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Evidence That Hydride Transfer Precedes Proton Transfer in the Liver Alcohol Dehydrogenase Catalyzed Reduction of *trans*-4-(*N*,*N*-Dimethylamino)cinnamaldehyde†

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ABSTRACT: The kinetics of the decay of enzyme-bound intermediate to products for the horse liver alcohol dehydrogenase (LADH) catalyed reduction of *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA) by NADH as a function of pH are reported. The intermediate has previously been shown to be an E(NADH,aldehyde) complex involving inner-sphere coordination of the carbonyl oxygen of DACA to the active-site zinc ion [Dunn, M. F., Biellman, J.-F., & Branlant, G. (1975) *Biochemistry 14*, 3176]. The reaction was carried out in the presence of 20 mM pyrazole, a potent LADH inhibitor, to drive the reaction to completion via formation of the LADH-NAD-pyrazole adduct. Under conditions of $[DACA]_0$, $[E]_0 \gg [NADH]_0$, intermediate decay was found to be an apparent first-order process over the pH range 4.33-9.00. The decay of the intensely chromophoric intermediate was found to be markedly pH dependent. Under conditions of rate saturation (i.e., when $[DACA]_0$, $[E]_0 \gg K_D$, the apparent dissociation constant for the intermediate), the decay rate was found to decrease with increasing pH. Substitution of (4*R*)-4-deuterionicotinamide adenine dinucleotide (NADD) for NADH gave a primary kinetic isotope effect of 2.8 at pH 4.33. The isotope effect decreases with increasing pH and was found to be $\simeq 1.0$ above pH 7. Analysis of the pH-rate profiles for NADH and NADD gave apparent pK_a values of 6.00 ± 0.20 and 6.51 ± 0.20, respectively. The pH-independent decay rate constants for NADH and NADD were 7.2 s^{-1} and 2.3 s^{-1}, respectively. The observed pH-independent spectrum of the intermediate and the pH dependencies of the decay process and the isotope effect could not be explained by mechanisms involving either a rapid preequilibrium protonation of the intermediate or a proton transfer concerted with hydride transfer. Although unlikely, a concerted mechanism involving an as yet undetected proton transfer via a protonic enzyme residue with $pK_a > 10.6$ could not be ruled out. The simplest mechanism that predicts both the pH dependencies of the isotope effect and the decay rates and explains the pH-independent spectrum of the intermediate involves (1) aldehyde activation for hydride attack via inner sphere coordination to zinc ion, (2) proton transfer to zinc-coordinated alcoholate ion in a step subsequent to hydride transfer, and (3) release of products from the site.

Previous studies from this laboratory (Dunn & Hutchison, 1973; Dunn et al., 1975; Angelis et al., 1977; Dunn et al., 1977) have shown that a transient chemical intermediate (λ_{max} 464 nm; ϵ_{max} 6.2×10^4 M^{-1} cm^{-1}) is formed during the liver alcohol dehydrogenase (LADH)[1] catalyzed reduction of 4-*trans*-(*N*,*N*-dimethylamino)cinnamaldehyde (λ_{max} 398 nm; ϵ_{max} 3.10×10^4 M^{-1} cm^{-1}), viz., Scheme I.

The intermediate (NADH)E(I) is formed in a rapid, reversible, and pH-independent step with $k_f = 4 \times 10^7$ M^{-1} s^{-1} and $k_r = 280$ s^{-1} (Dunn & Hutchison, 1973; Dunn et al., 1975). Preliminary studies (Dunn & Hutchison, 1973; Dunn et al., 1977) have shown that the rate of decay of the intermediate to products is markedly pH dependent; above pH 9, the intermediate is extremely long-lived, while, at lower pH values, the intermediate is a transient species. When the 1,4,5,6-tetrahydronicotinamide analogue of NADH (H_2NADH) is substituted for NADH, reaction yields a chromophore with a visible spectrum nearly identical with that of the intermediate (Dunn et al., 1975). This species is stable over a broad range of pH values. On the basis of these studies, a structure for the intermediate involving inner-sphere coordination of the carbonyl oxygen of DACA to the LADH active site zinc ion (Scheme II) was proposed.

Studies of model Lewis acid complexes with DACA and derivatives of DACA resulted in the formation of species with spectral properties remarkably similar to the spectrum of the

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[1] Abbreviations used: DACA, *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde; LADH, horse liver alcohol dehydrogenase; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADD, (4*R*)-4-deuterionicotinamide adenine dinucleotide; H_2NADH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; ADPR, adenosine diphosphoribose.

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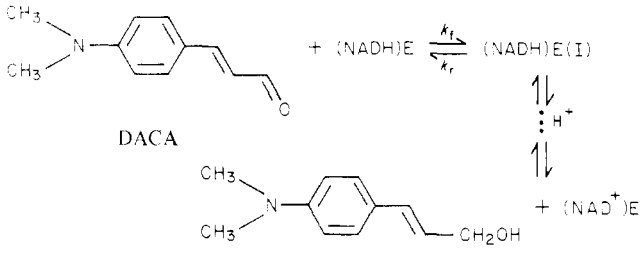
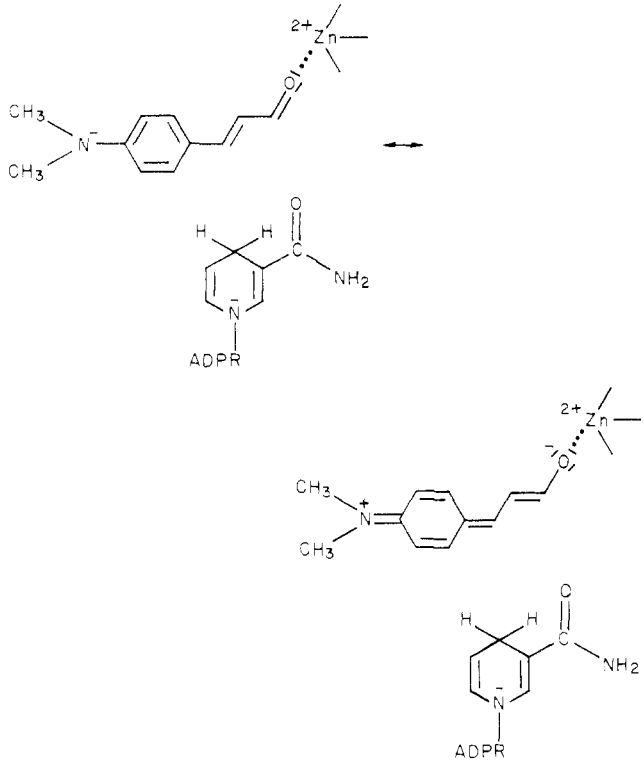
Scheme I



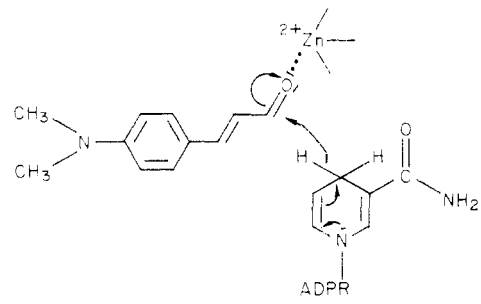
Scheme II



intermediate (Angelis et al., 1977). It was shown that the characteristically red-shifted spectra of these complexes are a direct consequence of the bonding of the carbonyl oxygen to the Lewis acid (as proposed in Scheme II). More recently, the X-ray crystallographic studies of the $(H_2NADH)E$-(DACA) complex at 3.7-Å resolution (Brändén et al., 1979; J.-P. Samama and E. Zeppezauer, personal communication) have confirmed the structural assignment proposed in Scheme II. Therefore, these findings provide strong evidence in favor of a Lewis acid catalytic role for the LADH active site zinc ion as depicted in Scheme III.

In an effort to learn more about the involvement of ionizable groups in the LADH catalytic mechanism in this study, we have carried out a detailed kinetic investigation of the pH-dependent decay of the intermediate (viz., Scheme I). By employing "pH-jump" techniques, we have extended the pH range over which the formation and decay of the intermediate can be studied. The investigation of kinetic deuterium isotope effects as a function of pH has been used to detect a pH-dependent change in the rate-limiting step for the decay process. As will be shown, these results, when combined with results of previous work, restrict the possible mechanisms for proton transfer during LADH catalysis. In particular, the data presented do not support mechanisms in which reaction is facilitated by a proton transfer prior to, or concerted with, hydride transfer. A preliminary account of this work has appeared elsewhere (Dunn et al., 1977).

Scheme III



Experimental Section

Materials. All reagents used in these studies were obtained commercially and were the finest available. Buffer solutions were prepared from crystalline salts by using water doubly distilled through a glass still. Whenever possible, buffer solutions of the desired pH were obtained by mixing the appropriate ratios of a salt and its conjugate acid. The pH of all pyrophosphate buffers was adjusted to the desired value by the addition of solid, monobasic sodium phosphate. Since chloride ion interferes with LADH activity (Sund & Theorell, 1963), all buffers used in these studies were free of this ion.

LADH was obtained from Boehringer Mannheim and prepared for use and assayed for activity as described previously (Bernhard et al., 1970). NADH and NAD^+ were obtained from Sigma Chemical Co. as the highest purity grades. NADH stock solutions were prepared in alkaline buffer immediately prior to use to minimize acid-catalyzed decomposition. Both (4*R*)-4-deuterionicotinamide adenine dinucleotide (NADD) and isotopically normal NADH were prepared enzymatically for the deuterium kinetic isotope studies as described previously (Rafter & Colwick, 1957; Dunn & Hutchison, 1973). NADD and NADH were further purified by the method of Silverstein (1965). DACA (Aldrich) was purified by vacuum sublimation prior to use; pyrazole (Aldrich) was used without further purification. The 1,4,5,6-tetrahydronicotinamide adenine dinucleotide (H_2NADH) was prepared as previously described (Dunn et al., 1975).

Methods. All static spectroscopy was carried out on a Varian 635 dual-beam spectrophotometer thermostated at 25 °C with a Lauda temperature bath. Transient kinetic studies were carried out and analyzed on a computerized Durrum-Gibson Model D-110 stopped-flow spectrophotometer equipped with a Kel-F flow system and 2-cm light path. The characteristics of the computer system and our data analysis program have been partially described previously (Dunn et al., 1979).

A pH-jump method combined with stopped-flow spectrophorometry was used to determine the pH dependence of the rate of decay of the DACA transient species. LADH and NADH were premixed in dilute (10 mM), chloride ion free sodium pyrophosphate buffer, pH 8.75. The substrate solutions were prepared by pipetting small aliquots of concentrated pyrazole and concentrated DACA (in CH_3CN) into 0.1 M buffers at varying values of pH. The pH of the final reaction solution (after mixing) was determined by combining equal volumes of substrate and enzyme solutions and measuring the pH of the resulting mixture. For calculation of rate constants and amplitudes, the data from each time course were fitted to the rate law describing a single, first-order decay.

Results

In the alkaline pH range, the reaction of DACA with the E(NADH) complex proceeds to equilibrium mixtures consisting of significant amounts of reactants, products, and in-

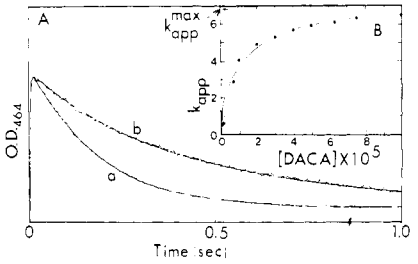


FIGURE 1: (A) Effects of deuterium substitution for the (4*R*)-4-hydrogen of NADH on the time course for intermediate decay measured at 464 nm. Conditions (after mixing): NADH (trace a) or NADD (trace b), 5.0 μM; LADH, 30 μM; pH 8.75 sodium pyrophosphate buffer, 10 mM (syringe 1); DACA, 99.6 μM; pyr, 20 mM; pH 3.93 sodium acetate buffer, 0.1 M (syringe 2) at 25.0 ± 0.2 °C. The final pH was 4.33. Computer best fit parameters (smooth curves drawn through experimental time courses, see Experimental Section) for trace a (NADH): k_{app}, 6.92 s^{-1}; amplitude, 0.289 OD; χ_ν^2, 1.26. Computer best fit parameters for trace b (NADD): k_{app}, 2.40 s^{-1}; amplitude, 0.217 OD; χ_ν^2, 1.12. Note that the data points corresponding to the rapid initial rise in OD were edited off before computer analysis. The inset (B) shows the dependence of k_{app} on the concentration of DACA. The conditions at DACA concentrations ≥ 10 μM were analyzed the same as for trace a except that the concentration of DACA was variable. The data points at lower concentrations were corrected for deviations from apparent first-order behavior by plotting the apparent first-order rate constant, obtained by analysis of the final ~10% of the time course vs. [DACA]–[E(NADH)]. The solid line is the computer-drawn best fit line assuming a hyperbolic dependence of k_{app} on [DACA]; K_D = 8.1 ± 1.1 μM and k_{app}^{max} = 6.97 ± 0.15 s^{-1}.

termediates (Dunn & Hutchison, 1973). Above pH 8.5 the amount of intermediate present at equilibrium is stoichiometrically significant, and above pH 9.5 the intermediate is the most stable species. At lower pH values, the conversion of DACA to the corresponding alcohol is essentially quantitative. Consequently, the effects of hydrogen ion on the relative stabilities of reactants, intermediate(s), and products complicate the study of the pH dependence of the kinetics of intermediate decay. To circumvent this complication, we measured the kinetics of intermediate decay in the presence of 20 mM pyrazole (pyr), a potent LADH inhibitor. The inhibitory action results from the rapid quasi-irreversible reaction of pyr with the E(NAD+) complex to form an adduct (Theorell & Yonetaini, 1963; McFarland & Bernhard, 1972). As indicated in eq 1, pyr only enters into the reaction after

$$E(NADH) + S \rightleftharpoons E(NADH,I) \rightleftharpoons^{H^+} \cdots \rightleftharpoons E(NAD^+,PH^+) \rightleftharpoons E(NAD^+) + PH \xrightarrow{pyr} E(NAD\text{–}pyr) \quad (1)$$

the alcohol product has dissociated from the site. Pyr is a mild competitive inhibitor of aldehyde binding; however, at 20 mM pyr and the DACA concentrations used in these studies this inhibitory effect is negligible. Thus, the presence of 20 mM pyr limits reaction to a single turnover of E(NADH) sites (McFarland & Bernhard, 1972) and, hence, drives the reaction of the intermediate to completion (viz., eq 1).

Since the formation of the adduct is quasi-irreversible and rapid relative to the rate-limiting step for the decay process, the presence of pyr (as will be shown in the following paragraphs) reduces the complexity of the decay kinetics in the alkaline pH range and thereby makes possible the comparison of the apparent rate constants for intermediate decay as a function of pH.

Figure 1 illustrates the observed transient kinetic time courses at 464 nm when a large excess of LADH and limiting amounts of NADH or NADD are mixed with a solution containing both excess DACA and 20 mM pyrazole. Since formation of the intermediate is complete within 20 ms under these conditions (Dunn & Hutchison, 1973; Dunn et al., 1975), the observable reaction (Figure 1) represents the decay of intermediate to products during a single turnover. Note in Figure 1 that when (4*R*)-4-deuterionicotinamide adenine dinucleotide (NADD) is substituted for NADH, the rate of decay to products is slowed. Computer fitting of the data via a nonlinear least-squares reiterative method (see Methods) demonstrated that the time courses are well described by the equation for an apparent, first-order decay process (eq 2)

$$OD_t = OD_\infty + Ae^{-kt} \quad (2)$$

where OD_t and OD_∞ are, respectively, the optical densities at time t and ∞, A is the reaction amplitude, and k is the rate constant. Here, OD_∞ has been treated as an unknown and fitted along with A and k.

Table I: pH Dependence of the Kinetic Isotope Effect for the LADH-Catalyzed Reaction of NADH and NADD with *trans*-4-(*N*,*N*-Dimethylamino)cinnamaldehyde Measured as the Rate of Decay of the Intermediate at 25 °C

pH[a]	k^H/k^D	pH	k^H/k^D
4.33	2.84	7.01	1.01
5.00	2.53	7.64	1.00
5.50	2.04	8.08	0.98
5.90	1.47	8.50	1.07
6.50	1.12	9.00	1.0

[a] See Figures 1 and 2 for conditions.

The magnitude of the apparent first-order rate constant was found to vary with the concentration of DACA (see the inset to Figure 1). The best fit to a rectangular hyperbola for these data yields an apparent dissociation constant (K_D) at pH 4.33 of 8.1 ± 1.1 μM and a k_{app}^{max} of 6.97 ± 0.15 s^{-1}. This K_D value is nearly identical with those reported at higher pH values for the directly measured (essentially pH-independent) equilibrium constant for the dissociation of DACA from the intermediate (Dunn & Hutchison, 1973).

The earlier studies by Dunn & Hutchison (1973) indicated that intermediate formation is rapid, reversible, and pH independent, while intermediate decay is pH dependent. Also, it was found that the rate of decay of intermediate is relatively insensitive to the substitution of NADD for NADH at high pH. Figure 2 shows detailed pH–rate profiles for the decay of the DACA intermediate over the pH range 4.33–9.00 for NADH and for NADD. The stopped-flow kinetic studies below pH 6 were carried out via the pH-jump technique (see Methods). At all pH values studied, the decay time courses were found to be apparent first-order processes. The k_{app}^{max} values in Figure 2 refer to the apparent first-order rate of intermediate decay measured in the presence of 20 mM pyrazole with a saturating concentration of DACA.

Comparison of the pH–rate profiles in Figure 2 for NADH and NADD reveals that the apparent rate of decay is subject to a primary kinetic isotope effect at low pH (k^H/k^D = 2.8). However, at high pH the isotope effect decreases with increasing pH and approaches a value of ≃1.0 (see Table I and Figure 2B). Plots of k_{app}^{max} vs. $k_{app}^{max}/[H]^+$ [see Jencks (1969)] for each profile (Figure 2A) yield apparent pK_a values (the slopes of the plots in Figure 2A) for NADH and NADD of 6.0 ± 0.2 and 6.51 ± 0.2, respectively. At pH 9.00, the enzyme retains a residual, pH-independent k_{app}^{max} (0.011 s^{-1}) that is approximately 650-fold slower than the k_{app}^{max} at low pH. Figure 2C presents the pH dependence of the apparent rate constants (k_{pyr}) for the formation of the E(NAD–pyr) adduct for comparison with the decay rates presented in Figure 2B. Note that above pH 5.5 in the presence of 0.9 mM NAD^+ and 20 mM pyr, $k_{pyr} \gg k_{app}^{max}$ and, therefore, the E(NAD^+)

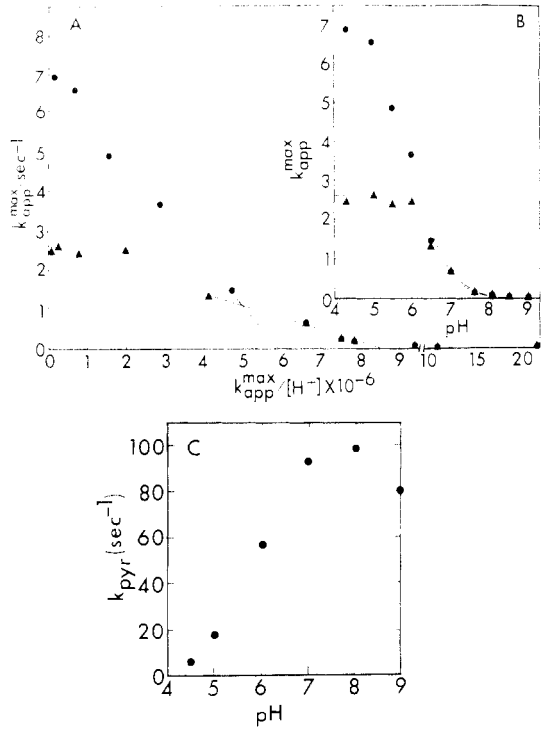


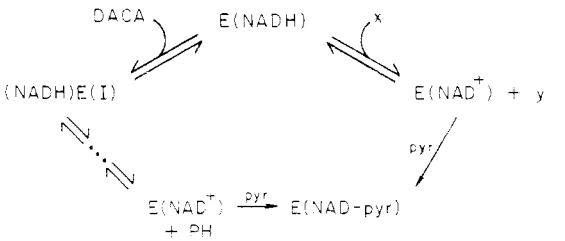
FIGURE 2: (A) Plot of k_{app}^{max} vs. $k_{app}^{max}/[H^+]$ for the decay of the (NADH)E(I) intermediate at 25.0 ± 0.2 °C [(●) NADH; (▲) NADD]. See Figure 1A for conditions and experimental method used. The buffers were as follows: pH 4.33–5.50, 0.1 M sodium acetate; pH 5.90–6.50, 0.1 M sodium cacodylate; pH 7.01–8.08, 0.1 M sodium phosphate; pH 8.50–9.00, 0.1 M sodium pyrophosphate. The concentration of DACA was 100 μM. The inset (B) shows the dependence of k_{app}^{max} on pH [same experimental data as in (A)]. The solid lines in (A) and (B) are the best fit lines assuming a single ionization is involved. (C) Plot showing the pH dependence of the apparent first-order rate constant for the formation of the E(NAD–pyr) adduct measured at 300 nm and 25.0 ± 0.2 °C. Conditions: (syringe 1) LADH, 10 μN; pH 8.75 sodium pyrophosphate buffer, 10 mM; Conditions: (syringe 2) NAD^+, 0.9 mM; pyr, 20 mM in the same buffers employed in (A).

complex is efficiently trapped by reaction with pyr. Below pH 5.5, $k_{pyr} \simeq k_{app}^{max}$ and the pyr reaction does not provide an efficient means for trapping the E(NAD^+) complex. However, below pH 7 in the absence of pyr, the conversion of intermediate to products when [DACA], [E] ≫ [NADH] is essentially quantitative (Dunn & Hutchison, 1973; these studies). When [DACA], [NADH] ≫ [E] and in the absence of pyr at pH 4.59, the intermediate decays in a rapid (burst) process corresponding to a single turnover. The burst reaction is then followed by a steady-state phase in which more DACA and NADH are converted to alcohol and NAD^+ (data not shown).

Since detailed kinetic studies involving LADH previously have not been extended to pH values below pH 5 (and only rarely below pH 6), it was necessary to investigate the stabilities of NADH and the intermediate relative to the rates of intermediate conversion to products at low pH to establish the validity of these kinetic studies.

In accord with the studies of Alivisatos et al. (1965) at 25 °C and in the same pH 4.39 buffer used for the decay studies, the acid-catalyzed decomposition of NADH was found to occur with an apparent first-order rate constant of 1.57×10^{-4} s^{-1}. The rate of decomposition of NADH in the presence of excess enzyme was found to occur at a slightly slower rate. For estimation of the stability of the intermediate, the stability of the ternary complex formed in the reaction of DACA with the 1,4,5,6-tetrahydronicotinamide adenine dinucleotide (H_2NADH)–enzyme complex was examined. Dunn et al.

Scheme IV



(1975) have shown that the reduced coenzyme analogue H_2NADH substitutes for NADH in the formation of the intermediate (as evidenced by a nearly identical red-shifted visible spectrum, λ_{max} 464 nm vs. 467 nm). However, since H_2NADH is not chemically functional as a donor of hydride in LADH catalysis (Biellmann & Jung, 1971; Dunn et al., 1975), the reaction of DACA with the E(H_2NADH) complex proceeds only to the formation of the 467-nm absorbing species. At pH values between 6 and 10.5, the stability of the 467-nm species is determined by the stabilities of H_2NADH and the enzyme and ranges from hours to days.

At pH 4.59, the reaction of DACA with E(H_2NADH) was found to give the 467-nm species as a long-lived transient that underwent decomposition (measured as the disappearance of the 467-nm absorption) with an apparent first-order rate constant of 2.6×10^{-3} s^{-1}. This process likely represents the rate of the acid-catalyzed decomposition of LADH since the acid-catalyzed decomposition of H_2NADH at this pH is 1 order of magnitude slower ($k_{obsd} = 3.8 \times 10^{-4}$ s^{-1}). These control experiments demonstrate that the enzymatic decay process is rapid relative to the acid-catalyzed decompositions of the intermediate and NADH at all pH values investigated.

Since the mechanism of the decay process could a priori occur via a step involving protonation of the enzyme-bound DACA chromophore, it was of interest to determine whether or not the spectrum of the intermediate is pH dependent. Experiments to investigate this possibility were carried out by reconstructing the spectrum of the intermediate at pH 7.34 and 4.59 from the wavelength dependence of the observed amplitudes of intermediate decay to products. These experiments (data not shown) yielded identical spectra for the intermediate in the spectral range 464–494 nm. Measurements were not made at lower wavelengths due to complications from the overlap of the spectra of intermediate with DACA and NADH. The pH independence of the spectrum of the intermediate also is supported by the observation that the amplitude of the decay process, measured at 464 nm, is almost invariant over the pH range 4.33–9.00 (data not shown).

A comparison of reaction time courses at 400, 424.8, and 494 nm (corresponding respectively to the absorption maximum for DACA, the isosbestic wavelength for the interconversion of DACA and intermediate, and a wavelength where the intermediate is the only absorbing species) was carried out at different pH values to determine whether or not the disappearance of intermediate actually measures the conversion of intermediate to alcohol and NAD^+. Below pH 8, the reaction time courses at these three wavelengths indicate that the only reaction of significance involves the conversion of intermediate to alcohol and NAD^+. Above pH 8, a slow, competing reaction that appears to involve the oxidation of NADH to NAD^+ provides an alternative route for the disappearance of intermediate. We presume that this slow reaction involves the LADH-catalyzed reduction of a trace contaminant as is depicted in Scheme IV, where x is the contaminant and y is the corresponding reduction product.

This competing reaction accounts for approximately 20% of the observed rate of disappearance of the intermediate at pH 9. The rate data presented in Figure 2A have been corrected for the contributions of this competing reaction.

Discussion

Evidence for a Change in Rate-Limiting Step. The pH dependence of the rate of intermediate decay at saturating DACA concentrations (Figure 2) and the ratio of k^H/k^D as a function of pH (Table I) constitute strong evidence for a change in the rate-limiting step as a function of hydrogen ion concentration. At low values of pH, the rate of decay of intermediate to products exhibits a primary kinetic isotope effect (see Table I and Figure 2), thus establishing that hydride transfer is a component of the rate-limiting step. In contrast, at high values of pH the absence of a kinetic isotope establishes that a process other than hydride transfer is rate-limiting. Note also that the apparent pK_a values for NADH and NADD are different (see Results).

Analysis of the pH Dependence of Intermediate Decay. The results presented in this work restrict the possible mechanisms for the process of intermediate decay. The rate of formation of the enzyme-bound intermediate and the apparent dissociation constant are nearly pH independent over the pH range 4.33–10.60 (Dunn & Hutchison, 1973; Dunn et al., 1975; these studies). The spectrum of the intermediate is similarly pH independent despite the fact that the spectrum of the DACA chromophore and the spectra of Lewis acid complexes of DACA which resemble the intermediate are extremely sensitive to the polarity of the microenvironment (Angelis et al., 1977). Therefore, since protonation undoubtedly would have altered the spectrum of the intermediate, all mechanisms that involve direct protonation of the *intermediate* in a preequilibrium step (*in stoichiometrically significant amounts*) must be discarded.

A second class of mechanisms that also must be discarded are those in which protonation of the *enzyme* is postulated to occur in a preequilibrium step (eq 3) with $4.33 > pK_a > 10.6$:

$$\begin{aligned}&(NADH)E(I) + H^+ \overset{K_a}{\rightleftharpoons}\\ &(NADH)(H^+)E(I) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (NAD^+)(H^+)E(P) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}\\ &\qquad (NAD^+)E + PH \xrightarrow[pyr]{k_4} E(NAD\text{–}pyr)\end{aligned} \quad (3)$$

Assuming step k_2 is rate limiting and the protonation of the enzyme occurs in a rapid preequilibrium step, and assuming step k_3 is quasi-irreversible (i.e., $k_4[pyr] \gg k_{-3}$), the following rate expression is obtained

$$v = \frac{k_2[(NADH)E(I)]_0[H^+]}{K_a + [H^+]} \quad (4)$$

where $[(NADH)E(I)]_0$ is the initial concentration of the intermediate. Because this rate equation predicts a *pH-independent* primary kinetic isotope effect, in contradiction to the observed pH dependences shown in Figure 2, this mechanism must be rejected. The assumption of a concerted transfer of both hydride and a proton in a rapid preequilibrium step with step k_2 rate determining, as in eq 5, must be rejected also since

$$\begin{aligned}&(NADH)E(I) + H^+ \overset{k_1}{\rightleftharpoons}\\ &(NAD^+)E(PH) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (NAD^+)E + PH \xrightarrow[pyr]{k_3} E(NAD\text{–}pyr)\end{aligned} \quad (5)$$

a rate expression similar to eq 4 is obtained. Note that the rate expression for eq 5 predicts only an equilibrium isotope effect on the rate of intermediate decay.

Scheme V

$$(NADH)E(I) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (NAD^+)E(P) \quad (6)$$

$$(NAD^+)E(P) + H^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (NAD^+)E(P)(H^+) \qquad K_a = k_{-2}/k_2 \quad (7)$$

$$(NAD^+)E(P)(H^+) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E(NAD^+) + PH \xrightarrow[k_4]{pyr} E(NAD\text{–}pyr) \quad (8)$$

The simplest class of mechanisms that predicts both the pH dependencies of k^H/k^D and k_{app}^{max} (Figure 2 and Table I) and is consistent with the spectral properties of the intermediate includes those in which (1) zinc ion serves a Lewis acid catalytic role via direct inner-sphere bonding to the carbonyl oxygen of the substrate and (2) proton transfer occurs in a step subsequent to hydride transfer. Scheme V illustrates one of several possible but kinetically indistinguishable mechanisms in this class.

If it is assumed that (1) the Bodenstein steady-state approximation is valid for the products of step 6 (Dunn & Hutchison, 1973), (2) step 7 is in mobile equilibrium, (3) step 8 is quasi-irreversible (i.e., $k_4[pyr] \gg k_{-3}$), and (4) equilibrium greatly favors (NADH)E(I) in step 6 (Dunn & Hutchison, 1973), then the rate expression for this mechanism becomes eq 9 (where $K_a = k_{-2}/k_2$):

$$\text{rate} = \frac{-d[(NADH)E(I)]}{dt} = \frac{k_1[(NADH)E(I)][H^+]}{K_a(k_{-1}/k_3) + [H^+]} = \frac{k_1[(NADH)E(I)][H^+]}{K_{app} + [H^+]} \quad (9)$$

Note that when $[H^+] \gg K_a(k_{-1}/k_3)$, eq 9 reduces to the rate expression of eq 10:

$$v \simeq \frac{k_1k_3[(NADH)E(I)][H^+]}{k_{-1}K_a} \quad (10)$$

Since the specific rate constants for the transfer of hydride in the forward and reverse directions appear as a ratio (k_1/k_{-1}), the observed kinetic isotope effect at high pH is expected to be near unity (assuming k_1 and k_{-1} are subject to comparable primary kinetic isotope effects). Deuterium kinetic isotope studies on the reduction of benzaldehyde (McFarland & Bernhard, 1972) and on the oxidation of benzyl alcohol (Shore et al., 1974) have shown that the kinetic isotope effects for the observed forward (k_f) and reverse (k_r) hydride transfer processes are comparable ($k_f^H/k_f^D \simeq 3.0$ vs. $k_r^H/k_r^D = 4.5$).

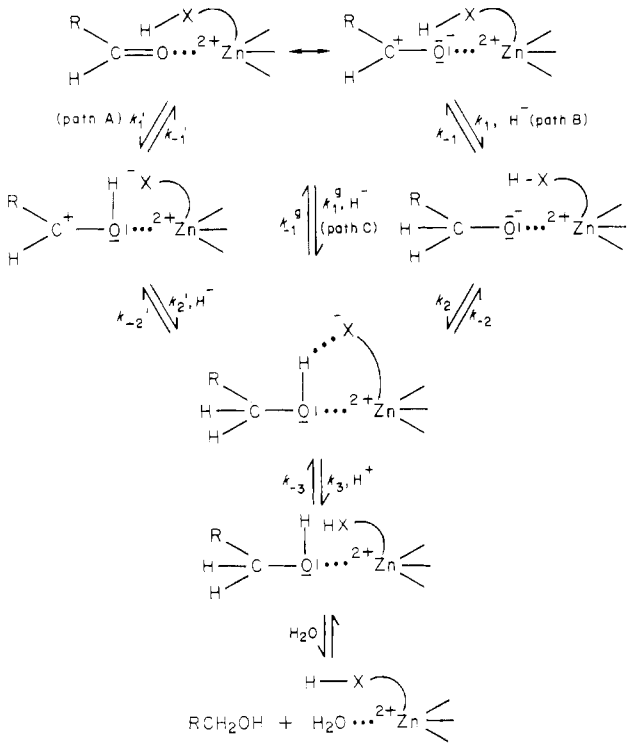
When $[H^+] \gg K_a(k_{-1}/k_3)$, eq 9 reduces to the rate expression of eq 11:

$$v \simeq k_1[(NADH)E(I)] \quad (11)$$

Consequently, at low pH the rate of hydride transfer becomes the rate limiting step and the kinetics of decay will be subject to a primary kinetic isotope effect. Thus, eq 11 predicts that the rate of decay of the intermediate will be subject to a pH-dependent kinetic isotope effect which decreases from a maximum (limiting) value at low pH to a value approaching the equilibrium isotope effect $(k_1^H/k_1^D)(k_{-1}^D/k_{-1}^H)$ at high pH (eq 6), a value expected to be near unity.

Because $K_{app} = K_a(k_{-1}/k_3)$, the apparent p$K_a$ will be subject to an isotope effect since k_{-1} but not k_3 is involved in hydride transfer. The apparent pK_a values obtained from the data in Figure 2 for NADH and NADD (6.0 ± 0.2 vs. 6.5 ± 0.2, respectively) are consistent with this prediction. The isotope

Scheme VI[a]



[a] Path A is the specific protonic acid catalyzed path; path B is the specific electrophilic catalysis by zinc ion; path C is the concerted path.

effect on K_{app} provides an estimate for $k_{-1}^{H}/k_{-1}^{D} \simeq 4.0$. The primary kinetic isotope effect on the apparent pK_a demonstrates that K_{app} is a complex quantity (viz., eq 9) composed of a protonic ionization and additional rate constants in the mechanism that includes the hydride transfer process. Therefore, K_{app} cannot be equated to the microscopic ionization constant of any group.

Mechanisms Which Involve a Protonic Acid Group. The transient kinetic studies of McFarland & Chu (1975) show that the "hydride transfer" step for β-naphthaldehyde is independent of pH for the pH range 6.0–9.9. The transient kinetic studies of Kvassman & Pettersson (1978) on benzyl alcohol oxidation indicate a similar pH independence for the hydride transfer process. Our studies (this work) show that the hydride transfer step for DACA reduction is independent of pH over the pH range 4.33 to ~7 (as discussed earlier, above pH 7.0 the kinetics are dominated by another process). The process of intermediate formation is pH independent over the pH range 4.33–10.6 (Dunn & Hutchison, 1973; Dunn et al., 1975; this work).

Clearly, if it is postulated that there is a protonic acid group which ionizes with p$K_a \geq 10.6$, then mechanisms can be written to involve this group in catalysis with transfer of the proton either prior to or concerted with hydride transfer. With p$K_a \geq 10.6$, the effect(s) of ionization of this group would not be easily detectable in the aforementioned pH studies. The data presented here do not a priori rule out the involvement of such a protonic acid catalyst in the hydride transfer process. However, for reasons stated below, we conclude that such a mechanism is unlikely, and a mechanism of this complexity is not required to explain any of the kinetic/mechanistic information in the LADH literature.

Stepwise vs. Concerted Mechanisms: Theoretical Considerations. On the basis of indirect evidence, a number of investigators have proposed that the hydride transfer step in the LADH mechanism is facilitated by protonic general acid–base catalysis [i.e., Shore et al. (1974), Sloan et al. (1975), and Dworschack & Plapp, 1977). Jencks (1972) has presented an elegant discussion of the factors which determine whether or not catalysis of nucleophilic addition to carbonyl centers occurs by a general acid–base (concerted) mechanism or by specific acid or base (stepwise) mechanisms. While the arguments which Jencks has presented are concerned exclusively with protonic acid–base catalysis, the nomenclature and concepts can be extended to include electrophilic catalysis by metal ions or other Lewis acids (Dunn & Bernhard, 1974). For purposes of discussion, Scheme VI presents two stepwise mechanisms (reaction via k_1 and k_2 or k_1' and k_2') and a concerted mechanism (step k_1^g, k_{-1}^g) for the transformation of the DACA intermediate to products.

When the criteria set forth by Jencks (1972) are applied to the three competing mechanisms presented in Scheme VI, it becomes apparent why catalysis does not involve either specific or general protonic acid catalysis. Thus, in Scheme VI the specific protonic acid-catalyzed mechanism involves the formation of a highly unstable intermediate in step k_1': a protonated, zinc-coordinated carbonyl oxygen. Therefore, reaction by this mechanism is improbable on chemical grounds.

For reaction to occur by the concerted mechanism (step k_1^g, k_{-1}^g) rather than by the remaining stepwise mechanism (steps k_1, k_{-1} and k_2, k_{-2}), the free energy change for proton transfer from the protonic acid catalyst to the substrate oxygen in the transition state must be highly favorable (Jencks, 1972). The basicity of the substrate oxygen in this transition state would be intermediate between that of a zinc-coordinated alkoxide ion and that of a zinc-coordinated carbonyl oxygen. The putative protonic acid catalytic group has been suggested to be a zinc-coordinated water molecule (Dworschack & Plapp, 1977). If a water molecule remains coordinated to zinc ion during catalysis in a pentacoordinate complex, then it seems unlikely that the water molecule would be sufficiently acidic to render favorable the free energy change for the transfer of a proton to the substrate oxygen in the transition state. In this context it is important to note that in the 3.7-Å resolution structure of the E(H_2NADH,DACA) complex, the carbonyl oxygen of DACA appears to have displaced the water molecule and the active-site zinc ion appears to be coordinated in a tetrahedral ligand field (Brändén et al., 1979; J.-P. Samama and E. Zeppezauer, personal communication). It seems far more likely that the protonated transition state would be a stronger acid than a coordinated water molecule.

On the basis of the above-stated arguments and since there appears to be no strong protonic acid at the LADH active sites (Brändén et al., 1976), we conclude that for LADH the direct participation of a protonic acid catalyst in the hydride transfer mechanism is unlikely on both experimental and theoretical grounds.

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Investigation of a Novel Liver Alcohol Dehydrogenase Catalyzed Redox–Elimination Reaction Involving Arylnitroso Substrate Analogues†

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ABSTRACT: The kinetics, product identifications, stoichiometries, and pH dependencies of the reaction of horse liver alcohol dehydrogenase (LADH) with two arylnitroso substrate analogues *p*-nitroso-*N*,*N*-dimethylaniline (NDMA) and *p*-nitroso-*N*-phenylaniline (NPA) are reported. A preliminary account of the reaction with NDMA has been published [Dunn, M. F., & Bernhard, S. A. (1971) *Biochemistry 10*, 4569]. Under conditions of [E(NADH)] ≫ [S], the second-order rate constant for the bleaching of the intense absorbance at 440 nm of both substrates is independent of pH in the range 4–10. NPA shows an apparent diminution in rate above pH 9, but this is attributable to substrate ionization to the nonreactive anion. The rate constants are 6.6×10^7 M^{-1} s^{-1} for NPA and 2.5×10^7 M^{-1} s^{-1} for NDMA. Isolation and derivitization followed by IR, UV, and mass spectral analysis of the product of NPA reduction implicate *N*-phenylbenzoquinonediimine as the immediate enzymatic product. The identification of the immediate enzymatic product of NDMA reduction is complicated by an ensuing nonenzymatic reduction by (excess) NADH, allowing a dismutation equilibrium to generate a previously described transient intermediate [Schack, P., & Dunn, M. F. (1972) paper presented at the 8th Federation of European Biochemical Societies Meeting, Amsterdam, Holland, Aug 1972] now assigned to the radical cation of *N*,*N*-dimethyl-*p*-phenylenediamine. However, a chemical trapping technique using 4-chloro-1-naphthol shows that the *N*,*N*-dimethylbenzoquinonediiminium cation produced is chemically and kinetically competent to be the enzymatic product. A mechanism is proposed to account for the novel reaction of both substrates. Upon substrate binding, coordination of the nitroso oxygen to the active-site zinc activates the nitroso nitrogen for nucleophilic attack by NADH, giving the corresponding zinc-coordinated hydroxylamine and NAD^+ at the active site. Then, the hydroxylamine undergoes rapid hydroxide elimination to form the benzoquinonediimine product. The lack of a kinetic isotope effect when (4*R*)-4-deuterionicotinamide adenine dinucleotide is substituted for NADH in transient experiments argues that these nitroso compounds are unique in that the rate-determining step for the chemical transformation appears to be diffusion to the enzyme active site.

In 1971 Dunn and Bernhard published a preliminary account of the reaction of NADH[1] with the intensely chromophoric substrate analogue *p*-nitroso-*N*,*N*-dimethylaniline (NDMA; λ_{max} 440 nm; ϵ_{440} 3.54×10^4 M^{-1} cm^{-1}) mediated by equine liver alcohol dehydrogenase (EC 1.1.1.1, LADH). Because the optical density changes at 330 nm were found to lag considerably behind those at 440 nm, it was suggested that stoichiometrically significant amounts of a nonenzymatic intermediate were generated in the reaction. A transient species

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[1] Abbreviations used: DPD, *N*,*N*-dimethyl-*p*-phenylenediamine; LADH or E, horse liver alcohol dehydrogenase (EC 1.1.1.1); NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; NADD, (4*R*)-4-deuterionicotinamide adenine dinucleotide; NDMA, *p*-nitroso-*N*,*N*-dimethylaniline; NPA, *p*-nitroso-*N*-phenylaniline; 4-CN, 4-chloro-1-naphthol; S, substrate; RNO, arylnitroso substrate analogue; QDI^+, quinonediiminium ion; QDI, quinonediimine.

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