Evidence for Site Equivalence in the Reaction Mechanism of Horse Liver Alcohol Dehydrogenase with Aromatic Substrates at Alkaline pH†

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ABSTRACT: The reaction mechanism of liver alcohol dehydrogenase with benzyl alcohol and benzaldehyde substrates was studied at pH 8.75 using transient kinetics. Oxidation of saturating concentrations of alcohol resulted in a transient burst of enzyme-bound NADH production equal to 46% of the enzyme site concentration. At high alcohol concentration (24 mM) resulting in extensive abortive complex formation, and in the presence of isobutyramide, the amplitudes of the transient burst reactions were respectively equal to 87 and 97% of the enzyme site concentrations. These results indicate that less than complete burst amplitudes are due to the ratio between the rate constants for aldehyde dissociation and reverse hydride transfer. In the presence of benzaldehyde as a product inhibitor, the burst of bound NADH production due to alcohol oxidation was almost completely abolished. In the reverse reaction direction, at least 90% of enzyme-bound NADH is rapidly oxidized by benzaldehyde with the remaining slow reaction readily accounted for by equilibrium considerations. An extrapolation of steady-state reaction amplitudes with NADH concentrations greater than enzyme indicated that a concentration of coenzyme equal to the enzyme site concentration was rapidly oxidized prior to turnover. It was possible to directly determine all rate and equilibrium constants in the mechanism with the exception of aldehyde binding, which could be estimated from the Haldane relation. No evidence for site nonequivalence was found, and the results are most readily explained by independently functioning active sites of the enzyme.

The broad substrate specificity of liver alcohol dehydrogenase has resulted in the use of aromatic aldehydes and alcohols for mechanistic studies. Substituent effects have been studied for aldehyde reduction (Jacobs et al., 1974) and alcohol oxidation (Hardman et al., 1974); proton equilibria (Dunn, 1974) and pH effects (McFarland and Chu, 1975) have been evaluated using aromatic substrates. These substrates have been useful since, particularly at alkaline pH, a number of rate constants of transient mechanistic processes become readily accessible to measurement in the stopped-flow time range.

A persistent problem in interpretation of transient intermediates with aromatic substrates is due to the current controversy regarding nonequivalence of the two active sites on the dimeric enzyme molecule. The first transient kinetic study using aromatic aldehydes (Bernhard et al., 1970) reported that, in the single turnover oxidation of limiting NADH[1] by excess enzyme and substrate, or the reduction of limiting substrate by excess of NADH and enzyme, biphasic reactions of equal amplitude occurred. These studies were subsequently expanded (Dunn and Bernhard, 1971; McFarland and Bernhard, 1972; Luisi and Favilla, 1972) and extended to the reaction in the opposite direction by the report that the oxidation of benzyl alcohol resulted in a burst of bound NADH formation equal to half the enzyme concentration (Luisi and Bignetti, 1974). The general theory which evolved from these findings was that the catalytic step induced a functional asymmetry so that, until product or coenzyme dissociated from the first subunit to react, the second subunit was unable to react.

In the case of liver alcohol dehydrogenase, results have been published which dispute this interpretation. Hadorn et al. (1975) reported that the concentration of substrate or coenzyme converted to product in the transient phase corresponded to the active site concentration of enzyme. This was further supported by the results of Tatemoto (1975), who used a steady-state analysis of burst amplitudes to determine that both active sites on the enzyme were turning over during the pre-steady-state period.

The present study was undertaken in an attempt to completely dissect the liver alcohol dehydrogenase reaction mechanism into its component partial reactions using an aromatic substrate at alkaline pH. Prior to doing this, however, it was necessary to evaluate carefully the plausibility of the half of the sites reactivity theory under these conditions.

Experimental Section

Materials. Liver alcohol dehydrogenase was prepared according to the method of Theorell et al. (1966). Samples of the crystalline enzyme were routinely dissolved in 0.05 M phosphate–ammonia buffer, pH 9.6, and dialyzed against 40 mM phosphate buffer (4 × 1000 mL), pH 7. The concentration of enzyme was determined by activity measurement (Dalziel, 1957) and fluorometric titration of NADH binding sites in the presence of isobutyramide (Theorell and McKinley-McKee, 1961) with results by the two methods always within 10% of each other. Coenzymes were purchased from Sigma Chemical Corp., and NAD^+ was purified by sulfuric acid elution from Dowex-1 (Stinson and Holbrook, 1973). Benzyl alcohol and benzaldehyde (Fisher Scientific Co.) were vacuum distilled prior to use. Isobutyramide and pyrazole were purchased from Eastman Organic Chemicals and used without further purification. Mitochondrial malate dehydrogenase was prepared in this laboratory from pig hearts by the method of Gregory

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[1] Abbreviations used: NADH, reduced nicotinamide adenine dinucleotide; NAD^+, oxidized nicotinamide adenine dinucleotide; E or LADH, horse liver alcohol dehydrogenase; alc or BzOH, benzyl alcohol; ald or Bzald, benzaldehyde; Iba, isobutyramide; Pyr, pyrazole; LDH, lactic dehydrogenase.

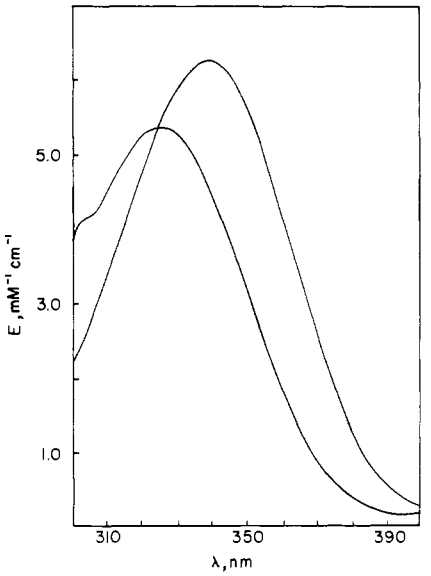

FIGURE 1: Absorption spectrum of NADH and enzyme-bound NADH at pH 8.75; [NADH] = 28.9 μM and [LADH] = 40 μN.

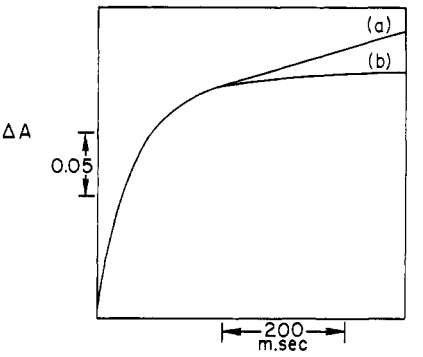

FIGURE 2: Burst of bound NADH at 330 nm. (a) Syringe 1, 49.4 μN LADH; syringe 2, 2 mM NAD^+ and 48 mM BzOH. (b) Syringe 1, 49.4 μN LADH; syringe 2, 2 mM NAD^+, 48 mM BzOH and 200 mM Iba.

et al. (1971) and lactate dehydrogenase was purchased from Sigma Chemical Corp. All kinetic experiments were carried out in 0.05 M pyrophosphate buffer, pH 8.75.

Methods. Ultraviolet-visible spectra were recorded on a Cary Model 17 spectrophotometer. Stopped-flow experiments were performed on a Durrum-Gibson spectrophotometer with fluorescence attachment. The dead time was determined to be 3.5 ± 0.5 ms as measured by the reaction of ascorbic acid with 2, 6-dichloroindophenol (Gutfreund, 1972). Kinetic experiments were performed at 25 °C. Data (2048 samples per reaction) were collected with a Transidyne Neurograph Model N-3 waveform recorder. Rate constants were computed using an analog device designed by Dr. D. Ballou, consisting of a logarithmic converter and a variable potentiometer for setting the end point of the reaction to obtain a linear first-order plot. Fluorescence experiments to monitor the oxidation of NADH or the reduction of NAD^+ were carried out by exciting at 335 nm, and observing emission at wavelengths higher than 400 nm using a PBL GL-42-01 filter. Reactions monitored in the spectrophotometric mode which did not exhibit simple first-order kinetics were analyzed on a Nova 2/10 computer. A double Guggenheim plot (Gutfreund and Sturtevant, 1956) was employed to obtain exponential burst rate constants.

Results

Extinction Coefficient of Bound NADH. Since the evaluation of amplitudes of transient reactions in the liver alcohol dehydrogenase mechanism requires accurate values for the extinction coefficient of free and bound NADH, the absorbance of these species was determined under the reaction conditions of our kinetic experiments. Figure 1 shows the absorption spectrum of free and bound NADH at pH 8.75 in pyrophosphate buffer. The isosbestic point is at 325 nm, and the peak wavelength for bound NADH is 325 nm, showing a hypochromicity compared with free coenzyme. The difference extinction coefficient for bound NADH minus enzyme at 330 nm, the wavelength used in our kinetic studies, was 5.3 ± 0.13 $\times 10^{+3}$ M^{-1} cm^{-1} determined by experiments at various ratios of enzyme to coenzyme with corrections for the small amount of free NADH due to the K_D of 0.5 μM at this pH (Taniguchi et al., 1967; Theorell et al., 1970). Formation of ternary complex with isobutyramide or benzyl alcohol had a negligible effect on the binary complex absorbance at 330 nm. In the stopped-flow instrument, the extinction coefficient for bound coenzyme was 5.1 ± 0.1 $\times 10^3$ M^{-1} cm^{-1} with a 1-mm slit, being slightly lower due to the optics of the instrument. This value was used in calculation of reaction amplitudes of our kinetic studies.

Benzyl Alcohol Oxidation. Figure 2 shows typical traces of the reaction of liver alcohol dehydrogenase with saturating concentrations of NAD^+ and benzyl alcohol, in the presence and absence of isobutyramide. The exponential formation of enzyme-bound NADH is followed by a steady-state rate which is inhibited due to abortive enzyme-NADH-benzyl alcohol complex formation, and more extensively diminished by the presence of isobutyramide. The amplitude of the exponential burst phase obtained from a double Guggenheim plot was corrected by the method of Gutfreund (1972). This method takes into account the rates of both the hydrogen-transfer step and coenzyme dissocation from the enzyme. The corrected amplitude is 87% of the enzyme site concentration in the absence of isobutyramide and 97% in the presence of inhibitor. The rate constants of the exponential phase were 20 s^{-1} in the absence of isobutyramide and 18.2 s^{-1} in its presence. The rate constant for the exponential phase was independent of alcohol concentration in the concentration range 1 to 24 mM. When the same reaction, in the presence of isobutyramide, was measured by monitoring the fluorescence of bound NADH, the rate constant for the exponential burst phase was also 20 s^{-1}.

The effect of product on the transient kinetics of alcohol oxidation was also determined. Increasing the concentration of benzaldehyde in the reaction mixture produced three effects on the observed transient: the amplitude of the exponential phase decreased, the apparent rate constant of the exponential phase increased, and the slope of the steady-state phase was decreased. Typical results are presented in Figure 3. The amplitude of the exponential phase could be diminished by more than 95% in the presence of 0.2 mM benzaldehyde.

Benzaldehyde Reduction. The reaction in this direction can be studied by single turnover experiments, in which enzyme concentration is slightly greater than NADH, and benzaldehyde is in large excess. Figure 4 shows the results of three typical experiments. Curve 4a is the reaction with NADH in the same syringe as aldehyde, while in curve 4b enzyme was premixed with NADH. Curve 4c shows the reaction with 0.5 mM benzaldehyde. In no case was the amplitude of the rapid phase equal to that of the slow phase. The slower apparent reaction rate of the fast phase in curve 4a was due to the bi-

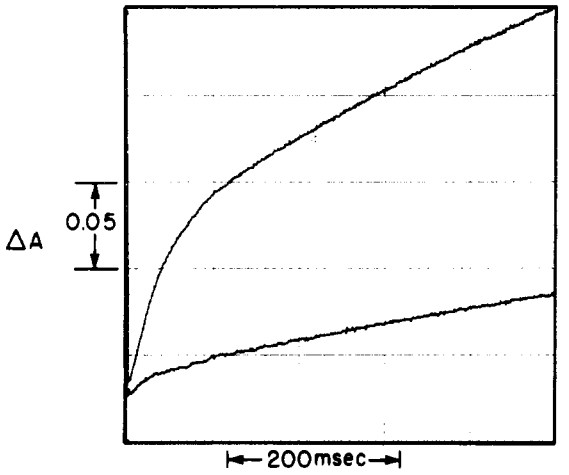

FIGURE 3: Burst of bound NADH with added Bzald at 330 nm. Syringe 1, 51.9 μN LADH; syringe 2, 2 mM NAD^+, 0.96 mM BzOH and no Bzald (upper trace) or 0.4 mM Bzald (lower trace).

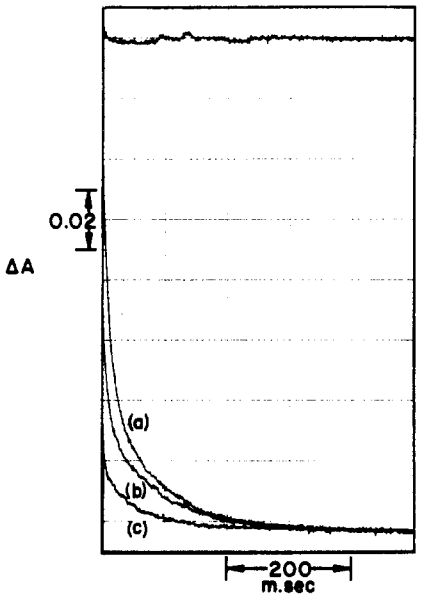

FIGURE 4: Single turnover experiment at 330 nm. (a) Syringe 1, 52 μN LADH; syringe 2, 39 μM NADH and 0.2 mM Bzald. (b) Syringe 1, 52 μN LADH and 39 μM NADH; syringe 2, 0.2 mM Bzald. (c) Syringe 1, 52 μN LADH and 39 μM NADH; syringe 2, 1.0 mM Bzald.

molecular rate of NADH binding to the enzyme. When coenzyme was premixed (Figure 4b), more than half the reaction amplitude was lost in the instrument dead-time. With 0.5 mM aldehyde, 80% of the reaction was not seen and the slow reaction accounted for only 10% of the total amplitude.

The large fraction of the amplitude lost during the instrument dead-time agrees with the results of Hadorn et al. (1975) who reported rapid rates for the hydrogen-transfer reaction under these conditions. Although the rate constant for this reaction was beyond the time resolution of our instrument, an extrapolated double-reciprocal plot of rate constants and aldehyde concentrations yielded an estimated value of 500–1000 s^{-1} at saturating aldehyde. Since the 0.5 mM concentration of aldehyde used in the experiment presented in Figure 4c is not saturating, it is probable that an even greater fraction of the rapid phase would occur within the instrument dead-time under saturating conditions, with a correspondingly smaller relative amplitude for the slow phase.

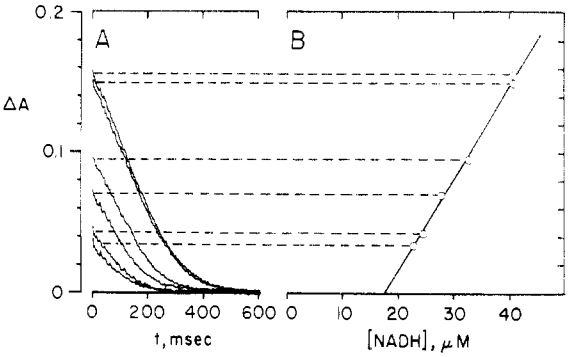

FIGURE 5: (A) Steady-state NADH oxidation; (B) plot of amplitudes vs. NADH concentration. Syringe 1, 36 μN enzyme and NADH; syringe 2, 24 mM benzaldehyde. NADH concentrations after mixing (top to bottom) were 40.9, 32.7, 27.8, 24.6, and 22.9 μM. Wavelength, 360 nm; time constant, 0.1 ms; slit width, 1 mm (4 nm HBW); path length, 1.6 cm.

As a further demonstration that at least 90% of the enzyme sites turn over very rapidly, we performed an experiment suggested to us by Dr. J. J. Holbrook. Enzyme and various NADH concentrations in excess over enzyme were mixed with benzaldehyde in a stopped-flow apparatus. Under these conditions, the rapid oxidation of enzyme-bound NADH is followed by the slower steady-state oxidation of free NADH. The amplitude of the pre-steady-state reaction indicates the coenzyme concentration oxidized prior to steady-state turnover. This rapid oxidation occurs almost totally within the instrument dead-time and is easily distinguishable from the steady-state reaction. If the amplitudes of the observed steady-state reactions are plotted against total NADH concentrations, the plot will intercept the abscissa at a concentration of NADH equal to the concentration oxidized in the rapid pre-steady-state phase.

Figure 5 shows the result of this experiment, which was performed with the Durrum instrument interfaced to a Data General Nova 2/10 computer by a system purchased from On-Line Instrument Co. Each reaction curve in Figure 5A represents 200 data points, with data collection initiated 1 ms after flow stopped. A benzaldehyde concentration of 12 mM after mixing was used to ensure saturation, and the enzyme concentration was 18 μN after mixing in all experiments. The plot in Figure 5B shows reaction amplitudes plotted against total NADH concentration. A least-squares treatment of the data yields an intercept of 17.75 $\pm$ 0.27 μM, with 95% confidence limits of $\pm$0.74 μM. This value is experimentally indistinguishable from the concentration of enzyme active sites. The slope of the line was 6.58 $\pm$ 0.2 D mM^{-1} which, considering the 1.6-cm path length of the cuvette, corresponds to an extinction coefficient of 4.11 $\pm$ 0.13 D mM^{-1} cm^{-1}. This value is in excellent accord with the value for unbound NADH at 360 nm presented in Figure 1 and provides a further check on the validity of the experiment. These results demonstrate that a concentration of NADH equal to the enzyme site concentration is oxidized by benzaldehyde prior to steady-state turnover. The method has the advantage that it is possible to determine the active enzyme sites without assuming values for the reaction starting point, extinction coefficients, or instrument dead-time.

Rate Constants for Partial Reactions. In order to dissect the reaction mechanism, it is necessary to obtain rate constants for partial reactions. The rate constant for NADH dissociation was determined by addition of another enzyme and its sub-

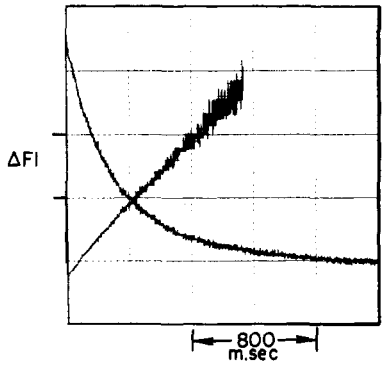

FIGURE 6: Rate of dissociation of NADH from LADH. Syringe 1, 40 μN LADH and 10 μN NADH; syringe 2, 48 μN LDH and 7.5 mM pyruvate; excitation λ 335 nm and emission $\lambda > 400$ nm.

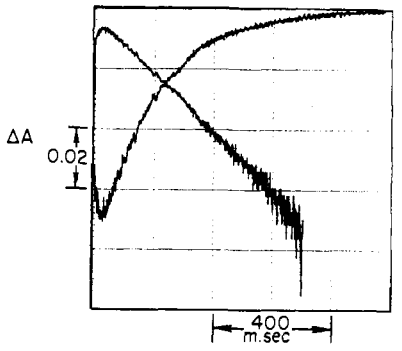

FIGURE 7: Rate of dissociation of BzOH from ternary enzyme-NAD^+-alcohol complex. Syringe 1, 49.8 μN LADH; syringe 2, 41.4 μM NADH, 0.2 mM Bzald and 20 mM Pyr. λ = 300 nm.

strate. The result is demonstrated in Figure 6 in which lactic dehydrogenase and pyruvate were added to the liver alcohol dehydrogenase-NADH complex. The rate constant for dissociation of the enzyme-NADH complex was 3.0 s^{-1}. The rate of binding of NADH to the enzyme was determined fluorimetrically in the presence of isobutyramide and was 8.8 $\times$ 10^6 M^{-1} s^{-1}, resulting in a calculated value of 0.34 μM for the dissociation constant of the enzyme-NADH complex, in good agreement with directly determined values at this pH (Taniguchi et al., 1967; Theorell et al., 1970). The rate constant for NAD^+ binding to the enzyme was determined in the stopped-flow instrument by protein fluorescence quenching in the presence of pyrazole, which forms a tightly bound ternary complex with enzyme-NAD^+ and was 6 $\times$ 10^5 M^{-1} s^{-1}. Addition of NADH to binary enzyme-NAD^+ complex was used to determine fluorimetrically the rate constant for NAD^+ dissociation, which was 7.4 s^{-1}. The calculated dissociation constant of 12.4 μM was in good agreement with literature values for NAD^+ binding at this pH (Taniguchi et al., 1967).

Since the site nonequivalence proposed for liver alcohol dehydrogenase is contingent upon a product release rate which is slow relative to the catalytic step, the rate constant for dissociation of alcohol from the ternary enzyme-NAD^+-alcohol complex was determined by the method of McFarland and Bernhard (1972). Figure 7 shows the results of this experiment, in which the rate of dissociation of alcohol from ternary complex generated by a single turnover experiment was followed by the absorbance at 300 nm due to enzyme-NAD^+-pyrazole complex formation. The rate constant for benzyl alcohol dissociation was 5.2 s^{-1}. To determine the approximate bimolecular rate constant for alcohol binding, a series of solutions containing enzyme-NAD^+ complex was mixed with 5.5 μM benzyl alcohol, and the rate of bound NADH formation was observed. The binary complex concentrations and respective pseudo-first-order rate constant are: 56.9, 49.5, 40.2, 37.1 μM; k_{obsd} = 8.6, 7.7, 6.1, 5.9 s^{-1}. Analysis of the data using two consecutive reversible first-order reactions (Frost and Pearson, 1953), i.e., alcohol binding followed by hydrogen transfer, gave 1.5 $\times$ 10^5 M^{-1} s^{-1} as the bimolecular rate constant for alcohol binding and, consequently, a dissociation constant of 3.5 $\times$ 10^{-5} M for the alcohol.

Discussion

The minimal mechanism required to describe the liver alcohol dehydrogenase reaction at pH 8.75, with the rate constants determined in this study, is as follows:

$$E + NAD^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{NAD^+} \qquad k_1 = 6 \times 10^5 \text{ L mol}^{-1} \text{ s}^{-1}, \quad k_{-1} = 7.4 \text{ s}^{-1}$$

$$E_{NAD^+} + \text{alc} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{NAD^+}{}^{alc} \qquad k_2 = 1.5 \times 10^5 \text{ L mol}^{-1} \text{ s}^{-1}, \quad k_{-2} = 5.2 \text{ s}^{-1}$$

$$E_{NAD^+}{}^{alc} \underset{k_{-h}}{\overset{k_h}{\rightleftharpoons}} E_{NADH}{}^{ald} \qquad k_h = 20 \text{ s}^{-1}, \quad k_{-h} = 500\text{-}1000 \text{ s}^{-1}$$

$$E_{NADH}{}^{ald} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_{NADH} + \text{ald}$$

$$E_{NADH} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + NADH \qquad k_4 = 3.0 \text{ s}^{-1}, \quad k_{-4} = 8.8 \times 10^6 \text{ L mol}^{-1} \text{ s}^{-1}$$

In an additional study, the overall equilibrium constant for the liver alcohol dehydrogenase reaction with benzyl alcohol at pH 8.75 was determined to be 2.02 $\times$ 10^{-2}. Use of the Haldane relationship permitted calculation of the dissociation constant of aldehyde from the ternary enzyme-NADH-aldehyde complex, which was 1.3 $\times$ 10^{-3} M if the hydrogen transfer rate is 1000 s^{-1} or 0.66 $\times$ 10^{-3} M if it is 500 s^{-1}.

Regarding the burst of bound NADH production due to adding saturating concentrations of NAD^+ and alcohol to enzyme, we consistently obtained values lower than the enzyme site concentration in the absence of isobutyramide. In Figure 2, the burst amplitude with 24 mM benzyl alcohol was 87% of the value expected for the enzyme concentration used, after correcting for the steady-state rate by the method of Gutfreund (1972). In Figure 3, at 0.5 mM benzyl alcohol, which should more than 90% saturate the enzyme-NAD^+ complex, we obtained a corrected burst amplitude equal to 46% of the expected value. Burst amplitudes less than 100% can be accounted for by the balance between the rate constants for aldehyde dissociation and the reverse hydrogen transfer, as expressed by the ratio k_3/k_{-h}. If this ratio is very large, with k_3 at least two orders of magnitude greater than k_{-h}, the burst amplitude at saturating alcohol, after correction for the steady-state rate, should be equal to the concentration of enzyme active sites. On the other hand, if k_3 is not very much larger than k_{-h}, the burst amplitude becomes a complicated function of several rate constants. We have recently derived an equation for the transient appearance of bound product without assuming irreversible hydrogen transfer and rapid aldehyde release, and the term in the equation for the amplitude of the exponential phase is:

$$\beta = E_0\left(\frac{1}{(1 + k_4'/k_h)(1 + k_{-h}/k_3)}\right)^2$$

The constant k_4' is the apparent rate constant for coenzyme dissociation from the binary complex and is equal to:

$$k_4' = \frac{k_4}{1 + ([\text{alc}]/K_I)}$$

where

$$E_{NADH}{}^{alc} \overset{K_I}{\rightleftharpoons} E_{NADH} + \text{alc}$$

The burst amplitude of 87% of enzyme sites at 24 mM benzyl alcohol and 46% at 0.5 mM substrate is consistent with a K_I of 3.7 mM for dissociation of the alcohol from the abortive ternary complex.

Our observation that the rate constant for the exponential burst in the presence of isobutyramide is the same when measured spectrophotometrically or by nucleotide fluorescence indicates that aldehyde dissociation is very rapid relative to k_h. This must be the case since the fluorescence yield of the enzyme-NADH-isobutyramide complex is much higher than enzyme-NADH and is the major signal in the fluorimetric experiment. The ternary complex with isobutyramide cannot form until aldehyde dissociates, and, since the fluorimetric burst rate constant is not slower than the spectrophotometric one, it may be assumed that aldehyde dissociation is very much greater than the 20 s^{-1} hydrogen-transfer rate. The experiment does not, however, provide any information on the reverse rate constant, k_{-h}.

Inspection of the equation for the reaction amplitude provides several insights. It can be seen that the ratio k_{-h}/k_3 will exert a large effect on the amplitude unless it is a very small number. In addition, anything which makes k_4 smaller will also tend to increase the amplitude. This explains the larger amplitudes seen at very high alcohol concentrations, and in the presence of isobutyramide. Formation of the abortive enzyme-NADH-alcohol complex, or the inhibitory enzyme-NADH-isobutyramide complex, decreases the apparent turnover rate constant, k_4. A physical description of the system would be that during turnover a large fraction of the enzyme exists as enzyme-NAD^+-alcohol complex due to the k_{-h}/k_3 ratio, and hence there is an incomplete exponential burst. If turnover is inhibited, either with isobutyramide or by benzyl alcohol substrate inhibition, complexes containing bound NADH accumulate and a full burst would theoretically be obtained when there is no turnover. The almost complete bursts obtained with high levels of benzyl alcohol, and isobutyramide present, appear to rule out the possibility of site nonequivalence despite the 46% burst amplitude obtained at noninhibitory benzyl alcohol levels. There is no a priori reason to expect the exponential burst amplitude to be equal to the enzyme site concentration unless product release rates greatly exceed the rate constant of the reverse catalytic step.

The ability of aldehyde product to diminish the amplitude of the exponential phase (Figure 3) to a negligible value indicates that there is no discernible slow isomerization of enzyme-NADH-aldehyde complex. The ratio k_h/k_{-h} indicates that approximately 2–4% of the enzyme will be in the form of enzyme-NADH-aldehyde and 96–98% in enzyme-NAD^+-alcohol at saturating concentrations of aldehyde and alcohol, in good agreement with our results. The increasing apparent rate constant for the exponential burst with increasing aldehyde is due to the reversible reaction proceeding to an equilibrium, with the rate constant for reverse hydrogen transfer affecting the observed rate constant. The equation which describes this process is:

$$\lambda - k_4' = \frac{k_h k_3}{k_{-h} + k_3} + \frac{k_{-h}}{k_{-h} + k_3} k_{-3}[\text{ald}]$$

where λ = burst rate constant. This is illustrated in Figure 3. The apparent burst rate increases from 20 s^{-1} (no benzaldehyde present) to 57 s^{-1} at 0.2 mM Bzald with a 90% decrease in the burst amplitude. We carried out this experiment at several other concentrations of benzyl alcohol and found the same qualitative effect. If half-site reactivity were an operative mechanism the dimeric species, $E_{NAD^+}{}^{alc}$-$E_{NADH}{}^{ald}$ should accumulate at saturating benzaldehyde since this species cannot return to $E_{NAD^+}{}^{alc}$-$E_{NAD^+}{}^{alc}$ while alcohol is still bound to the other subunit. Our experimental evidence refutes this hypothesis since, under a variety of experimental conditions, we were able to displace the hydrogen-transfer equilibrium to a point where the amount of bound NADH produced in the exponential phase was less than 10% of the enzyme active site concentration.

The single turnover experiment shown in Figure 4 indicates that, when enzyme and NADH are premixed, at 0.5 mM aldehyde, 90% of the reaction amplitude occurs rapidly, with the remaining 10% at a much slower rate. A premixing effect has been reported (Luisi and Favilla, 1972) but is totally due to the bimolecular rate of NADH binding to the enzyme, and we have been able to simulate curve 4a by calculations based on a second-order binding rate constant of 8×10^6 L mol^{-1} s^{-1} followed by an exponential hydrogen transfer rate of 10^3 s^{-1}. Hijazi and Laidler (1973) also studied the effect of premixing on the transient-phase kinetics of two-substrate enzyme systems. They derived equations which predict that premixing coenzyme with enzyme should lead to a more rapid initial reaction and concluded that site nonequivalence is not required to explain the premixing effect. The oxidation of 90% of the limiting reagent, NADH, in the rapid phase contradicts previous reports of equal amplitude fast and slow reactions under similar conditions (Bernhard et al., 1970; Luisi and Favilla, 1972). Those studies, however, were performed at aldehyde concentrations below 0.1 mM and the results can readily be explained by failure to saturate with substrate. As demonstrated in Figure 4b, an aldehyde concentration substantially below saturation results in a slower observed rate for the rapid phase and an increase in the amplitude of the slow phase.

The effect of substrate concentrations significantly below saturation can be understood by considering the partial reaction scheme:

$$E_{NADH} + \text{ald} \overset{K_3}{\rightleftharpoons} E_{NADH}{}^{ald} \overset{K_H}{\rightleftharpoons} E_{NAD^+}{}^{alc} \xrightarrow{k_{-2}} E_{NAD^+} + \text{alc}$$

K_3 represents the equilibrium formation constant for aldehyde binding, while K_H represents the equilibrium constant for hydrogen transfer. In past studies (McFarland and Bernhard, 1972), k_{-2} has been reported to be between 5.1 and 6.5 s^{-1}; our result (Figure 7) of 5.2 s^{-1} is in good agreement with this and indicates that alcohol dissociation partially limits turnover. This step is much slower than the preceding steps of hydrogen transfer and aldehyde binding, which are therefore in a rapid linked equilibrium. The equilibrium constant for conversion of E_{NADH} to $E_{NAD^+}{}^{alc}$ will be equal to K_3(ald) K_H. From the Haldane relation, we have calculated that, if k_{-h} is 500 s^{-1}, K_3 would be the reciprocal of 0.65×10^{-3} M, or 1.54×10^3 M^{-1}, and K_H would be 25. The fraction of E_{NADH} converted to $E_{NAD^+}{}^{alc}$ in this rapid equilibrium would equal:

$$\frac{(1.54 \times 10^3)25[\text{ald}]}{1 + 1.54 \times 10^3[\text{ald}] + (1.54 \times 10^3)25[\text{ald}]}$$

Using this relationship with 0.1 mM aldehyde, 77% of the

E_{NADH} complex should be rapidly converted to $E_{NAD^+}{}^{alc}$, while with 0.5 mM aldehyde the rapid phase should account for 91.6% of the amplitude. This is in rather good agreement with the results presented in Figures 4b and 4c. Subsequent to the attainment of this equilibrium concentration of $E_{NAD^+}{}^{alc}$, the reaction proceeds in the thermodynamically favored direction at the rate constant for alcohol dissociation, which accounts for the slow phase.

Although equilibrium considerations are the primary factors in explaining biphasic single turnover reactions, particularly at low substrate concentrations, several other factors can make a minor contribution to this phenomenon. (1) With an equilibrium constant of 25 for the hydrogen transfer reaction, 4% of the total amplitude would occur in the slow second phase even at saturating aldehyde concentrations. This amount is within the error of the experiment in Figure 5 and may account for the value slightly lower than 18 μM for the abscissa intercept. (2) At low substrate concentrations, the rate of the fast process becomes slower as would be expected. This enhances the difficulty of distinguishing between the rapid phase and slow phase for accurate amplitude determinations. (3) It is extremely difficult to ensure that all NADH will be enzyme bound, despite the relatively low dissociation constant of 0.5 μM. For example, in the experiments in Figure 4, the drive syringe with binary complex would contain 1.3 μM free NADH and 14.3 μN free enzyme. After mixing, these concentrations would be halved and the psuedo-first-order rate constant for binding the free NADH would be 63 s^{-1}. Although this is faster than the alcohol dissociation rate constant, it is considerably slower than hydrogen transfer and would contribute to the difficulties of precise amplitude determination. 0.65 μM NADH is only 3.3% of the total concentration, but it should be kept in mind that the difference between the results of Figure 4c and those expected with saturating aldehyde is only 6% of the total amplitude.

The extrapolation of steady-state amplitudes (Figure 5) further demonstrates that a concentration of NADH equal to the enzyme site concentration is rapidly oxidized. With a slow rate constant for dissociation of alcohol product, which partially limits turnover, the results of Figures 4 and 5 incontrovertibly demonstrate that site nonequivalence based on product release is not observed with this substrate under these conditions. The experiment of Figure 5 is particularly significant since it requires no assumptions about starting levels, extinction coefficients or instrument dead-times and does not involve elaborate curve-fitting procedures.

Through the use of transient kinetic techniques, it has been possible to determine most of the rate constants for the liver alcohol dehydrogenase reaction under these conditions. The only values not experimentally accessible are the binding and dissociation rate constants for benzaldehyde. Using the Haldane relationship, it was possible to estimate that the dissociation constant of the aldehyde is between 0.65 and 1.3 $\times$ 10^{-3} M. Although this is a calculated value, it does not seem unreasonable. From the incomplete burst of alcohol oxidation, k_{-h} and k_3 must be of the same order of magnitude. If k_3 and k_{-h} are 10^3 s^{-1}, k_{-3} would be 7.7 $\times$ 10^5 L mol^{-1} s^{-1}, a rate constant similar to that for alcohol binding. The reasons for both alcohol and aldehyde binding at slower than diffusion limited rates, and for the very slow rate constant for alcohol dissociation from the ternary complex, are still obscure and will require further study.

Past studies have indicated biphasic single turnover reactions for NADH oxidation by benzaldehyde, with equal amplitude fast and slow phases (Bernhard et al., 1970; McFarland and Bernhard, 1972; Luisi and Favilla, 1972; Luisi and Bignetti, 1974). Inspection of the conditions for these studies indicates that the substrate concentrations used were well below the aldehyde dissociation constant. This may have been due to the incorrect use of the steady-state K_m as a basis for estimating the concentrations needed for saturation. A rapid phase substantially greater than half of the total amplitude has been reported (McFarland and Bernhard, 1972) but was dismissed as an unusual effect due to excessive high substrate concentration. We have demonstrated that these results can be anticipated for an ordered ternary complex mechanism without subunit interactions.

Our results place severe constraints on proposals of functional asymmetry in liver alcohol dehydrogenase (Bernhard et al., 1970; Luisi and Favilla, 1972; Luisi and Bignetti, 1974). The greater than 50% amplitude in the rapid phase, with alcohol dissociating at a very slow rate relative to hydrogen transfer, rules out any mechanism in which product dissociation from one subunit is required for the other subunit to react. This is certainly also confirmed by the results of Figure 5. The "flip-flop" or reciprocation mechanism (Luisi and Bignetti, 1974) proposed for this enzyme would require equal amplitude fast and slow single turnover reactions and a half burst in the presence of isobutyramide, which can be ruled out by the experiments of Figures 2 and 4. It would also be impossible to displace the burst reaction by aldehyde, as demonstrated in Figure 3, in this type of mechanism. A biphasic rapid oxidation of bound NADH cannot be entirely ruled out since hydrogen transfer occurs primarily within the instrument dead-time, but is not necessary to explain currently existing data.

Two things seem clear from our results and those of Hadorn et al. (1974). First, the results with the benzaldehyde–benzyl alcohol system do not mandate proposing subunit interactions and can readily be explained by an ordered ternary complex mechanism with independently functioning sites. Secondly, the requirement for product dissociation prior to chemical reactivity of the second subunit cannot be a generally relevant mechanism for liver alcohol dehydrogenase. Future studies purporting to demonstrate functional asymmetry with other substrates should be characterized by meticulous attention to such factors as saturation by coenzyme and substrate, the hydrogen-transfer equilibrium, and product dissociation rate constants.

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Lewis Acid Complexes Which Show Spectroscopic Similarities to an Alcohol Dehydrogenase Ternary Complex†

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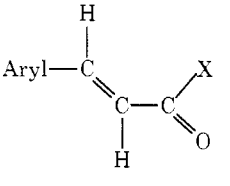
ABSTRACT: *trans*-4-*N*,*N*-Dimethylaminocinnamaldehyde (II) ($\lambda_{max}(H_2O)$ 398 nm, ϵ_{max} 3.15×10^4 M^{-1} cm^{-1}) reacts with the horse liver alcohol dehydrogenase–NADH complex to form a transient intermediate (III) (λ_{max} 464 nm, ϵ_{max} ~6.2×10^4 M^{-1} cm^{-1}) which breaks down to *trans*-4-*N*,*N*-dimethylaminocinnamyl alcohol and NAD^+. It was proposed that the intermediate has a dipolar quinodial structure that is stabilized by a coordination bond between the active site zinc ion and the carbonyl oxygen of II [Dunn, M. F., and Hutchison, J. S. (1973), *Biochemistry 12*, 4882]. In these studies, complexes of II or *trans*-4-*N*,*N*-dimethylaminocinnamaldehyde-2′-pyridinohydrazone (IV) with various Lewis acids in anhydrous aprotic solvents have been studied as spectroscopic and structural models for III. Both II and IV form complexes exhibiting red-shifted $\pi^* \leftarrow \pi$ spectral bands with a variety of Lewis acids. II forms complexes with $ZnCl_2$, $SnCl_4$, and $(C_2H_5)_3OBF_4$, and IV forms complexes with ZnI_2, $Zn(NO_3)_2$, $CoCl_2$, and $MnCl_2$. The magnitudes of the red shifts (39 to 110 nm) depend both on the chemical nature of the Lewis acid and on the properties of the solvent. The spectrum of the $ZnCl_2$ complex with II in diethyl ether (λ_{max} = 431 nm) is closely similar to the spectrum of III. The observed spectral red shifts are consistent with an inner sphere bonding interaction between the carbonyl oxygen of II, or the imine nitrogen of IV and the Lewis acid. The x-ray structure of the ZnI_2 complex of IV ($C_{26}H_{18}N_4ZnI_2$, mol wt 585.64 g/mol) which crystallizes in space group $P\bar{1}$ with unit cell dimensions of a = 13.47 (2) Å, b = 8.19 (1) Å, c = 9.69 (1) Å, α = 87.0 (1)°, β = 104.1 (1)°, and γ = 103.4 (1)° has been refined by full matrix least-squares to a final R of 0.092. An envelope conformation is observed for the five-membered ring created by chelation of zinc by the pyridine and imine nitrogens of the ligand, the coordination about zinc is distorted tetrahedral, and the π-bonding framework of the ligand suggests a quinoidal structure. The x-ray structure establishes that the spectral shift observed when zinc ion binds to IV is due to inner sphere coordination of the imine nitrogen. These studies support a structure for the enzyme-bound intermediate (III) involving the direct coordination of the carbonyl oxygen of II to the active site zinc ion.

The use of intense chromopheres in the study of enzyme catalytic mechanism can yield insight into the detailed chemical processes occurring at the enzyme site if the spectral changes are directly coupled to the chemical transformations(s). Substrate analogues based on the conjugated arylacryloyl moiety (I) have been particularly useful in the study of both proteases (Bernhard et al., 1965; Charney and Bernhard, 1967; Bernhard and Lau, 1971; Hinkle and Kirsch, 1970)



I

and dehydrogenases (Malhotra and Bernhard, 1968, 1973; Dunn and Hutchison, 1973; Dunn et al., 1975). The spectra of these chromophores are highly sensitive to: (1) the chemical nature of the group X attached to the carbonyl carbon; (2) the electronic nature of substituents on the aryl group ortho and para to the acryloyl residue; and (3) to the polarity of the microenvironment (Dolter and Curran, 1960; Charney and Bernard, 1967; Bernhard and Lau, 1971; Dunn and Hutchison, 1973). Hence the spectral properties of the arylacryloyl

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