{3}H]NADH actually formed is the difference between the [^{3}H]NADH formed in a preincubation experiment and the [^{3}H]NADH formed in a control experiment. The amount of [^{3}H]NADH formed in the control experiments was 10–25% of that formed in the pulse–chase experiment.

Sedimentation Velocity Binding Studies. Binding of NAD to aldehyde dehydrogenase was determined by sedimentation

velocity binding studies (Howlett et al., 1978). Enzyme, 16 IU/mL, and [^{3}H]NAD, 10–700 μM, were centrifuged in 80 μL of buffer A at 140000g for 1 h at 25 °C in an airfuge (Beckman Instruments, Inc.). The addition of gradient-stabilizing factors as has been recommended (Howlett et al., 1978) was not necessary at these protein concentrations.

Enzyme and NAD concentrations in the top 20 μL, which contained no enzyme, and the bottom 20 μL, which contained ~50 IU/mL of enzyme, were used to calculate the concentrations of free and bound [^{3}H]NAD. In addition, the concentrations of free and bound [^{3}H]NAD could be calculated by using the concentrations of enzyme and [^{3}H]NAD in the supernatant compared to the concentrations before centrifugation. Calculations by these two methods agreed and, therefore, indicated the absence of anomalies, such as binding of NAD to the walls of the centrifuge tube or loss of enzyme activity.

Isolation of Cyclopropanone Hydrate–Enzyme Complexes. Dissociation of Inhibitor and NAD. The binary aldehyde dehydrogenase–cyclopropanone hydrate complex was prepared and isolated as follows. Enzyme, 0.24 IU, was incubated with 2.8 mM [^{3}H]cyclopropanone hydrate in 0.01 mL of buffer A containing 15 mM 2-mercaptoethanol. The incubation was continued 1 h at 25 °C to 90% inactivation, and the inhibited enzyme was isolated by chromatography over Sephadex G-25 (medium, 0.5 × 24 cm) in buffer A. The enzyme eluted in a total volume of 0.4 mL. After correcting for incomplete inhibition, 5.5 mol of inhibitor was bound per mol of tetramer. The dissociation of cyclopropanone hydrate from the isolated complex was measured as recovery of enzyme activity.

The aldehyde dehydrogenase–cyclopropanone hydrate–[^{3}H]NAD complex was prepared similarly. Enzyme, 1.9 IU, was incubated with 5 mM cyclopropanone hydrate and 0.6 mM [^{3}H]NAD (15.9 Ci/mol) in 0.06 mL of buffer A containing 15 mM 2-mercaptoethanol. After 60 min at 25 °C (20 half-lives for inactivation), the enzyme–cyclopropanone hydrate–NAD complex was isolated by chromatography on Sephadex G-50 (fine, 0.5 × 25 cm) in buffer A. The 0.3-mL fraction containing the protein was shown also to contain 3.5 mol of [^{3}H]NAD/mol of enzyme. The enzyme complex was made 0.87 mM in unlabeled NAD and incubated at 25 °C. The dissociation of NAD was measured by chromatography of 75-μL aliquots over Sephadex G-50 as described above. Inhibited enzyme prepared similarly but with [^{3}H]cyclopropanone hydrate gave 4.2 mol of inhibitor/mol of inhibited tetramer.

Rate of Binding of [^{3}H]NAD to Aldehyde Dehydrogenase Inhibited with Cyclopropanone Hydrate. Enzyme, 1.6 IU, was incubated in 0.063 mL of buffer A containing 15 mM 2-mercaptoethanol and 7.1 mM cyclopropanone hydrate. Aliquots, 1 μL, were assayed for loss of activity. After 45 min, the inhibition had reached equilibrium, 93% inhibition. At this time, 0.060 mL was diluted with 4.5 mL of buffer A containing 0.75 mM cyclopropanone hydrate and varying concentrations of [^{3}H]NAD. Binding of [^{3}H]NAD was quenched by mixing 0.25-mL aliquots with 0.1 mL of 25 mM NAD in buffer A, and the enzyme was isolated by chromatography over Sephadex G-50 (fine, 0.8 × 25 cm) in buffer A. Enzyme-containing fractions were assayed for [^{3}H]NAD bound per milligram of protein.

Results

Binding of NAD to Free Enzyme. The rate constants describing the binding of NAD to free aldehyde dehydrogenase are k_1 and k_2 in eq 3. In order to evaluate these rate constants, it is necessary first to determine the steady-state kinetic parameters for aldehyde oxidation. Enzyme-catalyzed oxidation of benzaldehyde obeys the rate law of eq 4. The kinetic parameters determined in buffer A at 25 °C are as follows: k_{cat} = 26 s^{-1}, K_I = 1.3 × 10^{-5} M, K_2 = 7.7 × 10^{-6} M, and K_{12} = 3.1 × 10^{-10} M^2.

$$k_{cat}/k_{obsd} = 1 + K_I/[NAD] + K_2/[PhCHO] + K_{12}/[NAD][PhCHO] \quad (4)$$

A quantitative interpretation of isotope-trapping methods can be employed with knowledge of the above parameters to determine the rate of dissociation, k_2, of NAD from the binary enzyme–NAD complex (Rose et al., 1974). In these experiments, enzyme is incubated with [^{3}H]NAD, and this solution is diluted into a solution of benzaldehyde and a large excess of unlabeled NAD. The amount of [^{3}H]NADH formed after the addition to benzaldehyde is then a measure of the efficiency of trapping of enzyme-bound [^{3}H]NAD by benzaldehyde, i.e., of the rate of reaction of enzyme-bound [^{3}H]NAD with benzaldehyde vs. its rate of dissociation. When such a pulse–chase experiment was carried out, it was found that for high concentrations of [^{3}H]NAD in the preincubation (>0.33 mM) and for high concentrations of benzaldehyde in the trapping solution (>0.05 mM), a maximum of 4.4 mol of NAD was trapped per mol of enzyme (tetramer). Given the result that quantitative trapping of NAD is possible, the rate, k_2, of dissociation of NAD from the binary enzyme–NAD complex is given by (Rose et al., 1974)

$$k_2 = K_{1/2}k_{cat}/K_2 \quad (5)$$

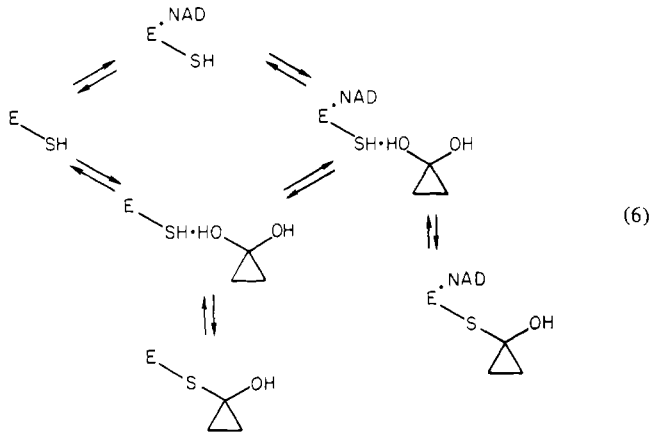
The constant $K_{1/2}$ is the concentration of benzaldehyde giving half-maximal trapping of NAD, and k_{cat} and K_2 are the kinetic constants from eq 4 (K_2 is the K_m for benzaldehyde). $K_{1/2}$ was found to be 1.7 × 10^{-5} M, and k_{cat}/K_2 = 3.3 × 10^{-6} M^{-1} s^{-1} as determined above. By use of these values, k_2 was calculated to be 56 s^{-1}.

The equilibrium dissociation constant for NAD was conveniently evaluated by sedimentation velocity measurements with an airfuge (Beckman Instruments, Inc.) as described by Howlett et al. (1978) (see Materials and Methods). The dissociation constant determined by this method was 75 μM, which compares to a published value of 120 μM determined by equilibrium dialysis and of 68 μM determined by a rate of dialysis experiment (Bostian & Betts, 1978a).

With the equilibrium dissociation constant for NAD and the rate constant for dissociation, k_2, the rate of binding of NAD, k_1 in eq 3, can be calculated: k_1 = 7.5 × 10^5 M^{-1} s^{-1}. If the mechanism of oxidation of aldehydes is ordered with NAD binding first, then k_1 can also be evaluated from the steady-state parameters k_{cat} and K_1 of eq 4 (Dalziel, 1957). In this case, $k_1 = k_{cat}/K_1$ = 1.9 × 10^6 M^{-1} s^{-1}, which is in good agreement with the value of k_1 calculated from k_2 and the equilibrium constant.

Kinetics of Inhibition by Cyclopropanone Hydrate. Rates of inhibition of aldehyde dehydrogenase as a function of both NAD and cyclopropanone hydrate concentrations are shown in Figure 1. The rates of inhibition show saturation with respect to both NAD and cyclopropanone hydrate concentrations. The mechanism of eq 3 was expanded as shown in eq 6 to be consistent with the observed saturation kinetics. Except for the immediate discussion, however, the model of eq 3 will be sufficient since it adequately parallels the binding steps for aldehyde substrates for this enzyme.

Rate constant k_5 in eq 3, for reaction of cyclopropanone hydrate with free enzyme, can be readily determined from the kinetics of inhibition in the absence of NAD. This rate constant is a second-order rate constant and represents the rate



(6)

of inhibition at below saturating concentrations of inhibitor. The value of k_5 is evaluated from the slope of the line at [NAD] = 0 in Figure 1: $k_5 = 1.1\ M^{-1}\ s^{-1}$. The rate of the reverse reaction, k_6 in eq 3, was measured directly. Direct measurement is possible since the dissociation of the enzyme–inhibitor complex is slow and the enzyme–inhibitor complex can be isolated by Sephadex chromatography. Aldehyde dehydrogenase was inactivated with cyclopropanone hydrate and the enzyme–inhibitor complex isolated by chromatography over Sephadex. Dissociation of inhibitor was assayed as recovery of enzyme activity. The rate of dissociation, k_6 in eq 3, was $6.0 \times 10^{-5}\ s^{-1}$. This rate constant had previously been determined under similar conditions by a different procedure (Wiseman & Abeles, 1979). The value presented here is in agreement with the previously determined value.

In order to evaluate k_3 in eq 3, the rate of reaction of cyclopropanone hydrate with the enzyme–NAD complex, it is necessary to decide whether inhibition in the presence of NAD proceeds via the upper or the lower pathway in eq 6. The data in Figure 1 imply that the upper pathway predominates. From Figure 1, the maximum observed rate of inhibition is $0.146\ s^{-1}$. This compares with the rate of dissociation of NAD from the binary enzyme–NAD complex, $k_2 = 56\ s^{-1}$, determined above. Since the rate at which equilibrium is established between NAD and free enzyme is the sum of k_1[NAD] and k_2, the equilibrium is clearly established rapidly on the time scale of the inhibition reaction. It follows that if the lower pathway in eq 3 predominated, then NAD would necessarily be inhibitory at high concentrations. NAD would bind the enzyme in the binary enzyme–NAD complex which would be nonproductive. The data in Figure 1 are inconsistent with this model. Rates of inhibition (the reciprocals of the slopes in Figure 1) increase with increasing NAD concentration to a maximum at ~0.2 mM. No inhibition by NAD is observed by increasing the NAD concentration further to 1.6 mM, even though this latter concentration is 20 times greater than the dissociation constant, k_2/k_1, for NAD. The upper pathway, however, is consistent with the observed kinetics. Rate constant k_3 in eq 3 is the rate of reaction of cyclopropanone hydrate, at below saturating concentrations, with the enzyme–NAD complex as described by this upper pathway. From the slope for the line in Figure 1 at the highest NAD concentration, a value $k_3 = 9.1\ M^{-1}\ s^{-1}$ can be calculated.

Binding of [^{3}H]NAD to Aldehyde Dehydrogenase Inhibited with Cyclopropanone Hydrate. The enzyme–cyclopropanone–NAD complex was found to be stable, with NAD dissociating only very slowly. Enzyme (1.9 IU) was incubated with 5 mM cyclopropanone hydrate and 0.6 mM [^{3}H]NAD (15.9 Ci/mol) in buffer A containing 15 mM 2-mercapto-

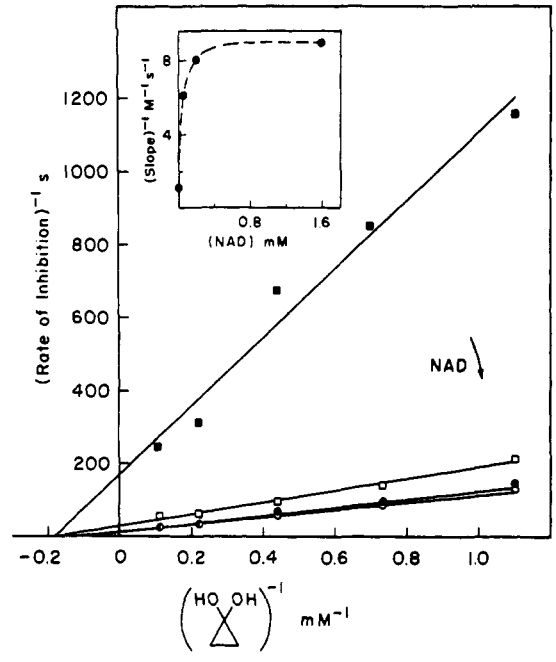


FIGURE 1: Inhibition of aldehyde dehydrogenase by cyclopropanone hydrate; effects of NAD and inhibitor concentrations. Enzyme, 0.15 IU, was incubated at 25 °C in 0.11 mL of buffer A containing varying concentrations of cyclopropanone hydrate and NAD. Aliquots, 0.02 mL, were removed periodically, and the inhibition reaction was quenched by diluting it into 2 mL of buffer B. NAD and benzaldehyde, 25 and 20 mM, respectively, in 0.05 mL were added to assay for remaining enzyme activity. (■) NAD = 0 mM; (□) NAD = 0.060 mM; (●) NAD = 0.20 mM; (○) NAD = 1.60 mM. Insert: slope^{-1}, second-order rate constant for inhibition at low inhibitor concentration, replotted as a function of NAD concentration.

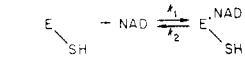
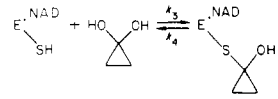
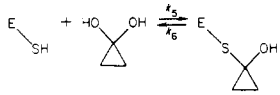
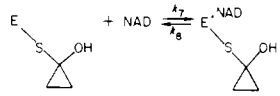
ethanol. After 60 min at 25 °C (20 half-lives for inactivation), the reaction mixture was passed through a Sephadex G-50 column (fine, 0.5 × 25 cm). The fraction containing protein also contained radioactivity: 3.5 mol of [^{3}H]NAD was bound per mol of protein (tetramer). The rate of release of [^{3}H]-NAD, k_8 in eq 3, was assayed as release of tritium from the protein fractions upon subsequent chromatography of aliquots of the complex over Sephadex. The rate of release observed was $k_8 = 1.3 \times 10^{-6}\ s^{-1}$. The dissociation rate measured for [^{3}H]NAD in the above experiment could truly represent k_8, dissociation of NAD from the ternary complex, or could actually represent rate-limiting dissociation of cyclopropanone followed by rapid dissociation of [^{3}H]NAD. In the latter case, the measured rate would represent k_4 instead of k_8 in eq 3. Rate constants k_4 and k_8 are related by the thermodynamic cycle as shown in eq 7. The constants on the right-hand side

$$\frac{k_8}{k_4} = \frac{k_2}{k_1}\frac{k_7}{k_3}\frac{k_5}{k_6} \qquad (7)$$

of eq 6 are known (k_7 is calculated below). By use of these constants, the ratio k_8/k_4 is calculated to be 120, i.e., dissociation of NAD from the ternary complex is faster than dissociation of cyclopropanone. The value given for k_8 is therefore correct.

In addition to the fact that NAD dissociates slowly from the ternary enzyme–inhibitor–NAD complex, it was also found that aldehyde dehydrogenase, which has been inactivated with cyclopropanone hydrate, binds NAD at a rate which is slow enough to be conveniently measured. Aldehyde dehydrogenase was incubated with 7.1 mM cyclopropanone hydrate until equilibrium was reached, in this case 93% inhibition. At this point, the inhibited enzyme was diluted into a solution containing [^{3}H]NAD. The concentration of [^{3}H]NAD was varied

Table I: Summary of the Rate Constants of Equation 3

binding reaction	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d[a]	binding stoichiometry[b] (mol/tetramer)
	1.9×10^6 [c]	56[d]	7.5×10^{-5}[e]	3.2[e]
	9.1	1.1×10^{-8}	1.2×10^{-9}	4.2
	1.1	6.0×10^{-5}	5.5×10^{-5}	5.5
	8.1×10^2	1.3×10^{-6}	1.6×10^{-9}	3.5

[a] $K_d = k_{off}/k_{on}$. [b] Based on a molecular weight of 240 000 for the tetrameric enzyme. Iodoacetamide binds with a stoichiometry of 4.0. [c] Determined from the kinetics of oxidation of benzaldehyde. [d] Determined by the isotope-trapping method. [e] Determined from sedimentation velocity binding studies.

from 3.5 to 90 μM in different experiments. Aliquots were diluted at varying times with a solution of unlabeled NAD so that the final concentration of unlabeled NAD was 7 mM. These aliquots were assayed for enzyme-bound [^{3}H]NAD by chromatography over Sephadex. The time dependences for binding at three different concentrations of [^{3}H]NAD are presented in Figure 2. A stoichiometry of binding of 3.6 NAD molecules/enzyme (tetramer) was obtained. The rate of binding clearly does not vary linearly with [^{3}H]NAD concentration but shows saturation kinetics; a 26-fold increase in concentration gives only a 3.6-fold increase in rate. From the rate of complex formation at low NAD concentrations (Figure 2), a second-order rate constant for the reaction of NAD with enzyme/cyclopropanone hydrate of 810 M^{-1} s^{-1} is obtained. This second-order rate constant corresponds to k_7 in eq 3. The half-saturating concentration of [^{3}H]NAD is calculated to be 11 μM, whereas for comparison the dissociation constant of NAD from free enzyme was found to be 75 μM (see above).

Given a value for k_7, the value of k_4 can now be calculated from eq 6: $k_4 = 1.1 \times 10^{-8}$ s^{-1}. This is the last of the rate constants of eq 3 to be determined. The rate and equilibrium constants applicable to eq 3 are summarized in Table I.

Inhibition by Iodoacetamide. The inhibition of aldehyde dehydrogenase by cyclopropanone was compared with the inhibition by iodoacetamide, a structurally dissimilar reagent which reacts with the active-site thiol. It has previously been shown that cyclopropanone hydrate reacts with the same thiol on the enzyme that is alkylated by iodoacetamide (Wiseman & Abeles, 1979).

NAD enhances the rate of inactivation by cyclopropanone hydrate approximately eightfold. We examined the effect of NAD on the rate of inactivation by iodoacetamide. When aldehyde dehydrogenase, 0.004 IU/mL, is incubated in buffer A at 25 °C with 1 mM iodoacetamide, loss of activity is first order with a half-life of 3.9 min. NAD retards the rate of inactivation. If NAD concentrations are varied, a maximum retardation of only 34% is observed, however. A half-maximal effect of NAD is observed between 10 and 100 μM, a closer estimation of this binding constant being unreliable due to the low magnitude of the rate effect.

NAD does not form a kinetically stable complex with iodoacetamide-inhibited aldehyde dehydrogenase, as it does with cyclopropanone hydrate inhibited enzyme. For example, 0.18

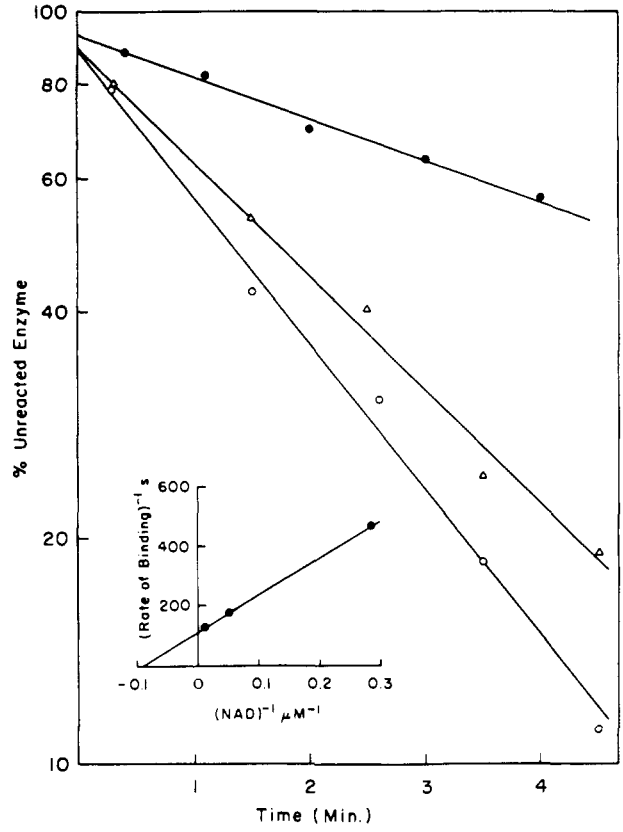


FIGURE 2: Rate of binding of [^{3}H]NAD to aldehyde dehydrogenase inhibited with cyclopropanone hydrate. Enzyme was inhibited with cyclopropanone hydrate and then incubated with [^{3}H]NAD (see Materials and Methods). Aliquots were quenched periodically with unlabeled NAD, and the enzyme–cyclopropanone hydrate–[^{3}H]NAD complex was isolated as described under Materials and Methods. Concentrations of NAD were 9×10^{-5} (○), 1.9×10^{-5} (△), and 3.5×10^{-6} M (●).

IU of aldehyde dehydrogenase was incubated at 25 °C in 107 μL of buffer A containing 6.5 mM iodoacetamide and 0.56 mM [^{3}H]NAD. The half-life for inactivation is 1 min under these conditions. After 12 min, this solution was chromatographed on Sephadex G-25 (medium, 0.5 × 25 cm) in buffer A. The protein-containing fractions contained only 0.01 mol of [^{3}H]NAD/mol of inhibited enzyme.

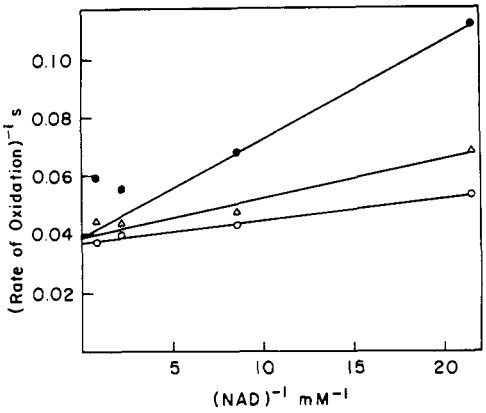


FIGURE 3: Substrate inhibition of benzaldehyde oxidation. Rates of oxidation were measured at 3×10^{-4} IU/mL aldehyde dehydrogenase in buffer A at 25 °C; benzaldehyde = (○) 1.34 mM, (△) 5.36 mM, and (●) 13.4 mM.

It was possible, however, to detect binding of NAD to iodoacetamide-inactivated aldehyde dehydrogenase by the sedimentation velocity method (Howlett et al., 1978). Enzyme, 8 IU, was inactivated with 1 mM iodoacetamide in 0.32 mL of buffer A. After 8.5 min, 1 μL of neat 2-mercaptoethanol was added to quench the reaction; 40-μL aliquots were diluted with an equal volume of buffer A containing [^{3}H]NAD in varying concentrations. The resultant solution was centrifuged for 45 min at 140000*g* at 25 °C. Aliquots of 20 μL, from the top and bottom of the centrifuge tubes, were assayed for both radioactivity and protein. A dissociation constant of 170 μM for NAD was determined. This value is little different from the dissociation constant of 75 μM observed in the absence of iodoacetamide.

Binding of Benzaldehyde to Free Enzyme. There is evidence from the steady-state kinetics of the oxidation of benzaldehyde for binding of the aldehyde to free enzyme. Aldehyde dehydrogenase is inhibited by aldehydes at high concentrations (Nirenberg & Jakoby, 1960). We have examined the effect of benzaldehyde on the rate of oxidation at relatively high substrate concentrations (Figure 3). For benzaldehyde, inhibition is marked at concentrations of NAD near 45 μM and benzaldehyde concentrations above 1 mM but decreases at higher NAD concentrations. The fact that inhibition by benzaldehyde is competitive with NAD binding can be interpreted as the formation of a nonproductive binary benzaldehyde complex (Dalziel & Dickinson, 1966). The results in Figure 3 give K_i = 4.1 mM for benzaldehyde. The data in Figure 3 deviate from linearity at high concentrations of both NAD and benzaldehyde. This additional inhibition would be interpreted as the formation of a nonproductive ternary complex. Similar results have been described in detail for alcohol dehydrogenase (Dalziel & Dickinson, 1966). The inhibition pattern observed for benzaldehyde is consistent with an ordered binding mechanism with NAD binding first (Dalziel & Dickinson, 1966).

Binding of NMN to Aldehyde Dehydrogenase. Several aspects of the binding of NMN were investigated in order to begin to assess how different regions of the NAD molecule contribute to the binding of cofactor to this enzyme. The kinetic parameters for benzaldehyde oxidation were determined with NMN in place of NAD. In terms of eq 4, the corresponding values in buffer A at 25 °C are k_{cat} = 1.2 s^{-1}, K_1 = 6.5×10^{-3} M, K_2 = 1.5×10^{-3} M, and K_{12} = 3.6×10^{-6} M^2. Compared to NAD, the effect of NMN is to decrease k_{cat} by a factor of 20 and to increase the K_m for cofactor and aldehyde by factors of 500 and 200, respectively.

In a manner exactly analogous to that for NAD, an isotope-trapping experiment was also carried out with [^{3}H]NMN. At 37 mM [^{3}H]NMN (sp act. 12.7 Ci/mol) and 9.4 mM benzaldehyde, no [^{3}H]NMNH was formed. Due to the limited solubility of benzaldehyde, experiments at higher aldehyde concentrations were not performed. The lack of [^{3}H]NMNH formation could be due to the rapid dissociation of NMN from the ternary complex or to the fact that NMN dissociation from the binary complex is more rapid than the combination of the complex with benzaldehyde (Rose et al., 1974).

NMN, in addition, does not exhibit the tight binding to cyclopropanone hydrate inhibited enzyme that is characteristic of NAD. The experiment is analogous to that for NAD. Enzyme was incubated with 5 mM cyclopropanone hydrate and 7.4 mM [^{3}H]NMN. After 1 h (93% inactivation) the solution was chromatographed on Sephadex G-25. The protein-containing fractions contained radioactivity equivalent to only 0.05 mol of NMN/mol of enzyme (tetramer).

An experiment was carried out to determine the effect of NMN on the rate of reaction of cyclopropanone hydrate and upon its dissociation from the enzyme–cyclopropanone hydrate complex. The reaction mixture consisted of aldehyde dehydrogenase, 0.2 IU, 1 mM cyclopropanone hydrate, and 30 mM NMN in a total volume of 0.1 mL of buffer A containing 30 mM 2-mercaptoethanol at 25 °C. The rate of inactivation was monitored by periodically assaying 10-μL aliquots for aldehyde dehydrogenase activity. The reaction reached equilibrium when 90% of the enzyme activity was lost with $t_{1/2}$ = 45 min. The second-order rate constant for reaction of cyclopropanone hydrate is 0.27 M^{-1} s^{-1}. The rate of dissociation of cyclopropanone hydrate from the ternary enzyme–NMN–cyclopropanone hydrate complex was also determined. A 20-μL aliquot of the inhibited complex was diluted into 2 mL of buffer A containing 30 mM NMN and 30 mM 2-mercaptoethanol. The rate of inhibitor dissociation, determined by assaying 100-μL aliquots of the dilute mixture for recovery of enzyme activity, is 2.7×10^{-5} s^{-1}. The equilibrium constant ($K_d = k_{off}/k_{on}$) of inhibition by cyclopropanone hydrate determined from these values is 1.0×10^{-4} M. Thus, NMN has no effect comparable to NAD on the on and off rates (Table I), although it appears to decrease both rate constants slightly.

Discussion

The kinetics of oxidation of benzaldehyde are consistent with an ordered mechanism with NAD binding first. The major new evidence which we present here is the isotope-trapping experiment. It is observed that [^{3}H]NAD which binds to free aldehyde dehydrogenase is trapped on the addition of benzaldehyde to give [^{3}H]NADH. This observation in itself shows that the binary enzyme–NAD complex is a viable intermediate. In addition, it is possible to trap the [^{3}H]NAD quantitatively at high concentrations of benzaldehyde. This implies that NAD does not dissociate from the ternary enzyme–benzaldehyde–NAD complex (Rose et al., 1974). This isotope-trapping experiment is in agreement with the recent conclusions of Bostian & Betts (1978a,b), who have postulated the same order of binding, and refutes the original characterization of this enzyme by Bradbury & Jakoby (1971), who postulated the opposite order of binding. The conclusions of the latter investigators were based primarily on their inability to detect binding of NAD to free enzyme by equilibrium dialysis. As these authors have discussed, however (Bradbury & Jakoby, 1971), this experiment is technically difficult for this enzyme, and these difficulties may be the source of the discrepancy. In this regard, the sedimentation velocity technique described

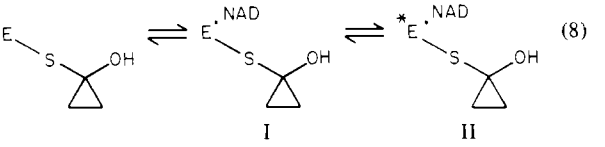
by Howlett et al. (1978) was particularly useful to us in evaluating the dissociation constant for NAD.

The rate constants for inhibition of aldehyde dehydrogenase by cyclopropanone hydrate are summarized in Table I. The striking results are the extremely tight binding of cyclopropanone hydrate to the enzyme and the very slow rate of formation of the ternary complex from NAD and enzyme–cyclopropanone hydrate, as well as the slow dissociation of NAD from the ternary complex. The dissociation of NAD from cyclopropanone hydrate inhibited enzyme has a half-life of 6 days and is slower by a factor of 10^8 than the rate of dissociation of NAD from the binary enzyme–NAD complex. In order to explain these slow rates, we propose the model for the binding steps shown in Figure 4. The upper pathway in Figure 4 is the preferred pathway for formation of the ternary enzyme–cyclopropanone–NAD complex. The binding of NAD in the first step causes a conformational change that enhances the binding constant for cyclopropanone hydrate by a factor of 5×10^4. According to our model, this is achieved in part by locking the group X in a position favorable for interaction with the –OH group of cyclopropanone thiohemiketal. This conformational change not only enhances the binding of cyclopropanone hydrate but also facilitates the rate of reaction with the –SH group 10-fold. It is very likely that this is achieved, at least in part, by making the –SH group more accessible. Such conformational changes are not unexpected for NAD-dependent dehydrogenases. Such changes have been documented for lactic dehydrogenase (White et al., 1976) and for liver alcohol dehydrogenase (Ekland & Branden, 1979).

The occurrence of a conformational change upon binding of NAD which makes the –SH group more accessible is supported by additional evidence. Neither benzaldehyde nor glycoaldehyde protect the enzyme against iodoacetamide inhibition. One would expect a reaction between the –SH group and the aldehyde simply on chemical grounds, since thiohemiacetal formation is thermodynamically reasonably favorable, i.e., $K_{eq} = 10^3\ M^{-1}$ (Kallen & Jencks, 1966). Lack of reactivity could therefore be due in part to inaccessibility of the SH group which is eliminated by NAD binding.

Along the lower pathway of Figure 4, cyclopropanone hydrate binds first and holds the enzyme in a conformation complementary to the binding of NAD. As a result of this conformational change, the binding constant of NAD to the enzyme–cyclopropanone hydrate complex is enhanced (5×10^4)-fold over that with uninhibited enzyme. The additional thermodynamic stability for the binding of NAD to the inhibited enzyme is the same factor of 5×10^4 observed above for the converse order of binding, a thermodynamical requirement. Despite the fact that the binding of NAD is thermodynamically favorable, however, the rate of binding is very slow, a factor of 2×10^3 slower than the binding to free enzyme. These slow rates are unexpected for a process that does not involve covalent bond breaking or formation. Our explanation for these slow rates, as presented in Figure 4, is that the presence of bound cyclopropanone presents a steric barrier to the binding of NAD. The steric hindrance may be due to cyclopropanone itself or to a fold of the enzyme which is locked into position through interaction with the cyclopropanone. Furthermore, the result of the thermodynamic stability of the complex and the slow rate of its formation is an extremely slow rate of dissociation of NAD from the ternary enzyme–cyclopropanone–NAD complex, $1.3 \times 10^{-6}\ s^{-1}$.

This model may be elaborated somewhat to account for all the features of the kinetics of binding of NAD. The binding of NAD to cyclopropanone hydrate inhibited enzyme, as described above, is defined as formation of the kinetically stable ternary complex. The rate of binding of NAD shows saturation kinetics with respect to NAD concentration. This result indicates a fast preassociation of NAD with the enzyme prior to the slower formation of the stable complex (II in eq 8). The



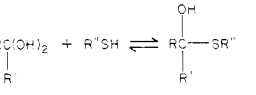
(8)

I II

K_m of NAD in this reaction is 11 μM, while the K_d for NAD dissociation from the cyclopropanone hydrate inhibited complex is 1 nM. Thus, the dissociation constant for complex I is 10^4-fold higher than that for the kinetically stable complex II. An interpretation of this result consistent with our model is that a significant portion of the NAD molecule in complex I is sterically excluded from its preferred binding domain. The transition between complex I and complex II would involve a transient conformational change, i.e., breathing of the enzyme, which allows the excluded NAD fraction access to its binding site.

The binding of cyclopropanone hydrate to the enzyme is unexpectedly tight. The equilibrium constant for cyclopropanone hydrate with free enzyme is 5.5×10^{-5} M and with the enzyme–NAD complex is 1.2×10^{-9} M. Equilibrium constants for the formation of cyclopropanone–thiohemiacetals from cyclopropanone and mercaptans are not known. However, data are available for the corresponding reaction with other ketones and aldehydes. Based on these data, we estimate[1] an equilibrium constant for the reaction of cyclopropanone hydrate with enzyme of $\sim 10^{-3}$ M. This equilibrium constant is 6 orders of magnitude (9 kcal) higher than the observed equilibrium constant for the reaction of cyclopropanone hydrate with enzyme–NAD. Thus, the formation of thiohemiacetal between cyclopropanone hydrate and the enzyme sulfhydryl group cannot account for the tight binding of cyclopropanone hydrate. Additional factors must be involved. We indicated above that the OH group of the thiohemiketal could form a hydrogen bond with a group on the enzyme. Hydrophobic interaction between the cyclopropanone ring and the enzyme could also contribute to the binding. Each of these interactions can only provide relatively small amounts of binding energy. However, as discussed by Jencks (1975),

[1] Consider the equilibrium between hydrated carbonyls and thiohemiacetals (-ketals):



An equilibrium constant for the binding of cyclopropanone hydrate to aldehyde dehydrogenase would be calculated from data for small molecules as follows. The binding of a series of thiols, varying in acidity by 7 orders of magnitude, to acetaldehyde has been shown to be independent of the nature of the thiol (Lienhard & Jencks, 1966; Barnett & Jencks, 1969). In addition, differences in the electrophilicity of different carbonyl groups should be compensated for by considering only the hydrated carbonyl. For example, the equilibrium dissociation constant for formaldehyde hydrate and 2-mercaptoethanol is 1.6×10^{-3} M (Kallen & Jencks, 1966), and the value for acetaldehyde hydrate and methoxyethanethiol is 3.1×10^{-2} M (Lienhard & Jencks, 1966). In comparison, the hydration constants for formaldehyde and acetaldehyde differ by a factor of $\sim 10^4$ (Bell & McDougall, 1960). Taken together, these results for model compounds imply that the equilibrium constant for the above reaction should be insensitive to the nature of both the thiol and the carbonyl compound. The equilibrium between cyclopropanone hydrate and enzyme would be expected, therefore, to have a dissociation constant on the order of 10^{-3} M.

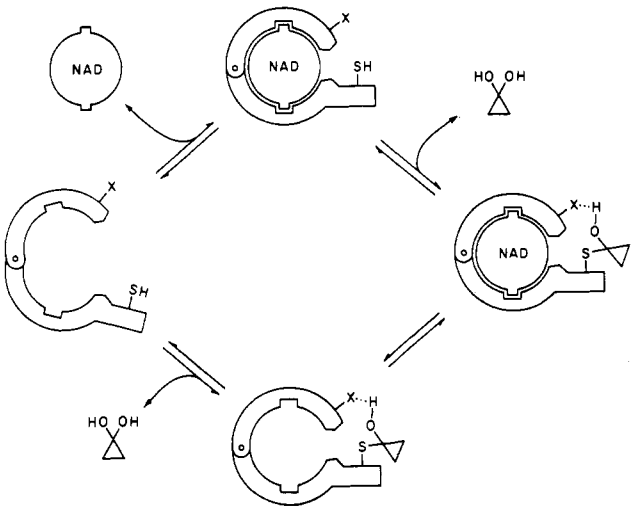


FIGURE 4: Model for binding of NAD and cyclopropanone hydrate to aldehyde dehydrogenase.

the simultaneous effect of these interactions could be large. In addition, the possible covalent interaction between cyclopropanone hydrate and NAD must be considered. This point will be further investigated. At this point, we do not have a satisfactory explanation for the tight binding of cyclopropanone hydrate.

Several close analogies exist between the reaction with cyclopropanone hydrate and the catalytic reaction. (1) Both reactions are preferentially ordered with the coenzyme binding first. (2) The rate of dissociation of NAD from enzyme–NAD–cyclopropanone hydrate is slow. In the catalytic reaction, dissociation of NAD from the ternary complex is slow relative to k_{cat} as shown by the isotope-trapping experiment. (3) NAD increases the binding of cyclopropanol hydrate to aldehyde dehydrogenase (5×10^4)-fold. A corresponding effect is seen in the catalytic reaction. NAD increases the binding of the aldehyde substrate to the enzyme. From the substrate inhibition observed with benzaldehyde, an equilibrium constant for benzaldehyde and enzyme was determined to be 4×10^{-3} M. This dissociation constant is only a lower limit because benzaldehyde at these concentrations does not form an thiohemiacetal with the active-site thiol. Benzaldehyde at these concentrations does not protect against inhibition of the enzyme by cyclopropanone hydrate (Wiseman & Abeles, 1979). The K_m for benzaldehyde, on the other hand, is 7.7×10^{-6} which is a factor of 500 lower than the above estimation of the dissociation constant.

Similar extrapolations can be made to the catalytic reactions in the presence of NMN from the interaction of NMN with the cyclopropanone hydrate inactivated enzyme. NMN does not enhance the rate of binding of cyclopropanone hydrate nor does it form a kinetically stable complex with inhibited enzyme. With NMN as the cofactor, K_m for benzaldehyde is 1.5×10^{-3} M. This is again 200-fold higher than the K_m for benzaldehyde in the NAD dependent oxidation. A parallel result is the observation that [^{3}H]NMN is not trapped by benzaldehyde in an isotope-trapping experiment. One interpretation of the isotope trapping is that NMN dissociates rapidly relative to turnover from the ternary enzyme–NMN–benzaldehyde complex.[2] These results must reflect binding energy lost by deleting a portion of the NAD molecule.

[2] The isotope-trapping experiment is subject to an alternate interpretation. NMN may not be trapped because it dissociates from the binary enzyme–NMN complex faster than benzaldehyde (at the concentrations used here) can bind.

In terms of the model of Figure 4, the result with NMN indicates that NMN is much less effective than NAD in bringing about the conformational change required to form the stable ternary complex. Therefore, it would be expected that it is less effective in forming the ternary complex in which hydrogen transfer occurs in the catalytic process so that the rate of the hydrogen-transfer step in the catalytic process should be reduced. Substitution of NMN for NAD reduces V_{max} for the catalytic reaction by a factor of 20 and V_{max}/K_m for cofactor and benzaldehyde by 10^4 and 3×10^3, respectively. However, the hydrogen-transfer step is not rate determining with NAD or NMN since no deuterium isotope effect is seen on V_{max} when deuteriobenzaldehyde is used. With deuteriobenzaldehyde and NMN, a V_{max}/K_m isotope effect of 5.5 is observed; no isotope effect is observed with NAD on V_{max}/K_m (G. Fendrich and R. H. Abeles, unpublished experiments). At this point, it can be concluded that substitution of NMN for NAD reduces the rate of the hydrogen-transfer step, but the magnitude of this reduction may be greatly underestimated from a comparison of V_{max} values.

The reduction of V_{max} in the presence of NMN indicates that NAD is present on the enzyme in the rate-determining step. Since there is no isotope effect on V_{max}, we conclude that product release must be rate determining. The data are inconsistent with the possibility that NAD dissociates first followed by rate-determining hydrolysis of acyl enzyme (see eq 2) and release of acid. The data do not distinguish among the remaining possibilities however. Until the nature of the rate-determining step and order of product release are determined, the effect of NMN on V_{max} cannot be further explained.

If cyclopropanone is a valid model for the normal substrates for this enzyme, then one would expect inhibition by structurally dissimilar thiol reagents to be different in many respects from the inhibition described above. The following results are consistent with this expectation. In contrast to cyclopropanone hydrate there is little effect of iodoacetamide on the binding of NAD. The equilibrium dissociation constant for NAD from iodoacetamide-inhibited enzyme is 170 μM. The corresponding dissociation constant from free enzyme is 75 μM and from cyclopropanone hydrate inhibited enzyme is 0.0016 μM. Although the rates of binding of NAD to, and dissociation from, iodoacetamide-inhibited enzyme could not be determined, it was demonstrated that NAD dissociated in the time it took to chromatograph the inhibited enzyme–NAD complex over Sephadex G-50, i.e., with a rate constant $>10^{-2}$ s^{-1}. This compares with rate constants of 150 s^{-1} for the dissociation from free enzyme and 1.3×10^{-6} s^{-1} for the dissociation from cyclopropanone hydrate inhibited enzyme.

The reaction of cyclopropanone hydrate with aldehyde dehydrogenase positions a hydroxyl at the active site, the hydroxyl of the thiohemiketal. This hydroxyl is absent in iodoacetamide-inhibited enzyme and may, as implied in Figure 4, contribute to the exceptional kinetic stability of the complex between NAD and cyclopropanone hydrate inhibited enzyme. The fact that this hydroxyl is also present in the hemiacetal between enzyme and normal aldehyde substrates is an important consideration in using cyclopropanone hydrate as a model for the normal aldehyde substrate.

We have concluded that the combination of enzyme, cyclopropanone hydrate, and NAD is an ordered process, i.e., NAD binds first. This conclusion arises from the fact that the rate of binding of NAD to the enzyme–cyclopropanone complex along the lower path is 3 orders of magnitude slower than the rate of binding to free enzyme. In contrast, the

binding of inhibitor to the enzyme–NAD complex along the upper pathway is eightfold faster than binding to free enzyme. Statements about order of binding, although conceptually useful, can be quantitatively meaningless, however. This is because expressions for the partitioning ratio between two possible pathways will contain second-order rate constants and, therefore, depend upon the concentrations of the reactants. The partition ratio between the upper and lower pathways in eq 3 is given by eq 9. One approach to defining what is meant

$$\frac{v(\text{upper})}{v(\text{lower})} = \frac{k_1k_3}{k_5k_7}\frac{k_6 + k_7[\text{NAD}]}{k_2 + k_3[\text{I}]} \qquad (9)$$

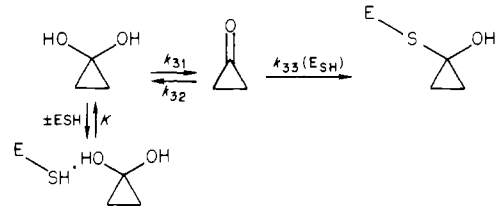
by an ordered mechanism is to consider the limiting case of low concentrations of NAD and inhibitor such that the expression for the partition ratio becomes concentration independent. This is equivalent to requiring $[\text{NAD}] \ll k_6/k_7 = 7.4 \times 10^{-8}$ M and $[\text{I}] \ll k_2/k_3 = 6.2$ M. These limiting concentrations are at opposite ends of, and well outside, the range of concentrations which would be used in any determination of the rate of inhibition. This result points out a severe flaw in any approach that considers only limiting concentrations of reactants. We prefer the following approach. Instead of limiting the concentrations of reactants and determining the resulting partitioning ratio, it seems more useful to calculate the reactant concentrations for which the partitioning ratio equals unity. In this case, since the term $k_3[\text{I}]$ is insignificant at any accessible concentration of inhibitor, i.e., below 6 M, eq 9 can be simplified to eq 10. According

$$\frac{v(\text{upper})}{v(\text{lower})} = \frac{k_1k_3}{k_2k_5k_7}(k_6 + k_7[\text{NAD}]) \qquad (10)$$

to eq 10, the rates via the upper and lower pathway would be equal at NAD concentrations of 10^{-5} M, and the lower pathway would, in fact, predominante at lower NAD concentrations.

It is not possible to quantitatively extrapolate the results for the inhibition reaction to the oxidation of aldehydes, although qualitative arguments may be made. A reliable binding constant for benzaldehyde with free enzyme is not known, but it is at least 100-fold less favorable than the binding of cyclopropanone hydrate. This result increases the probability of the reaction through the upper pathway as is observed for the inhibition reaction. This effect is particularly important at low concentrations of substrates. In contrast to the inhibition reaction, however, the reverse reactions k_4 and k_8 in eq 3 contribute to the flux of intermediates in the oxidation reaction. This effect is concentration dependent and is important at saturating substrate concentrations. The value of k_4 for the inhibition reaction is a poor estimate of the corresponding value for the oxidation reaction. The low value of k_4 observed here reflects to a large extent the exceptional kinetic stability of cyclopropanone adducts (Wasserman et al., 1974). There is insufficient data available to characterize the contribution of these reverse reactions. Attempts to detect reaction via the lower pathway by trapping of enzyme-bound benzaldehyde with NAD have been made by using the isotope-trapping method of Rose et al. (1974) but have so far been unsuccessful.

The mechanism of binding of cyclopropanone hydrate to aldehyde dehydrogenase requires some comment. Binding, i.e., the formation of a thiohemiketal with the active-site thiol, shows saturation kinetics. This is true both in the presence and in the absence of NAD. The observation of saturation kinetics has previously been interpreted to represent a mechanism in which there is preassociation of cyclopropanone hydrate with the enzyme (Wiseman & Abeles, 1979). According to this model, the cyclopropanone hydrate rapidly binds and is then dehydrated at the active site in an enzyme-catalyzed reaction; the cyclopropanone so formed would be trapped by the active-site thiol. There is, however, a kinetically equivalent mechanism which is equally, if not more, likely. This mechanism is represented in eq 11. In eq 11 cyclopropanone



(11)

hydrate binds to the enzyme, but this complex is nonproductive. Aldehyde dehydrogenase is inactivated by reaction directly with free cyclopropanone to give the hemithioketal.

It is possible to show that this alternate mechanism is kinetically competent, within the limits of the data now available, by demonstrating that rate constant k_{33} need not be faster than the diffusion-controlled limit. Rate constant k_3 from eq 3 is the faster of the two observed rate constants for the reaction of enzyme (in this case the enzyme–NAD complex) with cyclopropanone hydrate. This observed rate constant can be written in terms of the rate constants in eq 11: $k_3 = k_{31}k_{33}/k_{32}$. A lower limit of 10^5 for the hydration constant k_{32}/k_{31} has been determined for cyclopropanone (Wiseman & Abeles, 1979). Given a value of 9.1 M^{-1} s^{-1} for k_3, it is evident that k_{33} need not be greater than 10^5 M^{-1} s^{-1} in order for this model to be valid. Note, however, that this calculation depends critically on knowing the hydration constant for cyclopropanone so that the value for k_{33} is only a lower limit.

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Induced Circular Dichroism in Enzyme–Dye Complexes: Lactic Dehydrogenase–Bromphenol Blue†

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ABSTRACT: Bromphenol blue (BPB) binds to bovine lactic dehydrogenase (LDH). We have characterized the interaction with bovine H_4 LDH by using difference spectroscopy, equilibrium dialysis, steady-state enzyme kinetics, and circular dichroism. Binding to the enzyme leads to a red shift and hyperchromism in the visible band of bromphenol blue. Equilibrium dialysis indicates that three types of sites exist on each subunit, with K_d values of 5.8×10^{-5} M, 3.4×10^{-4} M, and 1.3×10^{-3} M. Steady-state kinetic studies show that the dye is a competitive inhibitor with respect to both the coenzyme, reduced nicotinamide adenine dinucleotide (NADH), and the substrate, pyruvate. When ν, the number of dye molecules bound per tetramer, is less than ~0.75, the circular dichroism (CD) of the LDH–BPB complex exhibits a single positive band in the visible region, near 600 nm, and three bands of alternating sign in the 300–400-nm region. At higher ν, the visible band splits into a positive band at longer wavelengths and a weaker negative band on the short-wavelength side. Both lobes increase in amplitude as ν increases. Only small changes are observed in the near-ultraviolet bands. Bromphenol blue forms ternary complexes with LDH saturated with NADH or adenosine 5′-monophosphate (AMP), the CD spectra of which have a negative 600-nm band. Bromphenol blue also binds to LDH modified at the essential thiol by *p*-(hydroxymercuri)benzoate, and the CD spectrum of this complex has a positive band at 600 nm. The two tight binding sites for bromphenol blue are identified as the coenzyme-binding (C) site and the substrate-binding (S) site, in order of increasing K_d. Two possible interpretations are suggested for the splitting of the visible band and the ν-dependent CD amplitudes. Dye molecules bound to the coenzyme- and substrate-binding sites could account for the couplet, while ligand-induced conformational changes must be invoked to explain the variation in amplitude. Alternatively, exciton coupling between dyes bound to the same or different subunits could explain the long-wavelength CD behavior.

Although X-ray diffraction studies are yielding detailed descriptions of protein structure in the solid state, we must continue to improve techniques for structure determination in solution. By the very nature of the phenomenon, circular dichroism (CD)[1] is aptly suited for such structural investigations. We report here studies that were designed to broaden our knowledge of the CD of enzyme-bound chromophores.

One method of exploring the enzyme active site uses molecular probes that are bound (covalently or noncovalently) to the active site. By studying the properties of the enzyme-associated probe, we can determine various physical and chemical properties of the protein environment surrounding the probe. The CD of an enzyme-bound chromophore contains information about the conformation or configuration of the chromophore itself and the stereochemical properties of the binding site. Moreover, it can aid in the identification of the chromophore if that is in doubt.

All proteins have CD spectra in the ultraviolet region due to the transitions of the peptide backbone and side-chain residues. The CD bands due to the protein itself are called *intrinsic* Cotton effects. However, many proteins have CD bands at wavelengths that do not overlap with the intrinsic Cotton effects. These bands are due to enzyme-bound chromophores such as coenzymes, prosthetic groups, metal ions, substrates, inhibitors, etc. and are called *extrinsic* Cotton effects.

Extrinsic Cotton effects are due to the inherent dissymmetry of the enzyme-bound chromophore (an *inherent* effect) and/or to the interactions of the chromophore with the encompassing dissymmetric environment (*interactive* effects). The inherent effects are those which the free chromophore would exhibit if its conformation were identical with that of the enzyme-bound form. The interactive effects result from protein–ligand interactions or ligand–ligand interactions. The main problem in interpretation of the CD of enzyme-bound chromophores is distinguishing between the inherent and the interactive effects.

We have studied the CD of enzyme–dye complexes for several reasons. (1) Glazer (1970) has noted the propensity of dyelike molecules to bind to functional sites on proteins. (2) The dyes considered here have an extensively conjugated but twisted π-electron system which creates strong inherent effects. Thus, we may be able to assume that interactive effects are not dominant (perhaps even negligible), particularly for transitions at energies far from those of the protein transitions and when the number of dyes bound per protein is small. (3)

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[1] Abbreviations used: CD, circular dichroism; LDH, lactic dehydrogenase; BPB, bromphenol blue; PMB, *p*-(hydroxymercuri)benzoate; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide; AMP, adenosine 5′-monophosphate.

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