Reaction of 4-*trans*-(*N*,*N*-Dimethylamino)cinnamaldehyde with the Liver Alcohol Dehydrogenase–Oxidized Nicotinamide Adenine Dinucleotide Complex†

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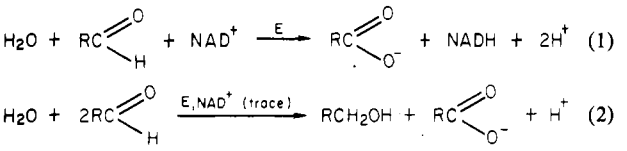
ABSTRACT: Evidence that horse liver alcohol dehydrogenase forms a ternary complex with 4-*trans*-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA) and oxidized nicotinamide adenine dinucleotide (NAD^+) is presented. Formation of the complex is characterized by a 97-nm red shift of the free chromophore to 495 nm ($\epsilon_{495} \sim 6.0 \times 10^4$ M^{-1} cm^{-1}). This shift is larger than the 66-nm red shift of the E(NADH,-DACA) complex (λ_{max} = 464 nm) previously reported by Dunn and Hutchison [Dunn, M. F., & Hutchison, J. S. (1973) *Biochemistry 12*, 4882–4892]. The large red shift of the E(NAD^+,DACA) complex is due to the combined effects of coordination of the carbonyl oxygen of DACA to the active-site zinc ion and to the close proximity of the positively charged nicotinamide ring of NAD^+. The stability of this complex is pH dependent and depends on a single apparent ionization with $pK_a = 7.6 \pm 0.3$. The pH-independent dissociation constant for binding of DACA to E(NAD^+) is 23 ± 6 μM. The stoichiometry of DACA binding to the E(NAD^+) complex is shown to be one per active site (two per enzyme molecule). Liver alcohol dehydrogenase is also shown to catalyze the NAD^+-mediated oxidation of DACA to the corresponding carboxylic acid with a very slow turnover rate. The possibility that the observed E(NAD^+,DACA) complex is an intermediate in the enzyme-catalyzed oxidation of DACA is discussed.

In studies of the catalytic mechanism for horse liver alcohol dehydrogenase (EC 1.1.1.1), the chromophoric substrate 4-*trans*-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA)[1] has been found to be a sensitive probe of individual steps in catalysis (Dunn & Hutchison, 1973; Dunn et al., 1975, 1982; Angelis et al., 1977; Dietrich et al., 1979; Morris et al., 1980). The reduction of this aldehyde is characterized by formation and decay of an intermediate E(NADH,DACA) complex where the π,π^* electronic transition of the aldehyde chromophore (λ_{max} = 398 nm) is red shifted 66 nm upon complex formation. Studies have shown that the intermediate has a structure involving inner-sphere coordination of the carbonyl oxygen of DACA to the active-site zinc ion, an interaction which activates DACA for hydride attack. This mode of DACA binding has recently been confirmed by determination of the crystal structure of the complex where NADH is replaced by the nonfunctional tetrahydro analogue H_2NADH (Cedergren-Zepperzauer et al., 1982). The oxime of DACA (the *Z* isomer) has recently been shown to be a sensitive probe for investigation of LADH(NAD^+) complexes (Sigman et al., 1982; Abdallah et al., 1984).

DACA has also been used with success in mechanistic studies of the cytoplasmic aldehyde dehydrogenase from sheep liver (Buckley & Dunn, 1982). These studies showed the rapid formation and slower decay of a transient intermediate with λ_{max} = 463 nm in the NAD^+-dependent oxidation. This intermediate was found to be an acyl-enzyme species tentatively identified as the cysteine thio ester.

It has long been known that liver alcohol dehydrogenase possesses aldehyde dehydrogenase activity (eq 1). This activity was first demonstrated as an aldehyde mutase reaction (eq 2). In the presence of catalytic amounts of NAD^+, the overall



stoichiometry corresponds to the formation of 1 mol each of acid and alcohol from 2 mol of aldehyde (Kendal & Ramanathan, 1952; Abeles & Lee, 1960; Dalziel & Dickinson, 1965). More recently, the aldehyde dehydrogenase reaction (eq 1) has been studied sperately by Hinson & Neal (1972) and by Tsai & Sher (1980). The recent proposal that oxidation of histidinol to histidine represents one of the physiological functions of this enzyme (Dutler & Ambar, 1983) has increased interest in the aldehyde dehydrogenase activity of LADH.

In the original investigation of the reaction of DACA with binary LADH–coenzyme complexes, it was reported that no significant amount of E(NAD^+,DACA) ternary complex was formed at pH 9.5 (Dunn & Hutchison, 1973). More recently, Andersson et al. (1983) have reported that DACA binds both to the enzyme–NAD^+ binary complex and to the coenzyme-free enzyme; however, they concluded that binding does not involve the direct coordination of DACA to the active-site zinc. In contradiction to these two studies, herein we report (1) evidence for the formation of an E(NAD^+,DACA) complex in which the carbonyl oxygen of DACA is directly coordinated to the active-site zinc and (2) that LADH catalyzes the oxidation of DACA by NAD^+ to the corresponding acid at a very

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[1] Abbreviations: LADH, horse liver alcohol dehydrogenase; LDH, rabbit muscle lactate dehydrogenase; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; H_2NADH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; ADPR, adenine diphosphoribosyl; E(NAD–pyrazole), enzyme-bound adduct of NAD^+ and pyrazole; DACA, 4-*trans*-(*N*,*N*-dimethylamino)cinnamaldehyde; DACA acid, 4-*trans*-(*N*,*N*-dimethylamino)cinnamic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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slow rate. We also investigate the relationship of the E-(NAD$^+$,DACA) ternary complex to the LADH-catalyzed oxidation of DACA.

Experimental Procedures

Materials. Buffer solutions were prepared from the crystalline, free acids with doubly glass-distilled water and NaOH. This kept the buffers free of anions which inhibit coenzyme binding (Dahl & McKinley-McKee, 1980; Oldén & Pettersson, 1982; Dietrich et al., 1983). LADH was obtained from Boehringer Mannheim and was assayed as described previously (Dalziel, 1957). Prior to use, LADH was first dialyzed extensively against phosphate buffer, pH 7.0, and then freed from phosphate by dialysis against two changes of 25 mM TES/Na$^+$ buffer, pH 7.0. LDH was also obtained from Boehringer Mannheim and used without further purification. The coenzymes NAD$^+$ and NADH were purchased from Sigma Chemical Co. as grade III. DACA, pyrazole, and isobutyramide were obtained from Aldrich. DACA and pyrazole were purified by vacuum sublimation while isobutyramide was recrystallized from water.

Methods. Static UV–visible spectra were obtained by using a Hewlett Packard 8450A spectrophotometer equipped with cuvette holders thermostated at 25 °C. Single-wavelength transient kinetic studies were performed with a Durrum Model D-110 stopped-flow spectrophotometer interfaced for on-line computer data acquisition and analysis as described previously (Dunn et al., 1979). Rapid-scanning stopped-flow (RSSF) spectrophotometry was carried out on a hybrid instrument consisting of a Durrum Model D-110 Kel-F flow system and a Princeton Applied Research (PAR) OMA-2 multichannel analyzer as described elsewhere (Koerber & Dunn, 1981; Koerber et al., 1983).

Results

Spectral Properties of the E(NAD$^+$,DACA) Complex. When LADH, NAD$^+$, and DACA are mixed at pH 7.0, the formation of an E(NAD$^+$,DACA) complex is evidenced by the appearance of a new spectral band with a maximum at 495 nm (Figure 1). The absorption of the free chromophore (λ_{max} = 398 nm, $\epsilon_{398} = 3.10 \times 10^4$ M^{-1} cm^{-1}) is red shifted to 495 nm on binding of DACA to the E(NAD$^+$) complex. The extinction coefficient for this complex is estimated to be $\epsilon_{495} = 6.0 \times 10^4$ M^{-1} cm^{-1}. This red shift (97 nm) is greater than the corresponding red shift which accompanies formation of the E(NADH,DACA) complex (66 nm) (Dunn & Hutchison, 1973). From spectrum b in Figure 1, it is clear that under these experimental conditions, saturation is not achieved, indicating that DACA has a lower affinity for E(NAD$^+$) than for E(NADH).

The addition of pyrazole to the E(NAD$^+$,DACA) complex (spectrum c, Figure 1) causes the disappearance of the 495-nm band and the reappearance of the spectrum of free DACA.[2] When the same experiment was repeated, but with the addition of pyrazole immediately following the addition of DACA, free DACA was quantitatively regained. This experiment shows

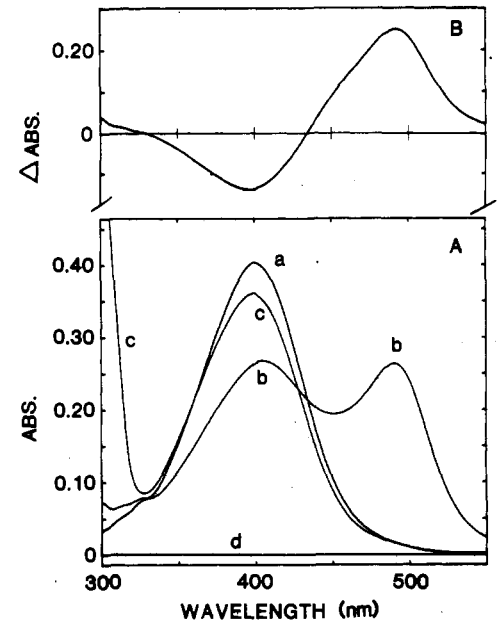


FIGURE 1: Spectra and the difference spectrum measured for the reaction of DACA with E(NAD$^+$). (A) (a) Free DACA; (b) mixture of free DACA and E(NAD$^+$,DACA); (c) spectrum after addition of pyrazole. (B) Difference spectrum of (b) – (a). The spectra were recorded as follows. The sample cuvette was a split cuvette with light path 2 × 0.438 cm. It contained in both compartments 100 μN LADH, 0.1 mg/mL LDH, and 13 mM pyruvate in 0.05 M TES/Na$^+$, pH 7.0. In addition, compartment 1 contained 2 mM NAD$^+$. After the spectrophotometer was balanced against a cuvette with water and the base line was recorded (d), 2 μL of DACA (final concentration 9.4 μM) was added to compartment 2, and spectrum a, which is the spectrum of free DACA, was recorded. Mixing compartments 1 and 2 gave spectrum b after 20 min. Adding 10 μL of pyrazole (final concentration 3.3 mM) gave spectrum c. All concentrations correspond to those after mixing.

that the addition of pyrazole quantitatively displaces DACA from the E(NAD$^+$,DACA) complex, presumably via the rapid and reversible formation of an E(NAD$^+$,pyrazole) complex which preceedes the quasi-irreversible formation of the E-(NAD–pyrazole) adduct (Theorell & Yonetani, 1963; Eklund et al., 1982).

Attempts to follow the formation of the 495-nm species in the rapid-scanning stopped-flow system showed that the formation of this species is complete during the ~10-ms interval prior to acquisition of the first spectrum (Figure 2, spectrum 1). However, these time-resolved spectra and difference spectra show that in the 460–470-nm region the absorbance undergoes a slow increase followed by a slower decrease (Figure 2A, traces 1–18). The difference spectra (Figure 2B) with λ_{max} = 470 nm and λ_{min} = 390 nm are very similar to the difference between the spectrum of DACA bound as the E(NADH,DACA) complex and the spectrum of free DACA, indicating that the increase in absorbance is due to the formation of small amounts of the E(NADH,DACA) complex (λ_{max} = 464 nm). The origin of this bound NADH is considered to be due to traces of an alcohol impurity which, in the presence of excess NAD$^+$, is oxidized (eq 3), and the resulting E(NADH) complex then binds DACA (eq 4). On

$$E(NAD^+) + RCH_2OH(\text{contaminant}) \rightleftharpoons E(NADH) + H^+ \quad (3)$$

$$E(NADH) + DACA \rightleftharpoons E(NADH,DACA) \quad (4)$$

a longer time scale, the NAD$^+$-regenerating system, consisting of the LDH and pyruvate present in the reaction mixture, reoxidizes the NADH thus formed, thereby accounting for the

[2] Under the conditions of Figure 1, free DACA is not quantitatively regained for two reasons: (1) During the elapsed time between the mixing of DACA with the E(NAD$^+$) complex and the addition of pyrazole, significant amounts of E(NADH) form via the oxidation of contaminating alcohol (viz., Figure 2 and eq 3). The E(NADH) thus formed binds and slowly reduces DACA (eq 4). Hence, the LDH and pyruvate present compete with DACA for the NADH produced. (2) The DACA bound to the E(NAD$^+$) complex slowly undergoes oxidation to the corresponding acid.

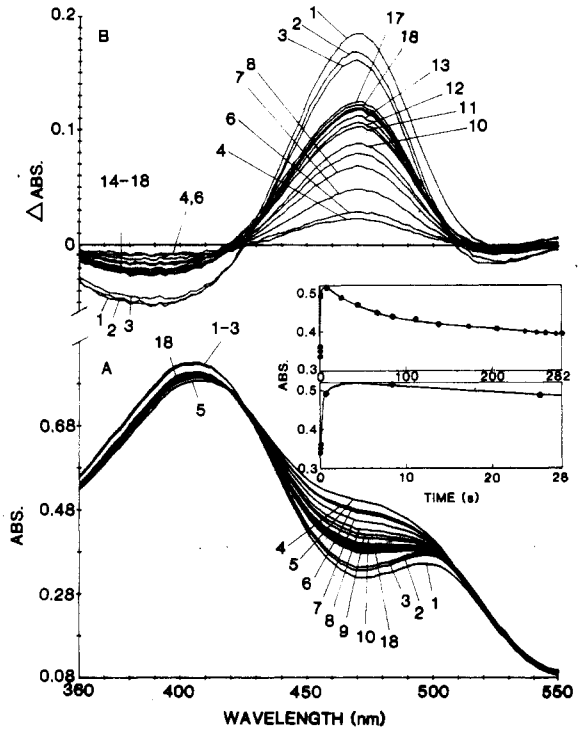


FIGURE 2: Rapid-scanning stopped-flow spectra (A), difference spectra (B), and the single-wavelength time course at 464 nm (inset to panel A) for the reaction of the E(NAD$^+$) complex with DACA. (A) The scan acquisition time was 8.605 ms/scan with delays introduced between scans to give the pattern of scans indicated in the inset. (Note that two time scales are shown, 0–28 s and 0–282 s.) Collection of the first scan was initiated approximately 10 ms after flow stopped. The conditions after mixing were the following: syringe A, 30 μN LADH and 1.0 mM NAD$^+$; syringe B, 20 μM DACA. Both syringes contained 0.05 M TES/Na$^+$, pH 7.0, 0.1 mg/mL LDH, and 1.0 mM pyruvate. (B) The family of time-resolved difference spectra were calculated from the data in (A) by subtracting spectra 1–4 and 6–18 from spectrum 4 and are numbered in chronological order.

slower decrease in absorption in the 460–470-nm region (viz., the inset to Figure 2A).

The identification of this transient ~470-nm band as the E(NADH,DACA) complex was verified by two additional experiments: (a) The mixing of E(NAD$^+$) vs. DACA (Figure 2) was repeated in the rapid-scanning stopped-flow instrument in the presence of 10 mM isobutyramide (data not shown). Here the rapid formation of the 495-nm species was observed, but no changes in the 460-nm region (such as those seen in Figure 2) were detected. Under the conditions used (10 mM isobutyramide), formation of the E(NADH,isobutyramide) complex would be rapid and thermodynamically favored; hence, any E(NADH) formed would immediately be trapped as E(NADH,isobutyramide), thus preventing the appearance of the 464-nm-absorbing E(NADH,DACA) species. (b) When 50 μM E, 2 mM NAD$^+$, and 10 μM DACA are mixed with 10 mM pyrazole, in either the rapid-scanning or the single-wavelength stopped-flow instrument (data not shown), the rapid disappearance of the E(NAD$^+$,DACA) spectral band around 500 nm was followed by the slower decay of a spectral band absorbing in the 460-nm region. These observations confirm the presence of trace amounts of the E(NADH,-DACA) complex (λ_{max} = 464 nm) formed upon incubation of the enzyme with NAD$^+$ and DACA. Pyrazole displaces DACA from the E(NAD$^+$,DACA) complex within the mixing dead time, while DACA bound to E(NADH,DACA) is reduced and the resulting E(NAD$^+$) is trapped as the E(NAD–pyrazole) adduct (Morris et al., 1980; Dunn et al., 1982). The observed decay rate for this 460-nm species of 1.5 s^{-1} at pH

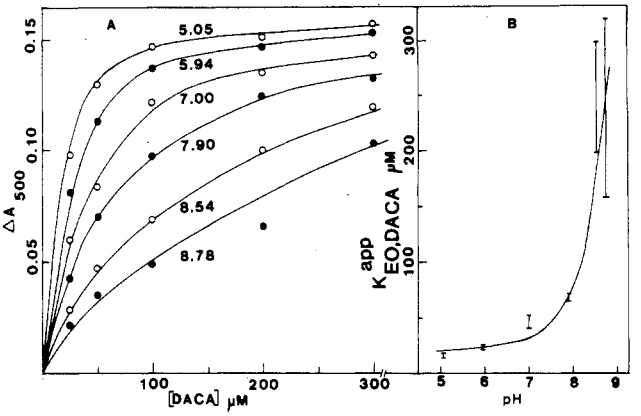


FIGURE 3: (A) Formation of the E(NAD$^+$,DACA) complex as a function of the DACA concentration at different pH values. The measurements were made 100 ms after the components were rapidly mixed in a stopped-flow instrument. Conditions were the following: syringe A, 5 μN LADH in 0.05 M buffer; syringe B, varied concentration of DACA and 2.0 mM NAD$^+$. The absorbance was read at 500 nm after the flow stopped, and the absorbance values have been corrected for the absorption of free DACA. Buffers were the following: pH 5.05 and 5.95, MES/Na$^+$; pH 7.00 and 7.90, TES/Na$^+$; pH 8.54 and 8.78, pyrophosphate/Na$^+$. At the different pH values, an apparent dissociation constant for the binding of DACA to E(NAD$^+$), $K^{app}_{EO,DACA}$, was calculted by using nonlinear regression. (B) pH dependence of $K^{app}_{EO,DACA}$. The solid line is the theoretical curve obtained by fitting the data to a single ionization (pK_a = 7.6 ± 0.3).

7.0 is in good agreement with the rate expected for the reduction of DACA (Morris et al., 1980).

Using a single-wavelength instrument (dead time ~3 ms), attempts were made to follow the kinetic time course for formation of the E(NAD$^+$,DACA) complex; however, this process was found to be too fast to measure with rapid-mixing stopped-flow methods. The rate of DACA dissociation from the ternary complex also was found to be too fast to measure in the stopped-flow apparatus. Upon mixing of the E(NAD$^+$,DACA) complex with 5 mM pyrazole, displacement of DACA (as measured by the apperance of the 398-nm spectral band) was essentially complete in the mixing dead time.

When 5 μN E(NAD$^+$,DACA) complex and excitation wavelengths of either 330 or 500 nm were used, no significant fluorescence was detected at wavelengths ≤700 nm.

Stability of the E(NAD$^+$,DACA) Complex. Due to the complications resulting from formation of the E(NADH,-DACA) complex via traces of oxidizable contaminants, the single-wavelength rapid-mixing stopped-flow technique was used to follow the appearance of the E(NAD$^+$,DACA) complex. By varying the concentration of DACA and by working on a time scale where E(NADH,DACA) complex formation is negligible (i.e., 100 ms), we determined the binding isotherms from the amplitude of the absorbance changes at 500 nm. The isotherms (Figure 3A) were found to be adequately fit with the assumption of a single class of noninteracting sites. Using this approach, the dependence of the apparent dissociation constant, $K^{app}_{EO,DACA}$, on the pH was investigated. Figure 3A shows that the binding of DACA to E(NAD$^+$) is subject to a noncooperative saturation effect and that the affinity depends on the pH. In Figure 3B, the apparent dissociation constants calculated from the data in Figure 3A are plotted against pH. Assuming a single ionization of the E(NAD$^+$) complex, and assuming that DACA binds only to the protonated form, the data have been fitted by nonlinear regression to

$$K^{app}_{EO,DACA} = K_{EO,DACA}(1 + K_a/[H^+]) \quad (5)$$

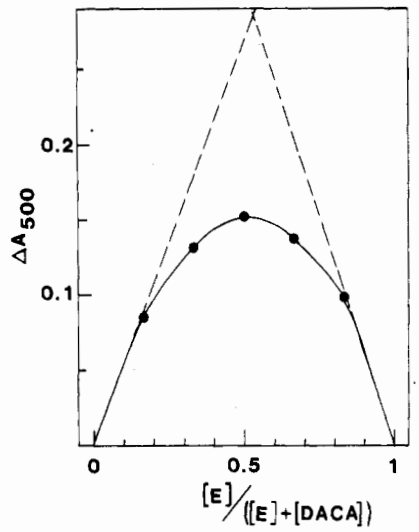


FIGURE 4: Stoichiometry of DACA binding to the E(NAD$^+$) complex determined by the method of continuous variation (Job, 1928). The measurements were carried out by rapid mixing in a stopped-flow spectrophotometer. The conditions were the following: syringe A, LADH in 0.05 M TES/Na$^+$, pH 7.0; syringe B, 2.0 mM NAD$^+$ and DACA. The concentrations of DACA and LADH were such that throughout the experiment, the sum [LADH] + [DACA] was always 30 μM, with the concentration of enzyme expressed as active sites. All concentrations given correspond to those after mixing. The absorbance at 500 nm was read immediately after the flow stopped. According to theory (Job, 1928), the curve should be symmetrical and the tangents should intersect at an abscissa value of 0.5 in order to confirm a binding stoichiometry ratio of 1:1 (i.e., one per enzyme active site).

$K^{app}_{EO,DACA}$ and $K_{EO,DACA}$ are the pH-dependent and pH-independent dissociation constants, respectively, for DACA binding to E(NAD$^+$), and K_a is the apparent ionization constant of the ionizing group within the E(NAD$^+$) complex. This gave $K_{EO,DACA}$ = 23 ± 6 μM and pK_a = 7.6 ± 0.3. In RSSF studies (data not shown), it was found that the spectrum of the E(NAD$^+$,DACA) complex is independent of pH over the pH range amenable to study (pH 6–8.5).

Stoichiometry of DACA Binding to E(NAD$^+$). The stoichiometry of DACA binding to E(NAD$^+$) was determined according to the method of continuous variation (Job, 1928). The data plotted in Figure 4 show that one molecule of DACA is bound per active site (i.e., two per enzyme molecule).

Catalytic Conversion of DACA to DACA Acid. Figure 5 provides evidence that LADH catalyzes the oxidation of DACA by NAD$^+$ to the corresponding acid at a very slow rate. When DACA is added to E(NAD$^+$), the absorbance of the rapidly formed E(NAD$^+$,DACA) complex (λ_{max} = 495 nm) and the absorbance due to free DACA slowly disappear, and a new species with λ_{max} = 323 nm slowly appears. When the final spectrum in Figure 5 (t = 120 h) is compared with that of free DACA acid, it is clear that LADH has converted all of the DACA initially present to the DACA acid. That the conversion is essentially quantitative is clear from the absorbance changes in the reaction (i.e., DACA $\epsilon_{398} = 3.10 \times 10^4$ M^{-1} cm^{-1} and DACA acid $\epsilon_{323} = 1.9 \times 10^4$ M^{-1} cm^{-1}; Buckley & Dunn, 1982). Since the reaction mixture also contains an NAD$^+$-regenerating system (LDH and pyruvate), the NADH formed in the oxidation of DACA does not accumulate. Consequently, NADH makes no detectable contribution to the absorbance changes in the 300–350-nm region. The spectrum of the final product in Figure 5 is also very different from the alcohol formed by reduction of DACA (with λ_{max} = 280 nm; Dunn & Hutchison, 1973). When the same experiment was performed with other enzyme concentrations (10 and 40 μN, data not shown), the rate of DACA oxidation was found to be proportional to the concentration of enzyme sites. Nevertheless, the oxidation of DACA is very slow, and from the time course in Figure 5, V/E_t is estimated to be 1.7×10^{-3} min^{-1}.

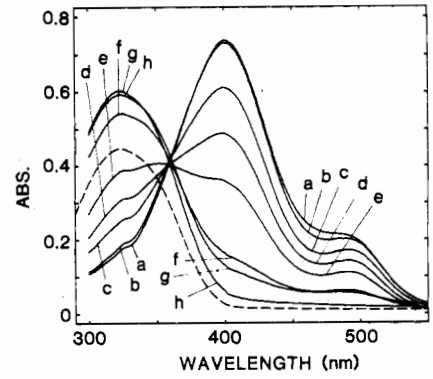


FIGURE 5: Time-resolved spectra for the LADH-catalyzed oxidation of DACA by NAD$^+$. The sample cuvette contained 20 μN LADH, 2.0 μM NAD$^+$, 0.025 mg/mL LDH, and 2.0 mM pyruvate in 0.05 M TES/Na$^+$, pH 7.0. The sample cuvette was balanced against a reference cuvette containing water. The reaction was then started by adding 5 μL of DACA, giving a concentration of 30 μM in a total volume of 2 mL. Spectra were then measured with the following time intervals (hours): 0.1 (a), 0.5 (b), 3.2 (c), 5.8 (d), 9.3 (e), 21.8 (f), 27.3 (g), and 120 (h). The spectrum of 30 μM 4-*trans*-(*N*,*N*-dimethylamino)cinnamic acid (---) is shown for comparison.

Since the DACA oxidating activity of LADH is so low, the possibility that this activity is due to some other enzyme present in trace amounts was tested as follows: (1) LADH treated with iodoacetic acid (which is known to specifically alkylate Cys-46 and to greatly reduce the alcohol dehydrogenase activity) was found to be unable to catalyze the oxidation of DACA. (2) Formation of the E(NAD–pyrazole) adduct, a reaction which is highly specific for liver alcohol dehydrogenase, was found to strongly inhibit the oxiddation of DACA. Consequently, it is likely that this activity is an intrinsic property of native LADH.

Discussion

Nature and Identity of the E(NAD$^+$,DACA) Complex. The data presented in Figures 1 and 2 demonstrate that an E(NAD$^+$,DACA) complex is formed. Our objective has been to study the nature of this complex and its possible role in the catalytic oxidation of DACA. The complex (Figures 1 and 2) is characterized by a large (97 nm) red shift in the spectrum of the DACA chromophore which is independent of pH. This red shift is quite characteristic and much larger than the red shift observed upon formation of the E(NADH,DACA) complex (66 nm). The identity of this complex is also proved by the addition of pyrazole which immediately bleaches the complex spectrum, while that of DACA is regained (eq 6 and 7). Pyrazole is known to form a tight abortive E(NAD–

$$E(NAD^+,DACA) \rightleftharpoons E(NAD^+) + DACA \quad (6)$$

$$E(NAD^+) + \text{pyrazole} \rightarrow E(NAD\text{–pyrazole}) + H^+ \quad (7)$$

pyrazole) adduct, and the addition of pyrazole has been used to limit the LADH-catalyzed reduction of aldehydes to a single turnover by trapping E(NAD$^+$) as the pyrazole adduct (Theorell & Yonetani, 1963; McFarland & Bernard, 1972; Dunn et al., 1982). The very rapid bleaching effect of pyrazole agrees with the identification of the 495-nm species as the E(NAD$^+$,DACA) complex. In contrast, the effect of pyrazole on the E(NADH,DACA) complex is to restrict the reduction

of DACA to one turnover (eq 8–10); consequently, bleaching

$$E(NADH,DACA) \rightleftharpoons E(NAD^+,P) \quad (8)$$

$$E(NAD^+,P) + H^+ \rightleftharpoons E(NAD^+,P)(H^+) \quad (9)$$

$$E(NAD^+,P)(H^+) \rightleftharpoons E(NAD^+) + PH \xrightarrow{\text{pyrazole}} E(NAD\text{–pyrazole}) \quad (10)$$

is a much slower process and DACA is not regained (Morris et al., 1980; Dunn et al., 1982). (In eq 8–10, PH represents the alcohol formed by reduction of DACA.)

In previous studies (Dunn & Hutchison, 1973; Dunn et al., 1975, 1982; Morris et al., 1980), the formation of an E(NAD$^+$,DACA) complex has been overlooked. In the original work (Dunn & Hutchison, 1973), evidence for this complex was sought at pH 9.5 where, due to the weak binding of DACA, the complex is difficult to detect (viz., Figure 3). Nevertheless, the small change in A_{460} reported on mixing DACA with E(NAD$^+$) (Dunn & Hutchison, 1973) very likely was due to in part to E(NAD$^+$,DACA) formation.

In the recent work by Andersson et al. (1983), evidence was sought for the existence of E(NAD$^+$,aldehyde) complexes involving DACA and other aldehydes. Although they conclude that E(NAD,aldehyde) complexes form, they failed to detect any red shift in the DACA spectrum. Since their studies spanned the pH range 6–10, it is not entirely clear to us why they failed to detect the 495-nm E(NAD$^+$,DACA) complex. If significant levels of contaminating alcohol were present in their enzyme preparations, then their static UV–visible spectra very likely were dominated by the E(NADH,DACA) complex. Our discovery of the 495-nm-absorbing E(NAD$^+$,DACA) complex was facilitated by investigation of the system via rapid-scanning UV–visible spectroscopy (viz., Figure 2).

Using a rapid-mixing stopped-flow spectrophotometer, the formation of E(NAD$^+$,DACA) at pH 7.0 is too fast to be measured. The process appears to be at least as fast as the formation of the E(NADH,DACA) complex, which by T-jump studies has been measured to be about 4×10^7 M^{-1} s^{-1} (Dunn & Hutchison, 1973).

The above observations agree with a mechanism for substrate binding to the LADH–coenzyme complex which involves displacement of a water molecule from the fourth ligand position of the active-site zinc ion, in the case of both the reduced and the oxidized coenzymes. Since the rate of formation of the E(NADH,DACA) complex is unaffected by substitution of Co^{2+}, Ni^{2+}, or Cd^{2+} for Zn^{2+} at the active site, it appears that the rate of complex formation is limited either by diffusion or by the formation of a pentacoordinate intermediate. The second-order rate constant is similar in magnitude to the apparent second-order rate constants determined for the substitution of ligands for inner-sphere-coordinated water in the aquated zinc ion (Wilkens & Eigen, 1964). Since $k_{off} = K_{EO,DACA}k_{on}$ and with the assumption of an association rate constant of $\sim 4 \times 10^7$ M^{-1} s^{-1}, the rate of dissociation of DACA from the E(NAD$^+$,DACA) complex (k_{off}) can then be estimated to be ~ 1000 s^{-1}. This value is in agreement with the finding that DACA dissociation is too fast to be measured by stopped-flow methods.

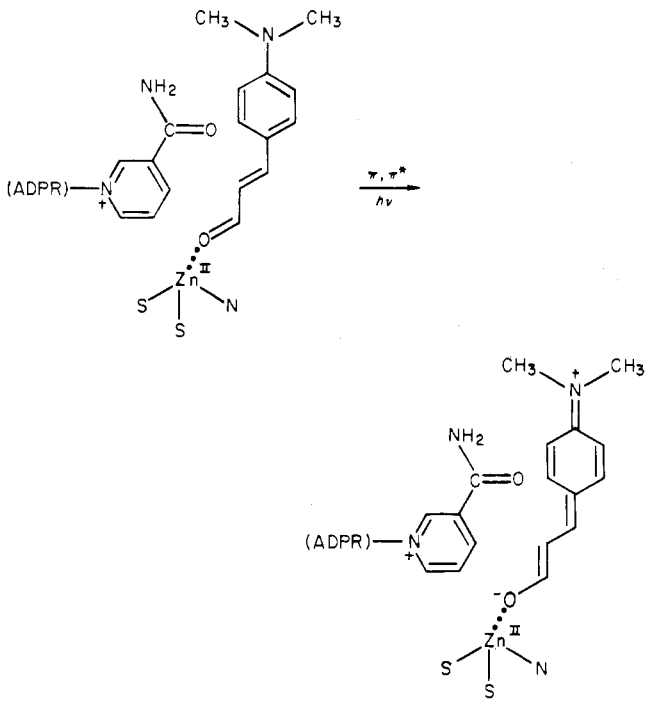
DACA is seen to bind to the E(NAD$^+$) complex with a stoichiometry of 1 per subunit (Figure 4) to a single class of sites which appear to be identical and independent. The pH-independent dissociation constant $K_{EO,DACA}$ was found to be 23 ± 6 μM (Figure 3). Previously, $K_{ER,DACA}$ was found to be 4.0 μM (using a similar method) or 7.0 μM (calculated from kinetic measurements) (Dunn & Hutchison, 1973). The relative magnitudes of these constants confirm previous studies showing that the regulation of enzyme affinity for ligands has its origins in a binding specificity which is determined by the coenzyme oxidation state (Theorell & Bonnichsen, 1951; Dunn & Hutchison, 1973). Since $K_{EO,DACA}$ is strongly dependent on pH while $K_{ER,DACA}$ is not, this specificity is pH dependent. At lower pH values where the difference between the dissociation constants is only about a factor of 4, the coenzyme oxidation state is not very selective for DACA, whereas at high pH DACA binds much more tightly to E(NADH) than to E(NAD$^+$).

In contrast to the behavior of the E(NADH,DACA) complex, the stability of the E(NAD$^+$,DACA) complex is strongly pH dependent in the pH range 6–10. The affinity for DACA decreases with increasing pH according to the relationship given in eq 5, implicating a group with a $pK_a = 7.6 \pm 0.3$. The data presented in Figure 5 indicate that DACA binds only to the protonated form. Since the same pK_a value has been observed for the combination of such substrate analogues as decanoate, trifluoroethanol, and pyrazole (Kvassman & Pettersson, 1980a,b; Andersson et al., 1981a,b), this pH dependence was expected. These substrate analogues also were found to combine mainly with the form of the E(NAD$^+$) complex where the group with apparent $pK_a = 7.6$ is protonated. This group has been proposed to be the zinc-bound H_2O, and a $pK_a = 9.2$ has been postulated for this group in the free enzyme, while pK_a values of 7.6 and 11.2 have been postulated respectively for the E(NAD$^+$) and the E(NADH) complexes (Shore et al., 1974; Andersson et al., 1981b). Since a group (or groups) with similar pK_a properties is observed also in the enzyme species lacking the active-site metal (Dietrich et al., 1983), the identification of the group (or groups) responsible for these apparent pK_a values as the metal-bound water has been questioned.

The pH dependence for the stability of the E(NAD$^+$,DACA) complex reported herein (Figure 3) contradicts the results recently reported by Andersson et al. (1983). They claim that the binding of both DACA and benzaldehyde to the E(NAD$^+$) complex is unaffected by pH changes over the pH range 6–10. The reasons for these conflicting results appear to be due to a combination of factors. (1) It is clear that in their UV–visible absorbance studies, Andersson et al. either overlooked or were unable to detect the 495-nm-absorbing E(NAD$^+$,DACA) complex. (2) Their methods for measuring aldehyde binding, by displacement of Auramine O or 2,2′-bipyridyl, are incapable of distinguishing between inner-sphere and outer-sphere coordinated complexes. (3) No special precautions were taken either to remove alcohol contaminants or to recycle the NADH formed in the oxidation of such contaminants. Thus, it is possible that their equilibrium measurements are skewed, and perhaps even dominated, by the presence of enzyme-bound NADH. Indeed, the (pH-independent) dissociation constants which they report for the E(NAD$^+$,DACA) complex only differ from the values reported for the E(NADH,DACA) complex (Dunn & Hutchison, 1973) by a factor of 2 or 3. The pH independence which they report is more consistent with the behavior of ternary complexes involving NADH.

Bonding Implications of the 97-nm Red Shift. Spectroscopic studies of DACA have shown that the absorption spectrum of this chromophoric molecule is critically dependent upon both the polarity of the solvent and the chemical nature of groups attached to the carbonyl oxygen (Angelis et al., 1977). These studies established that DACA is a sensitive probe of the microenvironment and provided strong evidence in support of the previous conclusion (Dunn & Hutchison,

Scheme I



1973) that DACA in the E(NADH,DACA) complex is bound to the active-site zinc ion via inner-sphere coordination of the carbonyl oxygen. Later it was found that the magnitude of the red shift upon formation of the E(NADH,DACA) complex depends upon the Lewis acid strength of the metal ion substituted in the active site (Dunn et al., 1982). When Zn^{2+} was substituted with other metals, the following λ_{max} values were observed: 478 nm (Co^{2+}), 475 nm (Ni^{2+}), and 457 nm (Cd^{2+}).

It is unlikely that the 97-nm (pH-indendent) red shift could be brought about without coordination of the carbonyl oxygen of DACA to a Lewis acid. Therefore, it seems certain that in the E(NAD$^+$,DACA) complex the carbonyl oxygen of DACA also is bound to the active-site metal via inner-sphere coordination. The finding that the λ_{max} for DACA is more red shifted (by 31 nm) in the E(NAD$^+$,DACA) complex than in the E(NADH,DACA) complex has important implications. The greater red shift must be due primarily to the additional positive charge within the site which resides on the nicotinamide ring of NAD$^+$. The long-wavelength spectral band of the DACA chromophore arises from a π,π^* transition where (relative to the ground state) the excited state carries a lower electron density on the amino group and a higher electron density on the carbonyl oxygen (Scheme I). Accordingly, this spectral band will be red shifted by interaction with charges and dipoles which stabilize the electron distribution of the excited state (relative to the ground state). In the E(NAD$^+$,DACA) complex, the excited form thus is stabilized not only by coordination to the active-site metal but also by the close proximity of the positively charged nicotinamide ring of NAD$^+$.

Catalytic Conversion of DACA to the Corresponding Acid. The experiments described in Figure 5 provide direct spectrophotometric evidence for the LADH-catalyzed oxidation of DACA by NAD$^+$. When the final spectrum is compared with that of the free DACA acid, the identity of the final product as the DACA acid is clear.

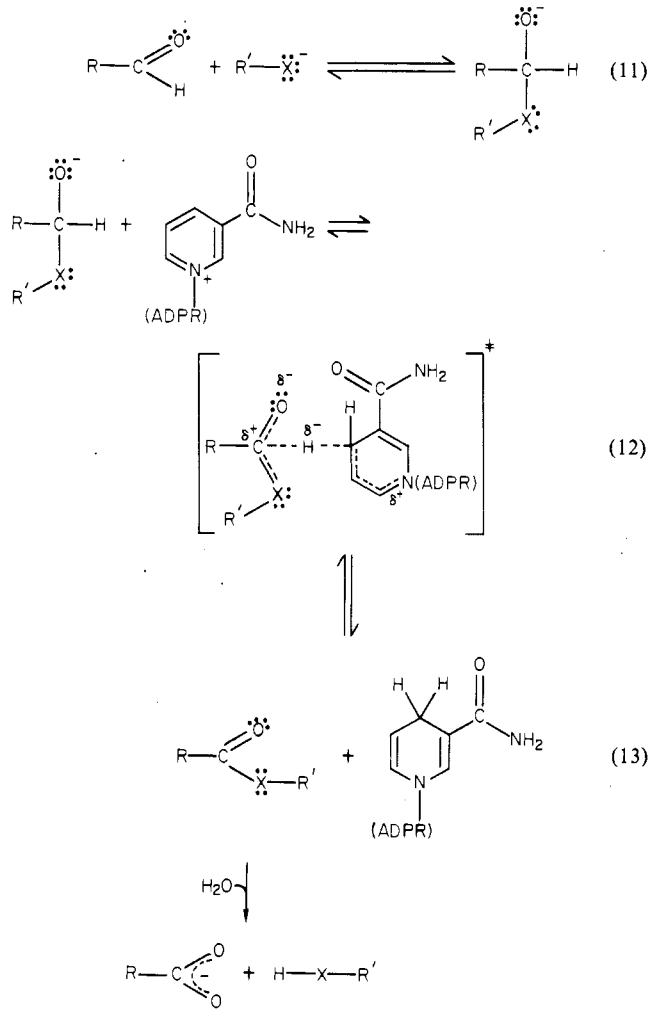
The rate of DACA oxidation is *very slow*. It is much slower than the rate reported for oxidation of octanal and even slower than that reported for acetaldehyde (Hinson & Neal, 1972). The rapid rate of formation of the E(NAD$^+$,DACA) complex relative to the rate of DACA oxidation ensures that the complex is kinetically competent to be an intermediate in the catalytic oxidation of DACA but does not demand that the complex is on the reaction path.

Catalytic Mechanism. In previous studies of aldehyde oxidation with LADH, several catalytic mechanisms for the oxidation of alcohol or aldehyde to the corresponding acid have been put forward. Kendal & Ramanathan (1952) and later Abeles & Lee (1960) proposed that the true substrate for LADH in the dismutase reaction is the hydrated form of the aldehyde. The hydrate has a structural resemblance to the alcohol, and oxidation of the hydrate would be a chemically reasonable pathway, whereas the direct oxidation of an aldehyde carbonyl is energetically difficult. However, Tsai & Sher (1980) *concluded* that hydrate structures are not essential intermediates for the oxidation of higher homologous aldehydes.

Recently, Dutler & Ambar (1983) proposed a mechanism for the LADH-catalyzed oxidation of histidinol to histidine. According to their scheme, histidinol is oxidized to histidinal which then forms a thiohemiacetal with one of the active-site cysteines involved in the ligation of zinc. The resulting thiohemiacetal is oxidized further to a cysteine thio ester which then is hydrolyzed in the final step. The present study provides no evidence for the involvement of a thiohemiacetal or a thio ester in the oxidation of DACA. Since conversion of the carbonyl carbon to an sp^3 center is expected to blue shift the spectrum of the chromophore as much as 100 nm in comparison to that of the free aldehyde, the 495-nm species could hardly be a thiohemiacetal. In the aldehyde dehydrogenase catalyzed oxidation of DACA by NAD$^+$, the putative thio ester intermediate absorbs at 463 nm (Buckley & Dunn, 1982); hence, a thio ester acyl-enzyme precursor of the DACA acid could possibly absorb at 495 nm. However, it is highly unlikely that the hydride transfer which would precede thio ester formation could occur as fast as the observed rate of formation of the 495-nm species. It is even more unlikely that the addition of pyrazole (viz., Figure 2) would cause a rapid reversal of thio ester formation.

On the basis of the spectral data presented in Figures 1 and 2, there is no doubt that the carbonyl of DACA is unhydrated in the E(NAD$^+$,DACA) complex and that the spectral properties of the complex show the aldehyde carbonyl to be intact and coordinated directly to the active-site zinc. Due to the highly conjugated structure of DACA, only a very small fraction is hydrated in aqueous solution. Clearly, it is the unhydrated aldehyde which actually binds to the LADH–coenzyme complex. However, this by no means implies that the intact aldehyde carbonyl is directly oxidized.

Indeed, the only chemically plausible mechanisms for aldehyde oxidation involve the following steps (eq 11 and 12): (1) An electron-rich tetrahedral species is formed (eq 11) via attack of a nucleophile ($R'-X^-$). (2) Hydride transfer (i.e., transfer of the equivalent of $H^+ + 2e^-$) occurs from the tetrahedral species to NAD$^+$ (eq 12). (3) If the nucleophile ($R'-X^-$) is a species other than H_2O/OH^-, then the resulting acyl intermediate must undergo hydrolysis to yield the acid product (eq 13). In this scheme, the carbocation-like transition state for the hydride transfer step (eq 12) is stabilized by the electron-rich substituents (X and O). The chemical and enzymological literature is replete with precedents for the mechanism outlined in eq 11–13. Therefore, we consider it likely that the LADH-catalyzed oxidation of DACA occurs via a similar pathway. If the E(NAD$^+$,DACA) complex is on the reaction path, then it must be a species which precedes



the formation of the reactive tetrahedral adduct; coordination of the DACA carboxyl oxygen to zinc then functions to activate the carbonyl carbon for attack by the nucleophilic group X^-. Consequently, the active-site zinc ion plays a Lewis acid catalytic role in this process, a role which is analogous to that played by zinc during aldehyde reduction (Dunn & Hutchison, 1973; Dunn et al., 1982).

Registry No. DACA, 20432-35-3.

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