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Roles of Zinc Ion and Reduced Coenzyme in Horse Liver Alcohol Dehydrogenase Catalysis. The Mechanism of Aldehyde Activation†

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ABSTRACT: 1,4,5,6-Tetrahydronicotinamide adenine dinucleotide (H_2NADH) has been investigated as a reduced coenzyme analog in the reaction between *trans*-4-*N*,*N*-dimethylaminocinnamaldehyde (I) (λ_{max} 398 nm, ϵ_{max} 3.15 × 10^4 M^{-1} cm^{-1}) and the horse liver alcohol dehydrogenase–NADH complex. These equilibrium binding and temperature-jump kinetic studies establish the following. (i) Substitution of H_2NADH for NADH limits reaction to the reversible formation of a new chromophoric species, λ_{max} 468 nm, ϵ_{max} 5.8 × 10^4 M^{-1} cm^{-1}. This chromophore is demonstrated to be structurally analogous to the transient intermediate formed during the reaction of I with the enzyme–NADH complex [Dunn, M. F., and Hutchison, J. S. (1973), *Biochemistry 12,* 4882]. (ii) The process of intermediate formation with the enzyme–NADH complex is independent of pH over the range 6.13–10.54. Although studies were limited to the pH range 5.98–8.72, a similar pH independence appears to hold for the H_2NADH system. (iii) Within the ternary complex, I is bound within van der Waal's contact distance of the coenzyme nicotinamide ring. (iv) Formation of the transient intermediate does not involve covalent modification of coenzyme. Based on these findings, we conclude that zinc ion has a Lewis acid function in facilitating the chemical activation of the aldehyde carbonyl for reduction, and that reduced coenzyme plays a noncovalent effector rôle in this substrate activating step.

The use of substrate and/or cofactor analogs to investigate mechanisms of enzyme catalysis often provides an experimental system where individual chemical steps in the overall reaction at the enzyme site can be studied in isolation. This happenstance arises when the free-energy profile for the overall reaction of the analog vis à vis that of natural substrates is substantially altered. Some notable examples which have been fruitfully investigated via this approach include the reaction of α-chymotrypsin with arylacryloyl imidazoles to form acyl-enzymes (Bender and Zerner, 1961; Bernhard et al., 1965), the reaction of glyceraldehyde-3-phosphate dehydrogenase with the pseudosubstrate β-(2-furyl)acryloyl phosphate to form acyl-enzyme intermediate

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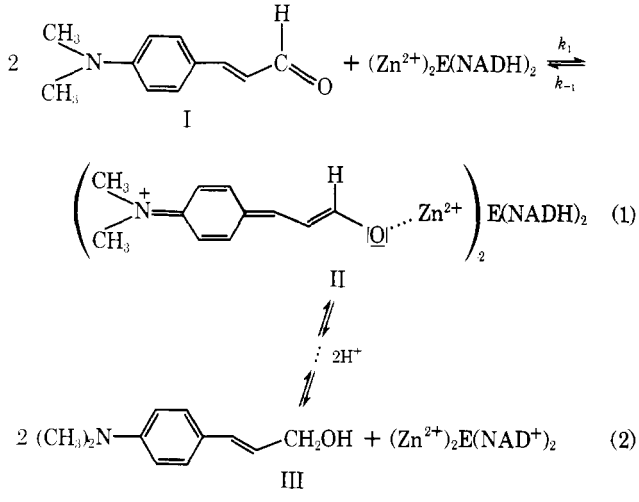
(Malhotra and Bernhard, 1968, 1973), the reaction of the quasi-substrate, 5-fluoro-2-deoxyuridylate, with thymidylate synthetase to form an active site nucleophile–adduct (Santi et al., 1974), and the elimination reaction between the pseudosubstrate β-chloro-D-alanine and D-amino acid oxidase (Walsh et al., 1971).

In our recent mechanism studies on the nicotinamide adenine dinucleotide requiring enzyme, horse liver alcohol dehydrogenase (EC 1.1.1.1) (Dunn and Hutchison, 1973), we have made use of the chromophoric quasi-substrate, *trans*-4-*N,N*-dimethylaminocinnamaldehyde (I), to examine the roles of both the essential zinc ion and the reduced coenzyme in the formation of chemical intermediate during catalysis. This work demonstrated that I and the E–NADH complex react in a rapid, reversible, pH-independent step to form a chromophoric transient intermediate (II) which decays to products, *trans*-4-*N,N*-dimethylaminocinnamyl alcohol (III) and NAD^+ in a much slower, pH-dependent step as indicated in eq 1 and 2. The spectral properties and the kinetic behavior of the intermediate led us to conclude that the transient intermediate has a dipolar quinodial structure (II) which is stabilized by a coordination bond between the active site zinc ion and the carbonyl oxygen of I. We further proposed that zinc ion functions as a Lewis acid catalyst in the activation of the aldehyde carbonyl for reduction via direct hydride transfer from NADH, and that NADH plays a heretofore unsuspected noncovalent effector role in this substrate activating step.



Examples of analogous cofactor-mediated noncovalent effector roles in the activation of substrate chemical bonds for reaction have been identified for (i) the NAD^+-mediated activation of the pseudo-acyl-enzyme intermediate, β-2-furylacryloylglyceraldehyde-3-phosphate dehydrogenase (Malhotra and Bernhard, 1973), (ii) the 5,10-methylenetetrahydrofolate-mediated formation of a nucleophilic adduct between the pseudosubstrate 5-fluoro-2-deoxyuridylate and the active site of thymidylate synthetase (Santi et al., 1974), and (iii) the AMP-mediated emission of yellow-green light in the luciferase-catalyzed oxidation of luciferyl adenylate by molecular oxygen (Lee and McElroy, 1971).

As we shall show in this paper, by making use of the NADH analog 1,4,5,6-tetrahydronicotinamide adenine dinucleotide (H_2NADH) (Biellmann and Jung, 1971), the reaction between I and the enzyme–coenzyme complex is limited to the reversible formation of the intermediate (eq 1). These experiments provide further evidence pertaining to the chemical role of zinc ion and to the noncovalent effector role of coenzyme in the horse liver alcohol dehydrogenase catalytic mechanism.

Experimental Section

Materials. The sources, purifications, and preparations of horse liver alcohol dehydrogenase, NAD^+, NADH, and *trans*-*N,N*-dimethylaminocinnamaldehyde, and the phosphate and pyrophosphate buffers used in this work were the same as previously described (Dunn and Hutchison, 1973). The AMP, ADP, and ADPR[1] employed in this work were purchased from Sigma (highest purity) and used without further purification. Isobutyramide (Aldrich) was used without further purification. Carbonate buffers were prepared from the analytical reagent grade sodium carbonate and sodium bicarbonate salts (Mallinckrodt).

The H_2NADH used in these studies was prepared by a modification of the method of Biellmann and Jung (1971). In this modification, 20–30 mg of 10% palladium on charcoal is added to a solution of 100 mg of NADH dissolved in 5 ml of water, and the resulting mixture is hydrogenated as previously described (Biellmann and Jung, 1971). The progress of the reaction is monitored by the uv spectral changes which accompany the formation of H_2NADH. Reduction is interrupted when a 265 nm/288 nm OD ratio of 0.90 is attained. The reaction mixture then is worked up by filtration over cellulose and lyophilization of the filtrate.

Enzyme active site normalities (N) were determined by the NAD^+–pyrazole binding site titration assay (Theorell and Yonetani, 1963). Normalities were found to range from 90 to 95% of the value predicted by the enzyme 280-nm extinction coefficient (Dalziel, 1957). The reported H_2NADH concentrations were calculated from optical density measurements at 263 nm (ϵ_{263} 1.85×10^4 M^{-1} cm^{-1}, Biellmann and Jung, 1971).

Methods. The instrumentation and experimental procedures used in the temperature perturbation of equilibrium kinetic studies described under Results have been previously described (Dunn and Hutchison, 1973; Erman and Hammes, 1966).

A fluorimetric titration method similar to the method of Theorell and Winer (1959) was used to determine the relative affinities of the enzyme for H_2NADH and NADH. In a typical titration, aliquots of concentrated H_2NADH were added to a solution containing 2.90 μN enzyme and a fixed amount of NADH (5–10 μM). The final H_2NADH concentration was varied from 8 μM to 0.4 mM. The extent of displacement of enzyme-bound NADH by H_2NADH was determined after the addition of each H_2NADH aliquot by monitoring the change in fluorescence emission at 425 nm. Values of the ratio K^{H_2NADH}/K^{NADH} were calculated from the fluorescence changes according to

$$K^{H_2NADH}/K^{NADH} \simeq \frac{f}{(1-f)}\left\{\frac{[H_2NADH]}{[NADH]_0 - f[E]_0}\right\} \quad (3)$$

The notation [ ]$_0$ refers to total concentration, and f is the ratio $f = \Delta F/\Delta F_0$, where the change in fluorescence, $\Delta F = F_{E(NADH)} - F_{NADH}$ (F refers to the amount of fluorescence emitted in arbitrary units). Fluorescence measurements were carried out with a Turner 210 recording spectrofluorometer; excitation wavelength, 330 nm, emission wavelength, 425 nm, excitation and emission bandwidths,

[1] Abbreviations used are: H_2NADH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; ADPR; adenosine diphosphoribose.

Table I: A Comparison of Equilibrium Constants, Rate Constants, and Difference Extinction Coefficients for the Reaction of I with the Alcohol Dehydrogenase–H_2NADH and –NADH Complexes as a Function of pH.[a]

System pH[a]	K^{H_2NADH}/K^{NADH} [b,g]	K^{H_2NADH} [c,g] $\times 10^6$	$\Delta\epsilon_{max}$ [d,g] (M^{-1} cm^{-1}) $\times 10^{-4}$	Slope[e] (k_1) (M^{-1} sec^{-1}) $\times 10^{-7}$	Intercept[e] (k_{-1}) (sec^{-1}) $\times 10^{-3}$	k_{-1}/k_1 (M) $\times 10^5$	K_1 [d,g] (M) $\times 10^5$
H_2NADH							
8.72	12.8 ± 2.0	7.9 ± 1.5	5.1 ± 0.3	6.6 ± 0.5[h]	2.25 ± 0.3[h]	3.4[h]	1.7 ± 0.2
7.65				8.6 ± 0.5[h]	2.00 ± 0.3[h]	2.4[h]	
5.98	7.8 ± 1.0	1.6 ± 1.0	5.3 ± 0.5	8.5 ± 0.5[h]	2.05 ± 0.3[h]	2.4[h]	1.9 ± 0.3
NADH							
10.54				6.2 ± 0.5[h]	0.35 ± 0.07[h]	0.57[h]	
9.48[f]				5.3[i]	0.222[i]	0.42[i]	0.43 ± 0.06 (pH 9.50)
8.19[f]				3.8[i]	0.345[i]	0.91[i]	
7.65[f]				5.3[i]	0.280[i]	0.53[i]	
7.21[f]				4.4[i]	0.227[i]	0.52[i]	0.25 ± 0.1 (pH 6.75)
6.13[f]				3.6[i]	0.268[i]	0.74[i]	0.41 ± 0.05 (pH 6.04)

[a] Measured in 0.1 M sodium phosphate buffer below pH 8, in 0.1 M sodium pyrophosphate buffer between pH 8 and pH 9.5 and in 0.1 M sodium carbonate buffer above pH 9.5. [b] Values of K^{H_2NADH}/K^{NADH} are calculated from eq 3, see Methods. [c] Values of K^{H_2NADH} are calculated from literature values for K^{NADH} (Theorell and McKinley-McKee, 1961) and the K^{H_2NADH}/K^{NADH} ratios. [d] Values of λ_{max} and K_1 are calculated from the Benesi–Hildebrand equation (Benesi and Hildebrand, 1949), see Methods. [e] Slope (k_1) and intercept (k_{-1}) values are taken from plots of $1/\tau$ vs. $([E]_f + [S]_f)$, see Figure 3. [f] Data taken from Dunn and Hutchison (1973). The accuracy of individual rate constants, as determined by the maximum deviation from the mean value, is estimated to be better than ±18%. [g] Data collected at 25.0 ± 0.1°. [h] Data collected at ~38.2°. [i] Data collected at ~31.2°.

100 nm. Equation 3 is derived assuming (a) NADH and H_2NADH compete for the same enzyme site, (b) there is no interaction between binding sites in the dimeric enzyme, and that all coenzyme sites have identical affinities, (c) that under the experimental conditions employed (i.e., an excess of H_2NADH), $[H_2NADH]_0 \simeq [H_2NADH]$, (d) that $[E] \simeq 0$, i.e., $[E]_0 \simeq [E(NADH)] + [E(H_2NADH)]$, and (e) that $f[E]_0 = [E(NADH)]$. The values of H_2NADH reported are calculated from literature values for K^{NADH} and the K^{H_2NADH}/K^{NADH} ratios are calculated from eq 3 (see Results).

The difference extinction coefficients, $\Delta\epsilon_{470}$, for the intermediate and the apparent equilibrium constants for the dissociation of I from the ternary complex formed with H_2NADH were determined spectrophotometrically from difference spectra measurements by the Benesi–Hildebrand method (Benesi and Hildebrand, 1949; Van Holde, 1971) assuming (a) a stoichiometry limit of 2 mol of I bound per enzyme molecule (Dunn and Hutchison, 1973); and (b) the binding of I to both sites is adequately described by a single (hyperbolic) dissociation process. Uv–visible spectra were obtained with a Varian 635 recording spectrophotometer.

Results

The affinity of liver alcohol dehydrogenase for H_2NADH was investigated at pH 5.98 and pH 8.72 in order to define the concentrations of H_2NADH necessary for the virtual saturation of the enzyme sites in the experiments with I described below. The enzyme–H_2NADH dissociation constant was determined by utilizing a fluorimetric assay (see Methods) for the titration of enzyme with H_2NADH in the presence of NADH. The relative affinity values, K^{H_2NADH}/K^{NADH}, derived from these competitive binding titration isotherms together with literature values (Theorell and McKinley-McKee, 1961) for K^{NADH} were used to calculate K^{H_2NADH}. These values are summarized in Table I. Inhibition constants for H_2NADH (K_i values) calculated from the effects of H_2NADH on the steady-state rate of substrate turnover are given in Table II.

The data show that the affinity of the enzyme site for H_2NADH is roughly an order of magnitude lower than for NADH. Owing to a much lower apparent affinity, attempts to measure K^{H_2NADH} at pH 10.54 were unsuccessful.

When I (λ_{max} 398 nm) is mixed with the enzyme–H_2NADH complex, there occurs the rapid, reversible formation of a new chromophoric species characterized by an intense absorption band at 468 nm. The spectral properties of the new species are summarized in Figure 1. We (Dunn and Hutchison, 1973) have previously described similar (albeit transient) spectral changes when I and the enzyme–NADH complex are mixed under similar conditions. *In contrast to the transient nature of this spectral change for the NADH system, a stable species is produced when H_2NADH is substituted for NADH.*[2]

The uv–visible spectral properties of the H_2NADH system and the NADH system (measured at pH 9.63 where the complex is stable) are presented in Figure 1A–D. The spectra in Figure 1A and in B compare the spectrum of I with the spectrum of the aldehyde-dinucleotide–enzyme mixture minus the spectrum of the dinucleotide–enzyme complex for each dinucleotide. The double difference spectra in Figure 1C and in D compare the spectrum of the ternary complex to the spectrum of the binary dinucleotide–enzyme complex and the spectrum of I. From inspection of these data it is evident that both the H_2NADH–enzyme complex and the NADH–enzyme complex react with I to yield chromophoric species which are characterized by nearly identical (long wavelength) absorption bands. The complex with H_2NADH, Figure 1A and C, displays a λ_{max}

[2] The relatively high stability of the H_2NADH ternary complex is attested to by the observation that the characteristic spectrum of the complex persists for more than a month's time at 4° both at pH 5.98 and pH 8.72. The transient species produced with NADH has half-lives of 0.5 and 23 sec at pH 5.98 and pH 8.72, respectively (Dunn and Saliman, unpublished results).

[3] Solutions containing *only* I and these adenosine derivatives exhibit uv–visible difference spectra with maxima of apparent low intensities at ~430 nm. For example, the difference spectrum which results from the comparison of a sample solution containing 10 μM I and 2.5 mM AMP with a reference solution containing only 10 μM I gives a difference OD of ~0.01 OD at 430 nm. The properties of this spectral transition suggest the occurrence of a weak molecular interaction between I and these adenosine derivatives of the donor–acceptor type.

Table II: Steady-State Inhibition Constants (K_i Values) for H_2NADH Inhibition of Horse Liver Alcohol Dehydrogenase.

pH	K_i (M)	Dinucleotide Varied[c]
8.42[a]	1.1×10^{-5}	NADH[d]
7.00[b]	1.2×10^{-5}	NADH[d]
8.70[b]	6.7×10^{-5}	NAD^+[f,h]
8.42[a]	2.6×10^{-5}	NAD^+[e]
7.60[b]	7.6×10^{-5}	NAD^+[f,g]
7.60[b]	9.0×10^{-5}	NAD^+[f,g]
6.90[b]	1.3×10^{-5}	NAD^+[f,h]

[a] Measured in 0.1 M sodium pyrophosphate buffer at 25 ± 0.2°. [b] Measured in 0.1 M sodium phosphate buffer at 25 ± 0.2°. [c] Measured in the presence of an (saturating) excess of substrate and variable but less than saturating amounts of coenzyme. The values of K_i are based on initial rate data collected at 0, 44.7 μM, and 170 μM H_2NADH. [d] Using 4-nitroso-*N*-phenylaniline (M. F. Dunn and A. M.-J. Au, unpublished results) as substrate. [e] Using *trans*-cinnamyl alcohol as substrate. [f] Measurements were carried out as described by Biellmann and Jung (1971). [g] Isoenzyme III (a gift from J. P. Von Wartburg) was used in this experiment. [h] Alcohol dehydrogenase (equine liver) purchased from Sigma was used in this experiment.

at 468 nm ($\epsilon_{max} \simeq 5.8 \times 10^4\ M^{-1}\ cm^{-1}$) and a difference spectrum λ_{max} at 470 nm ($\Delta\epsilon_{max} \simeq 5.2 \times 10^4\ M^{-1}\ cm^{-1}$), while the spectrum of the complex with NADH, Figure 1B and D, displays a λ_{max} at 464 nm ($\epsilon_{max} \simeq 6.2 \times 10^4\ M^{-1}\ cm^{-1}$), and a difference spectrum λ_{max} 468 nm ($\Delta\epsilon_{max} \simeq 5.8 \times 10^4\ M^{-1}\ cm^{-1}$). Note that the spectrum of enzyme-bound NADH, as evidenced by the shoulder at 367 nm in the NADH ternary complex–binary complex difference spectrum (Figure 1B), is perturbed.

The Benesi–Hildebrand equation (Benesi and Hildebrand, 1949; Van Holde, 1971, see Methods) has been used in the analysis of difference spectra titrations to determine both the apparent equilibrium constants for the dissociation of I from the H_2NADH ternary complex (eq 4) and the maximum difference extinction coefficient, $\Delta\epsilon_{max}$. In eq 4 the binding sites for I are assumed to be identical and independent, hence, $K_1 = k_{-1}/k_1 = [\text{sites vacant}][I]/[\text{sites occupied}]$. These parameters, along with the corresponding values taken from Dunn and Hutchison (1973) for the NADH system at lower pH values, are summarized in Table I.

$$(H_2NADH)E(H_2NADH) + I \underset{k_{-1}}{\overset{2k_1}{\rightleftharpoons}} (H_2NADH)E(H_2NADH, I) \quad (4)$$

$$I + (H_2NADH)E(H_2NADH, I) \underset{2k_{-1}}{\overset{k_1}{\rightleftharpoons}} (I, H_2NADH)E(H_2NADH, I)$$

The kinetic traces shown in Figure 2A and B are typical examples of temperature-jump relaxation spectra for the H_2NADH and NADH systems, respectively. These studies extend the previous range of measurements on the NADH system from pH 6.13–9.48 (Dunn and Hutchison, 1973) to pH 10.54. Note that the pH 10.54 data are in every aspect qualitatively similar to the data previously obtained at lower pH values.

The OD resolving capabilities of the instrument ($\sim 1 \times 10^{-3}$ ΔOD) and the useful time interval accessible to study ($\sim$50 μsec to 200 msec) place limitations on the amplitudes and the time constants of detectable relaxations. Within these limits of detection, each system is characterized by a

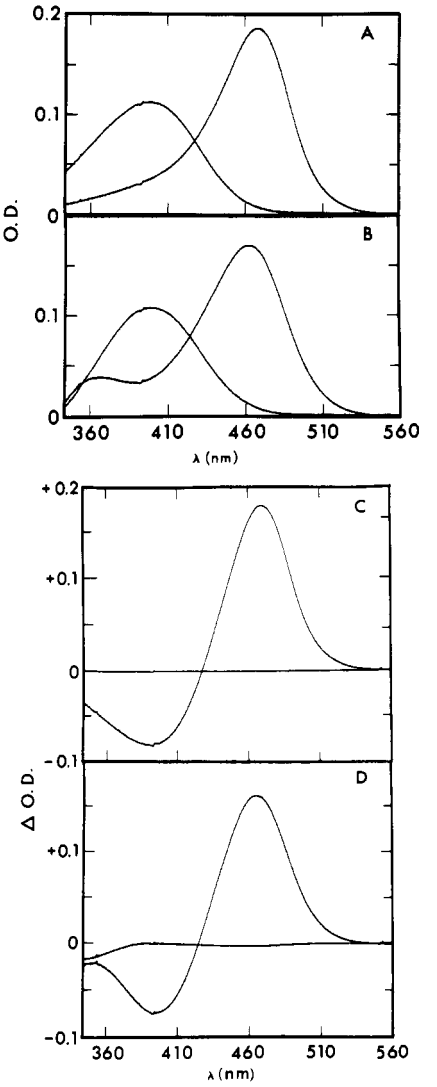


FIGURE 1: Uv-visible spectral properties of the I–enzyme–H_2NADH and I–enzyme–NADH systems. The traces in (A) compare the spectrum of I (λ_{max} 398 nm) with the spectrum of the intermediate (λ_{max} 468 nm) derived from the reaction of I with the enzyme–H_2NADH complex in 0.1 M sodium phosphate buffer (pH 6.98) at 25 ± 1°. These spectra were measured using double difference (split compartment) cuvets as follows. (A) For the spectrum of I, the sample cuvet contains enzyme, 85.5 μN, and H_2NADH, 286 μM, in one compartment (total volume 1.1 ml), and I, 8.02 μM, in the second compartment (total volume 1.1 ml); the reference cuvet contains an equal volume of the identical enzyme–H_2NADH solution in one compartment, and 1 ml of buffer in the other compartment. The spectrum of the intermediate was obtained by mixing the contents of the sample cuvet and recording the spectrum of the resulting reaction mixture. Note that under these experimental conditions, the conversion of I to intermediate is nearly complete. The traces in (B) compare the spectrum of I with the spectrum of the intermediate (λ_{max} 464 nm) derived from the reaction of I with the enzyme–NADH complex in 0.1 M sodium carbonate buffer (pH 9.63) at 25 ± 0.1°. The spectrum of I and the spectrum of the intermediate were obtained as in (A). The sample cuvet before mixing contains enzyme, 85.5 μN, and NADH, 310 μM, in one compartment, and I, 7.80 μM, in the other compartment; the reference cuvet contains an equal volume of the identical enzyme–NADH solution in one compartment and buffer in the other compartment. As in (A), the conversion of I to intermediate is nearly complete under these conditions; furthermore, the net conversion of intermediate to products is negligible at this pH. The double difference spectra recorded in (C) and (D) were obtained by adding I in amounts identical with those initially present in (A) and in (B), respectively, to the buffer compartment of the reference cuvets and recording the difference between the mixed sample cuvets in (A) and (B) and the unmixed references. The base line in each spectrum was recorded after mixing the contents of the reference cuvet. (Each compartment of the split cuvets has a 0.436-cm light path.)

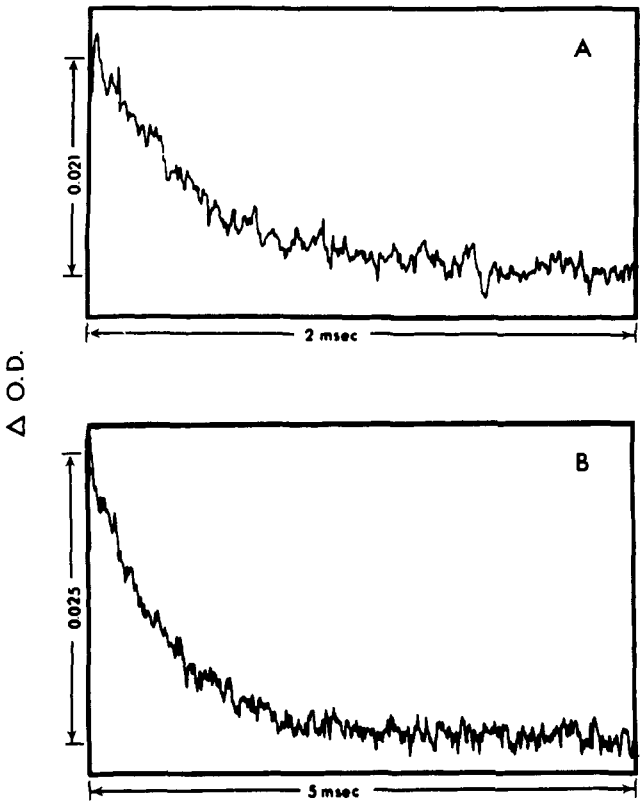


FIGURE 2: Temperature-jump kinetic traces for the enzyme-H_2NADH system in 0.1 M sodium phosphate buffer (pH 5.98) (trace A), and for the I-enzyme-NADH system in 0.1 M sodium carbonate buffer (pH 10.54) (trace B). The traces record the OD changes at 460 nm which accompany the relaxation of each system to the new equilibrium following the rapid change in system temperature produced by the heating pulse. Conditions: (A) enzyme, 4.10 μN; H_2NADH, 170 μM; I, 13.3 μM; (B) enzyme, 2.83 μN; NADH, 244 μM; I, 13.5 μM. Instruments settings: heating pulse width, 100 μsec; capacitor charge, 5 kV; electronic time constant, 10 μsec; initial temperature, 25.0 ± 0.2°; $\Delta T \sim 13.2°$; light path, 2 cm. Note that the net optical density changes reported are corrected to the values expected for a 1-cm light path.

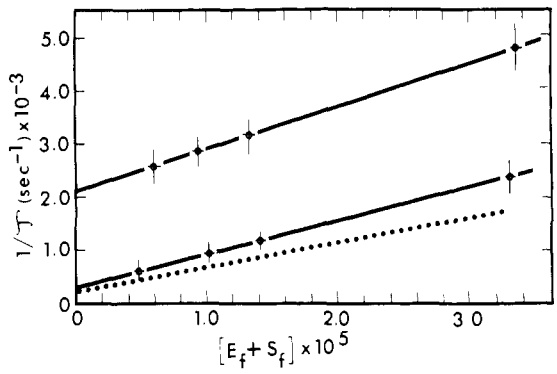


FIGURE 3: A composite summary of the dependence of $1/\tau$ on concentration and pH for the H_2NADH system and for the NADH system. (See Figure 2 for representative sets of the relaxation kinetic traces used in the determination of $1/\tau$ values.) The experimental conditions are the same as in the caption to Figure 2. Data for the H_2NADH system at pH 5.98, pH 7.65, and pH 8.72 are represented by the upper solid line. The fit of this line to these data is within the limits ±20%, as indicated by the error brackets. The lower solid line represents the best straight line fit of the data for the NADH system at pH 10.54 (0.1 M sodium carbonate buffer). The dotted line summarizes data for the NADH system taken from Dunn and Hutchison (1973). This line represents data covering the pH range 6.13–9.48 within the limits ±25%. Note that the present data were collected at a final system temperature of ~38.2° (initial temperature, 25.0 ± 0.2°, $\Delta T \sim 13.2°$) while the data of Dunn and Hutchison were collected at a final system temperature of 31.2° (initial temperature, 18.0 ± 0.5°, $\Delta T = 13.2°$). The slope (k_1) and intercept (k_{-1}) values taken from the individual plots are summarized in Table I.

single relaxation. Furthermore, over the aldehyde concentration range accessible to study, the relaxation time constant ($1/\tau$) in both systems increases with increasing aldehyde concentration. The dependence of $1/\tau$ on the free concentrations of aldehyde, $[S]_f$, and the dinucleotide-saturated enzyme sites, $[E]_f$, have been quantitated by constructing plots of $1/\tau$ vs. the sum ($[S]_f + [E]_f$), Eigen and de Maeyer (1963). A composite plot summarizing these data is presented in Figure 3 together with data taken from Dunn and Hutchison (1973) for comparison. The slope (k_1) and intercept (k_{-1}) data and calculated values of $K_1 = k_{-1}/k_1$ for each pH also are summarized in Table I.

It is apparent that at pH 10.54 the values for both k_1 and k_{-1} are slightly larger than the values previously obtained between pH 6.13 and 9.48 (see Figure 3 and Table I). However, this apparent difference in specific rate constants clearly results from the higher temperature (~38.2° vs. 31.2°) used in the present study. Correction for this temperature difference gives estimated values at pH 10.54 which are well within experimental error of the values reported for the lower pH's.

Discussion

In our previous studies (Dunn and Hutchison, 1973), *trans*-4-*N*,*N*-dimethylaminocinnamaldehyde (I) was found to react rapidly and reversibly with the horse liver alcohol dehydrogenase–NADH complex in a pH-independent step to form the above described 464 nm absorbing species as a transient intermediate. The intermediate decays to products in a much slower, pH-dependent step according to the kinetic sequence of eq 1 and 2. Only the decay step was found to be subject to a kinetic isotope effect when (4*R*)-4-deuterio-NADH is substituted for isotopically normal NADH. Furthermore, it was found that NAD^+ would not substitute for NADH in the process of intermediate formation even though we concluded that NADH is not chemically altered in this step. Therefore, we proposed that the specificity of the reaction for reduced coenzyme reflects a noncovalent effector role for NADH in facilitating the zinc ion mediated chemical activation of aldehyde for hydride attack *via* inner-sphere coordination of the aldehyde carbonyl oxygen as illustrated in eq 1.

The results of the present study further confirm and amplify the mechanistic conclusions of Dunn and Hutchison (1973). These studies extend the range of pH values investigated to pH 6.13–10.54 for the NADH system. The data presented on Figure 3 and in Table I clearly show that the process of intermediate formation is essentially pH independent over this entire range of pH values.

Since reduction of aldehyde by horse liver alcohol dehydrogenase involves a net consumption of protons from solution, the direct participation of hydrogen ion is required at some stage in the catalytic mechanism. As a consequence, it has been speculated that protonic acid–base catalysis plays a role in the transfer of hydride (or its equivalent) between coenzyme and substrate (Shore et al., 1974; Brooks et al., 1972; Wang, 1968). It therefore is noteworthy that, as previously argued (Dunn and Hutchison, 1973) and further emphasized by the results of the present study, *no such group is involved in the formation of the transient intermediate.*

Taniguchi et al. (1967) have proposed that the pH depen-

dence of the enzyme affinity for NAD^+ (apparent $pK_a' = 6.85$) can be attributed to the interaction of NAD^+ with a site residue with $pK_a' = 8.75$. Displacement of bound NAD^+ by NADH at pH 8.8 is accompanied by a net uptake of one equivalent of hydrogen ions from solution for every mole of NAD^+ displaced (Dunn, 1974). Moreover, transient kinetic studies with the substrate 4-(2′-imidazolylazo)benzaldehyde (Dunn, 1974) show that under single turnover conditions the stoichiometrically required amount of hydrogen ion uptake occurs at a rate which is much slower than the rate of aldehyde reduction. This "Bohr effect" for alcohol dehydrogenase is interpreted as resulting from a coenzyme oxidation-state dependent perturbation of the pK_a' of an enzyme site residue. Taken together the above findings suggest that involvement of a protonic acid–base group or groups in aldehyde reduction quite possibly is restricted to the modulation of site affinity for ligands.

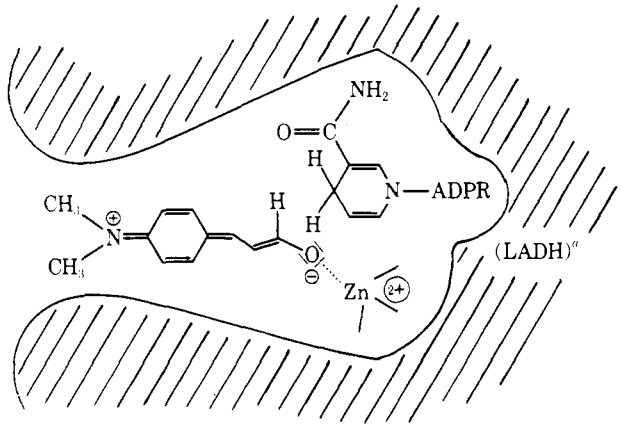
From the data presented in Figures 1–3 and in Table I it is apparent that H_2NADH will substitute for NADH in the process of intermediate formation *even though H_2NADH is chemically inert in the hydride transfer step* (Biellmann and Jung, 1971). The inertness of H_2NADH is consistent with the chemical properties expected of the 1,4,5,6-tetrahydronicotinamide ring. These chemical properties virtually exclude the possibility that intermediate formation involves covalent chemical bonding between I and the coenzyme.

The temperature-jump kinetic studies demonstrate that, just as in the case of the NADH system (Dunn and Hutchison, 1973), intermediate formation with the H_2NADH system is also a single-step, pH-independent kinetic process (viz., eq 4). It is noteworthy that the affinity of the E-NADH complex for I is tenfold greater than that of the E-H_2NADH complex. It is evident (see Table I) that the tenfold increase in k_{-1} accounts for the difference in affinity. Substitution of H_2NADH for NADH does not appreciably alter the nearly diffusion-limited rate of formation of the chromophore.

In view of the relatively subtle structural differences between the reduced nicotinamide moieties of H_2NADH and NADH, it seems most reasonable to view the decrease in affinity as arising from a steric hindrance effect at the site involving unfavorable interactions between I and H_2NADH introduced by the 1,4,5,6-tetrahydronicotinamide ring. The C_5–C_6 bond of H_2NADH certainly must be longer than the corresponding bond in NADH (Hope, 1969). Furthermore, the 1,4,5,6-tetrahydronicotinamide ring very likely assumes a nonplanar conformation with C_5 out of the plane defined by N_1, C_2, C_3, C_4, and C_6 (Hope, 1969), while the nicotinamide ring of NADH is almost certainly planar (Koyama, 1963; Karle, 1961). Assuming I is positioned within van der Waal's contact distance of the dinucleotide nicotinamide moiety, it is not surprising that these structural differences alter the affinity of the H_2NADH–enzyme complex for I.

Three additional pieces of evidence are relevant to this point: (1) isobutyramide, a potent competitive inhibitor of aldehyde reduction (Theorell and McKinley-McKee, 1961), is also a strong inhibitor of intermediate formation, (2) NADH fluorescence is virtually totally quenched in the ternary complex (M. F. Dunn and R. G. Morris, unpublished results), and (3) no such chromophoric species form in solutions containing I and the binary complexes involving enzyme and the coenzyme fragments AMP, ADP, or ADPR. These facts, together with the evidence that the spectrum of bound NADH is perturbed by I in the ternary complex (Figure 1), strongly argue that I is bound within van der Waal's contact distance of the coenzyme nicotinamide group. These facts also provide additional evidence in support of the proposed structure for the chromophore involving a coordination link between the carbonyl oxygen of I and the active site zinc ion (Scheme I).

Scheme I



[a] LADH = horse liver alcohol dehydrogenase.

The high resolution X-ray structure of native horse liver alcohol dehydrogenase (Brändén et al., 1973; Eklund et al., 1974) has revealed that the active site zinc ion is located at the point of convergence of two deep clefts in the surface of the subunit. One of these clefts has been identified as the coenzyme binding site (Brändén et al., 1973; Eklund et al., 1974). The other cleft is believed to be the substrate binding site, since the X-ray structural studies show that the competitive inhibitor *o*-phenanthroline occupies this cleft while coordinated to the zinc ion. The active site zinc ion in the native enzyme is four-coordinate, and exists in a warped tetrahedral ligand field geometry. Two of the four ligands are cysteinyl sulfhydryl residues, the third is a histidyl residue. The fourth ligand appears to be a water molecule. This water molecule is displaced on formation of the *o*-phenanthroline complex (Brändén et al., 1973; Eklund et al., 1974).

If the carbonyl oxygen of I is coordinated to the fourth ligand site of the active site zinc ion, as in Scheme I, then the inner sphere-coordinated water molecule must be displaced during complex formation. Wilkens and Eigen (1964) have shown that the apparent second-order rate constants measured for substitution of inner-sphere water in the zinc-aquo complex, $Zn(OH_2)_n^{2+}$, by other ligands is dominated by the rate of dissociation of inner sphere H_2O. Consequently, an upper limit is placed on the rate at which inner-sphere coordination to zinc ion can occur. So long as the ligand displaced is an H_2O molecule, the apparent second-order rate of displacement of water by a second ligand can be no greater than $\sim 10^7\ M^{-1}\ sec^{-1}$, unless, in the case of mixed complexes, the rate of water dissociation is influenced by other ligands coordinated to the metal ion (Wilkens and Eigen, 1964). Thus, acetate, sulfate, and chloride ions all combine with the aquo-zinc complex with apparent second-order rate constants $\sim 3 \times 10^7\ M^{-1}\ sec^{-1}$ at 25° (Wilkens and Eigen, 1964), while the neutral, tridentate ligand, pyridine-2-azodimethylaniline, gives a slightly lower value, $4 \times 10^6\ M^{-1}\ sec^{-1}$, at 15° (Wilkens, 1964).

The numerical similarity of these rate constants to the values of k_1 (Table I) is consistent with a mechanism for intermediate formation in which the rate-determining step in-

volves the dissociation of a water molecule from the fourth ligand position of the active site zinc ion. Alternatively, note that these rates are nearly large enough to be considered diffusion-limited processes.

Both NADH and H_2NADH bring about the same crystallographically identifiable conformational change in horse liver alcohol dehydrogenase (E. Zeppezauer, C.-I. Brändén, and H. Eklund, unpublished results). The above experiments and our previous work (Dunn and Hutchison, 1973) directly demonstrate that coenzyme binding stabilized the catalytically active conformation state of the enzyme and thereby plays a noncovalent effector role in chemical activation of substrate.

The regulation of both the enzyme affinity for ligand and the chemical activity of bound ligand via coenzyme oxidation state (Theorell and McKinley-McKee, 1961; Theorell and Yonetani, 1963; Dunn and Hutchison, 1973) appears to have its origins in a binding specificity which is determined by the charge on the nicotinamide ring. The differences in site conformation implied by the differential afffinities of the binary NAD^+- and the NADH-enzyme complexes toward various ligands, including substrates (Sund and Theorell, 1962; Theorell and McKinley-McKee, 1961; Dunn and Hutchison, 1973), demand that, in order to satisfy the principle of microscopic reversibility, the chemical transformation of substrate to product must be accompanied by a conformational transformation of the enzyme site.

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