On Site–Site Interactions in the Liver Alcohol Dehydrogenase Catalytic Mechanism†

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ABSTRACT: A reinvestigation of the single-turnover time course for the horse liver alcohol dehydrogenase (LADH) catalyzed oxidation of NADH and of (4*R*)-4-deuterio-NADH (NADD) by a series of substituted benzaldehyde substrates has been carried out in the alkaline pH range. The reaction time courses were monitored at 330 nm via stopped-flow rapid-mixing spectrophotometry. The pyrazole "suicide technique" [McFarland, J. T., & Bernhard, S. A. (1972) *Biochemistry 11*, 1486–1493] has been employed to limit reaction to a single turnover when enzyme sites are limiting. In all cases, the time courses were found to be remarkably biphasic under conditions of substrate and NADH (or NADD) saturation. As reported previously (McFarland & Bernhard, 1972), the two amplitudes of reaction at substrate saturation are not necessarily equal, but the ratio of amplitudes is not far from unity ($5 > A_{fast}/A_{slow} > 0.8$). On-line computer analysis demonstrated that the time courses were best described by the rate law for two simultaneous first-order processes [$OD = OD_\infty + B \exp(-k_1 t) + C \exp(-k_2 t)$]. Amplitude analysis [involving corrections for instrument dead time and for the OD changes contributed to the slow step by the formation of the E(NAD–pyrazole)adduct] gave the following percentage amplitudes for the rapid phase of the transient time course: *p*-CH_3O–, 62 ± 10%; *p*-CH_3–, 70 ± 10%; *p*-H–, 65 ± 10%; *p*-Cl–, 66 ± 10%; and *p*-NO_2–, 50 ± 10%. Control experiments in which the concentration of NADH rather than enzyme sites was limiting demonstrate that the rates and amplitudes which characterize the biphasic time course are unaffected by this change. Likewise, the presence of 0.44 mM NAD^+ in the stopped-flow syringe containing pyrazole and a saturating concentration of substrate was found to exert no influence on the biphasic time courses. The relative insensitivity of the ratio of amplitudes in the two phases to wide variations in the electronic substituents on the substituted benzaldehyde substrates argues strongly against a compulsory ordered mechanism as an explanation of kinetic biphasicity. The insensitivity of the amplitude ratio to the (rate-dependent) distinction between NADH and NADD adds further weight to the argument that the compulsory-ordered mechanism (although correct) is insufficient to account for kinetic biphasicity.

In 1970, Bernhard et al. (1970) reported that, when horse liver alcohol dehydrogenase (LADH)[1] reacts with NADH and various aromatic aldehydes, above pH 8 and under single-turnover conditions (e.g., when [NADH] > [E] > [S] or [S] > [E] > [NADH]), the observed transient time courses are biphasic and approximately equal in amplitude. The interpretation of these findings was that the biphasic rate behavior is the macroscopic manifestation of microscopic forces which render the two active sites of LADH catalytically nonequivalent and result in apparent "half-site" reactivity. McFarland & Bernhard (1972) extended the range of observations by reacting aromatic aldehydes with NADH and LADH in the presence of high concentrations of pyrazole. Since the reaction of LADH with substrate and coenzyme is ordered (NADH binds before aldehyde, while alcohol dissociates before NAD^+ (Theorell & Chance, 1951; Brändén et al., 1975)), and since pyrazole forms a tightly bound adduct with NAD^+ at the active site, aldehyde reduction in the absence of any added NAD^+ is limited to a single turnover (eq 1). By use of this "catalytic site suicide" technique, it

$$\begin{array}{l} E + NADH \rightleftharpoons E(NADH) \overset{RCHO}{\rightleftharpoons} E(NADH, RCHO) \rightleftharpoons \\ \qquad\qquad\qquad\qquad\qquad\qquad E(NAD^+, RCH_2OH) \\ \qquad\qquad\qquad\qquad\qquad\qquad \Big\downarrow \\ E(NAD^+) \underset{k_{-3}'}{\overset{Pyr}{\longrightarrow}} E(NAD\text{-}Pyr) \overset{Pyr}{\longleftarrow} E(NAD^+) + RCH_2OH \end{array} \qquad (1)$$

thus became possible to study the kinetics of a single turnover of enzyme sites over a much wider range of coenzyme and substrate concentrations. The introduction of the pyrazole "suicide" technique was a particularly important innovation since it provides a means for studying the single-turnover time course for aldehydes with low affinities for the E(NADH) complex.

The results of studies conducted in the presence of high pyrazole concentrations demonstrated that the biphasic character of the single-turnover time course persists even when [S], [NADH] ≫ [E], although in some instances the amplitude of the fast step was found to exceed the amplitude of the slow step (McFarland & Bernhard, 1972).

When the kinetics of the biphasic time course for aldehyde reduction were compared with the kinetics and stoichiometry of proton uptake under the conditions [E], [NADH] ≫ [S], it was found that the total change in hydrogen ion concentration predicted by the overall chemical reaction occurs in a single first-order process with a rate identical to the rate of the slow step (Dunn, 1974). No net uptake of protons from solution occurs in the fast process. Likewise, the formation of the spectrally observable E(NAD–pyrazole) complex

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[1] Abbreviations used: E or LADH, liver alcohol dehydrogenase (EC 1.1.1.1); NADH and NAD^+, reduced and oxidized nicotinamide-adenine dinucleotide; NADD, (4*R*)-4-deuterio-NADH; S and P, aldehyde and alcohol, respectively; Pyr, pyrazole.

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(McFarland & Bernhard, 1972) proceeds with the same slow, single-exponential time dependence that characterizes the slow step, although the redox kinetics are biphasic.

Together, the accumulated observations suggested that the state of liganding at one protomer regulates the catalytic activity of the other protomer. Subsequent to catalysis at one protomer, reaction of the second protomer is limited by one or more intermediary steps. It was postulated that these steps include dissociation of product from the first protomer and/or any possible conformational changes accompanying such dissociation (Bernhard et al., 1970; McFarland & Bernhard, 1972; Dunn, 1974).

The hypothesis that LADH exhibits a half-site reactivity that arises via ligand-mediated subunit interactions has been criticized by several other groups. Shore & Gutfreund (1970) found that other substrates such as ethanol and acetaldehyde yield apparent monophasic data. More recently, Kvassman & Pettersson (1976) and Hadorn et al. (1975) have reinvestigated the single-turnover time courses for benzaldehyde in the alkaline pH range using the pyrazole "suicide" technique. They each report that the benzaldehyde single-turnover time course is essentially monophasic when $[S] \gg [E] < [NADH]$. Thus, they contend that their experimental findings demonstrate alternative origins for the apparent "half-site" reactivity that do not involve ligand-mediated subunit interactions.

Since the experimental findings of Hadorn et al. (1975) and the conclusions of Kvassman & Pettersson (1976) are in substantial disagreement with the work of Bernhard et al. (1970) and McFarland & Bernhard (1972), we also have undertaken a reinvestigation of the transient time course for the LADH-catalyzed reduction of aromatic aldehydes using the pyrazole "suicide" technique, improved instrumentation, more refined analytical methods for data reduction, and a variety of para-substituted benzaldehydes as substrates. As will be shown, these studies demonstrate the persistence of the biphasic rate behavior for the single-turnover time courses for the reduction of benzaldehyde and a variety of para-substituted benzaldehydes under the conditions of limiting enzyme sites. This work constitutes a rebuttal both in response to the conclusions published by Kvassman & Pettersson (1976) and to the experimental analysis of Hadorn et al. (1975), as regards both the reduction of benzaldehyde by NADH and the generality of conclusions for other aromatic aldehyde reductions.

Materials and Methods

The LADH (Boehringer Mannheim) generally used in these studies was purified and the active site concentrations were determined as previously described (Bernhard et al., 1970). In several experiments, a highly purified "EE" isozyme was prepared (Anderson, Dahlquist, & Bernhard, to be published). This isoelectrofocused pure protein yielded virtually identical transient and steady-state rate constants by utilizing both benzaldehyde and *p*-nitrobenzaldehyde as substrates. The various para-substitued benzaldehyde and benzyl alcohols were obtained from Aldrich Chemical Co. and either sublimed or vacuum distilled prior to use. NADH and NAD^+ were obtained from Sigma Chemical Co. and were the best grades available. Pyrazole (Aldrich) was used without further purification. Both (4*R*)-4-deuterio-NADH (NADD) and isotopically normal NADH were prepared enzymatically for the kinetic isotope studies as described previously (Dunn & Hutchison, 1973; Rafter & Colowick, 1957). All other reagents used were obtained commercially and were the finest available.

In all experiments referred to herein "concentrations" of reactants, catalysts, and buffers are "final concentrations" after mixing. Transient kinetic studies were performed either with a Durrum-Gibson D-110 stopped-flow spectrophotometer (2-cm path length, dead time 2–4 ms, and 1.0-mm slit) or with an apparatus (1.0-cm path length, dead time ~1.0 ms) designed and constructed in the laboratory of Bernhard. Some of the characteristics and design of this instrument are described elsewhere (Seydoux & Bernhard, 1974). All of the herein reported absorbances are corrected to the values expected for a 1.00-cm light path. The 1.0-mm slit employed in the Durrum apparatus corresponds (according to the manufacturer) to a bandwidth of 8.6 nm. The investigations of the benzaldehyde and anisaldehyde time courses with NADH were carried out with both instruments in independent sets of experiments. The investigations of the *p*-chloro-, *p*-nitro-, and *p*-methylbenzaldehyde time courses presented in this paper were carried out with the Bernhard instrument only. The conversion, NADH + benzaldehyde + $H^+ \rightarrow$ NAD^+ + benzyl alcohol, was followed in the stopped-flow instruments under the conditions $[S]_0 \gg [NADH]_0 \gg [E]_0$ in order to check the readily measurable change in extinction in aqueous solution. All kinetic runs were at 25 °C. In all instances, preincubated enzyme and NADH (or NADD) solutions were mixed with aldehyde. The pyrophosphate buffers were prepared by dissolving $Na_4P_2O_7$ in double-distilled water and then adjusting the pH to the desired value with either NaH_2PO_4 or H_3PO_4. In each trace presented zero time refers to the time at which flow stops.

The phototube signal from the Durrum instrument was stored in a Biomation 810 transient recorder as 1024, 8-bit data points and continuously monitored on an oscilloscope. The stored data then were sent to a Hewlett-Packard 3000 digital computer via a Datos 305 interface. The computer was run under the control of an interactive Fortran program which allows the user to send, reduce, and store the data immediately. In addition, the program permits the user to plot both the raw data and the theoretical fits on an x–y plotter interfaced to the computer. (Details of the software and hardware for this system will be described elsewhere (R. G. Morris, S. Koerber, and M. F. Dunn, unpublished results). The phototube signal from the Bernhard instrument was collected in analog form via a high-speed magnetic tape recorder. Data were digitized via an A/D converter and sent to a Varian 620 computer for reduction and plotting.

Fortran programs developed for use with the Durrum-Gibson stopped-flow allowed us to test fit the entire observed time course to a variety of kinetic mechanisms. Using this empirical approach, we have searched for the simplest model and consequent differential equations which describe the transient kinetic data reported herein. Once the data are converted to ΔOD units, the converted data are then fitted to the desired differential equation. The method used to obtain the best fit to a given differential equation employs a gradient-expansion algorithm described by Bevington (1969) as the "CURFIT" subroutine. The standard deviation of the phototube "shot noise" for each set of experimental conditions was determined by collecting and analyzing a "straight line" from the stopped-flow spectrophotometer. The standard deviation then was used in the fitting subroutines to determine the "goodness of fit" as reflected in the reduced χ^2 value (χ_ν^2) (Bevington, 1969).

Results

Figure 1 shows the transient time courses obtained when LADH and NADH or NADD are mixed with benzaldehyde

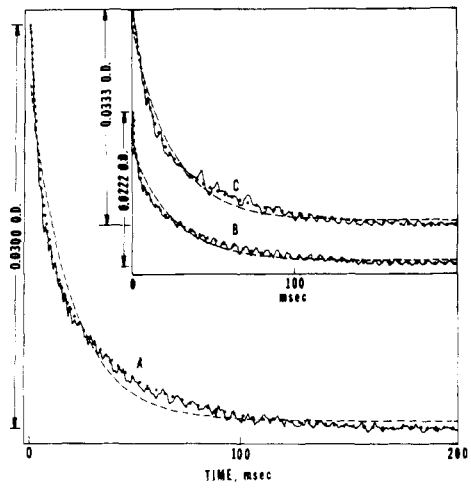


FIGURE 1: Stopped-flow kinetic traces comparing the time courses for the LADH-catalyzed reduction of benzaldehyde by NADH (trace A and inset, trace B) and by NADD (inset, trace C) in the presence of pyrazole at pH 8.50. Conditions (after mixing): (trace A) $[E]_0$, 45.2 μM; $[benzaldehyde]_0$, 1.96 mM; $[pyrazole]_0$, 20 mM; 0.1 M glycine–KOH, pH 8.50. (Traces B and C) $[E]_0$, 10 μN; $[NADH]_0$ or $[NADD]_0$, 43 μM; $[benzaldehyde]_0$, 0.807 mM $[pyrazole]_0$, 20 mM; 0.1 M sodium pyrophosphate, pH 8.50. (Trace A) Best fit parameters assuming a single first-order process (---): k, 53.0 s^{-1}; amplitude 0.0240 OD; χ_ν^2, 34.8. Best fit assuming two consecutive first-order processes (···); k_1, 192 s^{-1}; amplitude, 0.0160 OD; k_2, 27.9 s^{-1}; amplitude 0.0140 OD; χ_ν^2, 2.63. (Trace B) Best fit assuming a single first-order process (---): k, 40.3 s^{-1}; amplitude, 0.0163 OD; χ_ν^2, 45.1. Best fit assuming two consecutive first-order processes (···); k_1, 291 s^{-1}; amplitude 0.0106 OD; k_2, 25.1 s^{-1}; amplitude, 0.0116 OD; χ_ν^2, 5.87. (Trace C) Best fit assuming a single first-order process (---): k, 42.5 s^{-1}; amplitude, 0.0266 OD; χ_ν^2, 31.6. Best fit assuming two consecutive first-order processes (···); k_1, 102 s^{-1}; amplitude, 0.0202 OD; k_2, 19.8 s^{-1}; amplitude, 0.0131 OD; χ_ν^2, 0.981. Note that the conditions for trace A are similar to those used by Hadorn et al. (1975) in their Figure 1.

and pyrazole under the conditions of limiting site concentration in the Durrum-Gibson stopped-flow spectrophotometer at pH 8.50 under conditions similar to those employed by Hadorn et al. (1975) (see their Figure 1 for comparison). An attempt was made to fit the data in Figure 1 both to the rate law for a single first-order process (eq 2)

$$OD = OD_\infty + B \exp(-k_1 t) \quad (2)$$

and to the rate law for two simultaneous first-order processes (eq 3)

$$OD = OD_\infty + B \exp(-k_1 t) + C \exp(-k_2 t) \quad (3)$$

where OD and OD_∞ are respectively the optical densities at times t and t_∞, B and C are the reaction amplitudes, and k_1 and k_2 are the apparent rate constants (see Materials and Methods). Note that OD_∞ was treated as an unknown and fitted along with the other parameters.

The three fitted parameters for the single exponential decay (eq 2) and the five fitted parameters for the sum of two single exponential decays (eq 3) were used to generate theoretical time courses which then were overlaid onto the plot of the observed time course (see Figure 1). Examination of Figure 1 reveals that the fit of the transient time courses to the rate law for eq 3 is both qualitatively and quantitatively better than the fit obtained to the rate law for eq 2. Indeed, the theoretical curve for the two-step reaction coincides, within experimental error, with the observed transient, while the best fit theoretical curve for a one-step reaction shows significant systematic deviations from the observed time course. Clearly, *at high benzaldehyde concentrations (Figure 1), the time courses are noticeably biphasic.* At lower concentrations of substrate (50–100 μM, data not shown), the rate of the fast step is relatively slow. Consequently, the amount of OD change occurring in the dead time is negligible, and therefore the total OD change during a single turnover can be determined (to a good first approximation) by direct observation.

However, note in the inset to Figure 1 (trace B) that, at high benzaldehyde concentrations when NADH is used, the fast phase of the reaction is complete in 10–15 ms. The rate of this step is estimated to be 200–400 s^{-1}. Since the dead time of the Durrum-Gibson stopped-flow spectrophotometer used in this instance is 2–4 ms, a considerable portion of the OD change occurring in this step takes place during the mixing dead time. Hence, the determination of both the amplitude and the apparent rate constant for the fast process is subject to considerable error. Indeed, since most of the *observed* OD change occurs in the second step, the difficulty inherent in the analysis of the fast step is compounded by the presence of the second step. In order to partially circumvent the difficulties encountered in measuring the amplitudes and apparent rate constants for the fast process, we carried out all further studies with benzaldehyde and anisaldehyde using (4*R*)-4-deuterio-NADH (NADD). Earlier studies (McFarland & Bernhard, 1972) have shown that the use of NADD slows down the rate of the fast reaction approximately threefold (via a primary kinetic isotope effect). Therefore, by carrying out transient kinetic studies using NADD as coenzyme, a significantly greater proportion of the fast reaction can be observed at the higher benzaldehyde concentrations. This, in turn, allows a more accurate determination of both the rates and the amplitudes of the fast step in the biphasic reaction.

As can be seen in the inset to Figure 1 (traces B and C), the substitution of NADD for NADH decreases the rate of fast reaction approximately two- to threefold. Note, however, that, under these virtually saturating conditions, the total amplitudes in experiments B and C (corrected for instrument dead time) determined from the computer fit for both steps are, within experimental error (±15%), identical.

In addition to the correction for dead time, it is also necessary to correct for small changes in absorbance which occur at 330 nm due to the formation of the LADH–NAD–pyrazole adduct (Theorell & Yonetani, 1963). The studies of McFarland and Bernhard (1972) have established that, during a single-turnover reaction, the rate of formation of the LADH–NAD–pyrazole adduct is limited by the same process which limits the rate of the slow step. Accordingly, the total OD change for the formation of the LADH–NAD–pyrazole adduct (~13.3% of the corrected total OD change) must be added to the observed OD change for the slow step to obtain the corrected amplitude of the slow step. The necessary corrections were determined from control studies (Figure 2) in which the OD change for the formation of the E(NAD–Pyr) adduct was measured at 330 nm in the absence of substrate. The conditions used in Figure 2 allow the determination of this correction under conditions of concentrations of the various absorbing species which closely approximate the reaction conditions. (Note that the slow rate of NADH dissociation from the LADH–NADH complex limits the rate of formation of the LADH–NAD–pyrazole adduct in Figure 2.) These OD corrections are revelant to the results reported by Kvassman & Pettersson (1976). In their experiments, the observed kinetics were measured at a slightly shorter wavelength (328 nm) and hence require a slightly larger OD correction since $\lambda_{max}^{E\text{-}NAD\text{-}Pyr} < \lambda_{max}^{NADH}$ or $\lambda_{max}^{E\text{-}NADH}$

Figure 3 shows the concentration dependence of the rates and corrected amplitudes of the LADH-catalyzed reduction

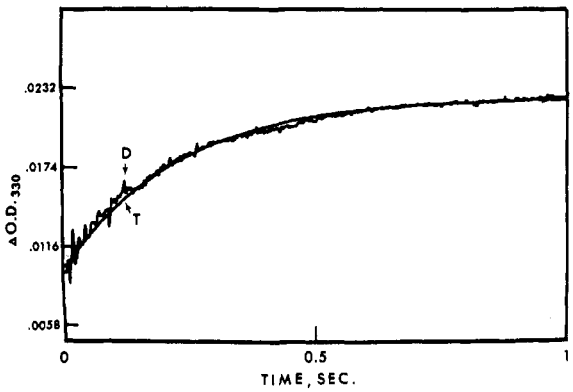


FIGURE 2: Optical density changes at 330 nm contributed by the formation of the LADH–NAD^+–pyrazole complex. The LADH–NADH complex was mixed with NAD^+ and pyrazole in the stopped-flow apparatus. The net optical density change and the apparent first-order rate constant (k_{obsd}) were determined by fitting the observed time course (trace D) to the rate law for a single first-order process (theoretical trace, T). Conditions: (syringe no. 1) LADH, 9.95 μN; NADH, 47.1 μM; (syringe no. 2) pyrazole, 20 mM; NAD^+, 0.47 mM; 0.1 M sodium pyrophosphate buffer, pH 8.74. k_{obsd} = 4.40 s^{-1}; amplitude, 0.0078 OD.

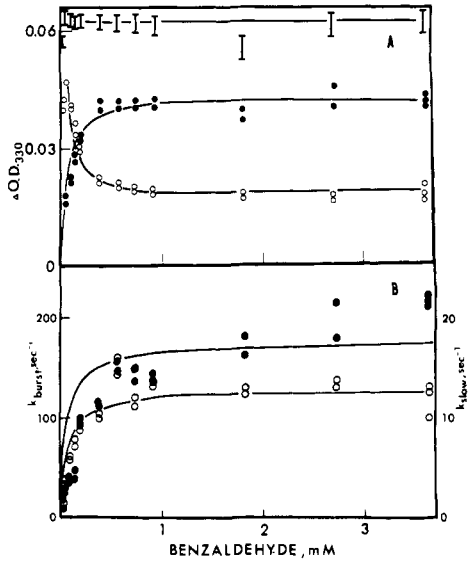


FIGURE 3: Dependence of the amplitude and rates of the fast and slow steps on the substrate concentration for the LADH-catalyzed reduction of benzaldehyde by NADD in the presence of excess pyrazole at pH 8.75. Amplitudes (A) and rates (B) were obtained by fitting each transient kinetic time course to the rate law for two simultaneous, irreversible first-order processes (see text). The amplitudes were corrected for instrument dead time and for the OD contribution due to formation of the LADH–NAD–pyrazole adduct. Conditions: $[E]_0$, 9.95 μN; $[NADD]_0$, 47.1 μM; 0.1 M sodium pyrophosphate buffer, pH 8.75. Fast step (●), slow step (○), and total amplitude (bars). Rate constants and amplitudes were obtained from the best fit of the time course to the rate law for two simultaneous first-order processes (viz. Figure 1).

of benzaldehyde under the conditions employed by Kvassman & Pettersson (1976) at pH 8.75, but using NADD as coenzyme. Since the reactions were performed in the presence of high pyrazole (20 mM), the total amplitude (corrected for both the instrument dead time and the E–NAD–pyrazole adduct OD changes) should remain constant if only a single turnover of enzyme sites takes place. The data in Figure 3A show that the total amplitude is essentially constant at all substrate concentrations so long as [S] ≥ [E]. Note also that the corrected amplitudes for both the fast and slow steps saturate at high (~1 mM) benzaldehyde concentrations with a fast-step-to-slow-step ratio of 65:35. The rates of the fast

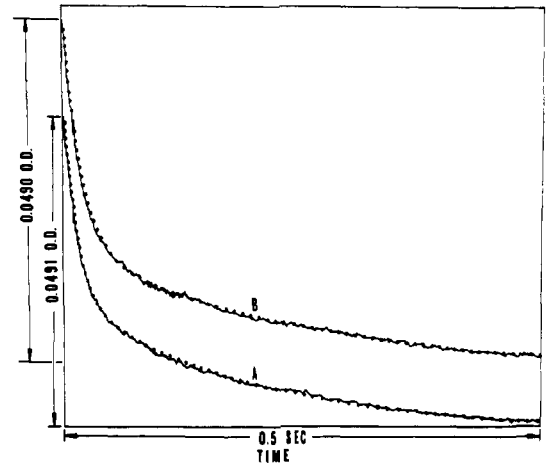


FIGURE 4: Stopped-flow traces comparing the time courses for the LADH-catalyzed reduction of anisaldehyde by NADD both in the presence (A) and in the absence (B) of NAD^+ in solutions containing 20 mM pyrazole. The experimentally observed data (solid lines) were fitted to the rate law for two simultaneous first-order processes (···). Enzyme and NADD were premixed in one syringe, anisaldehyde, pyrazole, and NAD^+ were premixed in the other syringe. Conditions: $[E]_0$, 10.6 μN; $[NADD]_0$, 49.1 μM; $[anisaldehyde]_0$, 3.64 mM; $[pyrazole]_0$, 20 mM; trace A, $[NAD^+]_0$, 0.44 mM; trace B, $[NAD^+]_0$, 0.0; 0.1 M sodium pyrophosphate buffer, pH 8.75; 25.0 ± 0.2 °C. Best fit parameters: (trace B) k_1, 75.2 s^{-1}; amplitude, 0.0292 OD; k_2, 5.53 s^{-1}; amplitude, 0.0198 OD; χ_ν^2, 2.80; (trace A) k_1, 59.4 s^{-1}; amplitude, 0.0317 OD; k_2, 4.45 s^{-1}; amplitude, 0.0174 OD; χ_ν^2, 2.98.

and slow steps also saturate with values of 180 ± 40 and 12 ± 2 s^{-1}, respectively (Figure 3B). The concentration dependence shown in the isotherms for the fast and slow step amplitudes and the rate of the slow step are adequately fit by a rectangular hyperbola with a hyperbolic constant K_{app} = 50 ± 20 μM [in good agreement with the values reported by Kvassman & Pettersson (1976)]. The isotherm for the rate of the fast step is not adequately fit by this hyperbolic constant nor can these data be fit to any isotherm described by a single hyperbolic constant. However, these rate data are not sufficiently accurate to define the nature of the isotherm. Control experiments in which 10 μM enzyme, premixed with 10 μM NAD^+ and 20 mM pyrazole, was mixed with 1 mM benzaldehyde and 70 μM NADH demonstrated that the steady-state rate of substrate turnover under these conditions is approximately 700-fold slower than the rate of the slow step.

Representative stopped-flow traces of the LADH-catalyzed reduction of anisaldehyde by NADD both in the presence and in the absence of 0.44 mM NAD^+ under conditions of limiting enzyme sites and in the presence of pyrazole are shown in Figure 4. Note that these single-turnover time courses are markedly biphasic; the best-fit theoretical time courses (not shown) assuming a single irreversible first-order process (eq 2) give large systematic deviations from the experimentally observed time courses. The fit to the rate law for two consecutive irreversible first-order processes yields theoretical time courses which are indistinguishable from the observed time courses. Note also in Figure 4 that at high concentrations of anisaldehyde the presence of 0.44 mM NAD^+ affects neither the amplitudes nor the rates of the biphasic transformation.

The dependence of the corrected amplitude and rates for the anisaldehyde system as a function of substrate concentration, both in the presence and in the absence of added NAD^+ in the substrate syringe, are presented in Figures 5A and 5B, respectively. At low anisaldehyde concentrations (below 1 mM), the amplitude of the slow step is attenuated (compare the isotherms for the dependencies of the slow step amplitude in the presence and in the absence of NAD^+, Figure

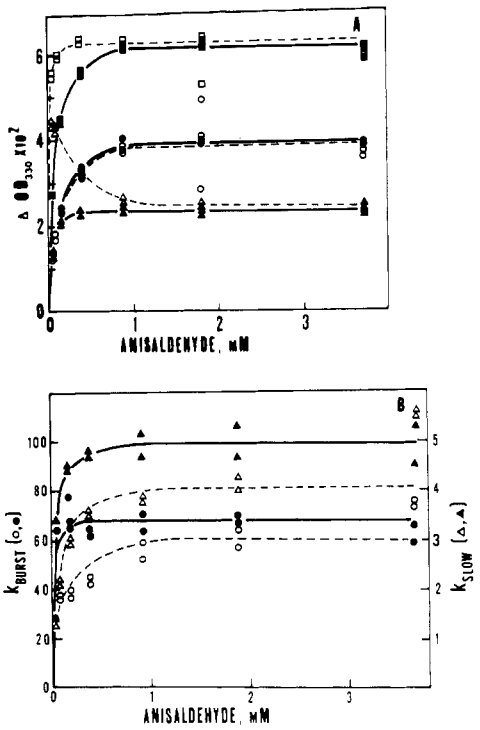


FIGURE 5: Dependence of the amplitudes and rates of the fast and slow steps on the concentration of substrate for the LADH-catalyzed reduction of anisaldehyde by NADD in the presence and in the absence of 0.44 mM NAD^+. The reaction amplitudes (A) and rates (B) for the biphasic time courses were obtained as described in Figure 4. The amplitudes were corrected for instrument dead time (3.3 ms) and for the OD contribution which results from formation of the LADH–NAD^+–pyrazole adduct. Conditions were the same as in Figure 4. Fast step plus NAD^+ (●) minus NAD^+ (○); slow step plus NAD^+ (▲), minus NAD^+ (△); total reaction amplitude plus NAD^+ (■), minus NAD^+ (□).

5A). The amplitude of the fast step is independent of added NAD^+ at all concentrations. When the anisaldehyde concentration is virtually saturating, neither the rates nor the amplitudes of the biphasic transformation are affected by the presence of 0.44 mM NAD^+ (Figures 4 and 5). Note that the dependencies of rates and amplitudes in the absence of added NAD^+ are similar to those already discussed for benzaldehyde. Detailed examination of the effects of added 0.44 mM NAD^+ on the benzaldehyde or *p*-chloro- or *p*-nitrobenzaldehyde time courses (data not shown) yielded results qualitatively analogous to the data presented in Figures 4 and 5. At saturation, the fast step amplitude accounts for 62 ± 10% of the total reaction.

The data presented in Figure 6A,B and Figure 6C,E are representative of the time courses obtained in the presence of pyrazole when LADH and NADH are mixed with *p*-chloro- and *p*-nitrobenzaldehyde, respectively, in the Bernhard stopped-flow spectrophotometer. The inset presented with each trace shows the fast phase of the reaction on an expanded time scale. Note that for both substrates the *observed* amplitude of the fast process decreases as the substrate concentration increases. The traces in Figure 6D,E demonstrate that the rate and amplitude of the slow step in the *p*-nitrobenzaldehyde single-turnover time course is *unaffected* when the limiting reactant is NADH (Figure 6E) rather than enzyme (Figure 6D). In contrast to benzaldehyde and anisaldehyde, the amplitude (and the apparent first-order rate constant) of the slow step observed for *p*-nitrobenzaldehyde is nearly independent of substrate concentration over the range investigated. Figure 7A,B summarizes the dependence of the burst and slow step amplitudes for *p*-chloro- and *p*-nitrobenzaldehyde re-

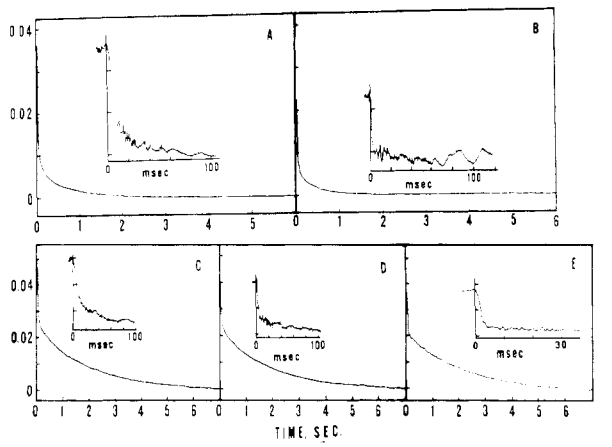


FIGURE 6: Representative stopped-flow traces for the LADH-catalyzed reduction of *p*-chlorobenzaldehyde (A and B) and *p*-nitrobenzaldehyde (C, D, and E) by NADH in the presence of pyrazole. Conditions: (traces A and B) $[E]_0$, 8 μN; $[NADH]_0$, 15 μM; $[pyrazole]_0$, 20 mM; [*p*-chlorobenzaldehyde]$_0$, (A) 24 μM; (B) 192 μM; (traces C and D) $[E]_0$, 12 μN; $[NADH]_0$, 15 μM; $[pyrazole]_0$, 20 mM; [*p*-nitrobenzaldehyde]$_0$, (C) 24 μM; (D) 96 μM; (trace E) $[E]_0$, 15 μN; $[NADH]_0$, 12 μM; [*p*-nitrobenzaldehyde]$_0$, 96 μM; pH 8.75 sodium pyrophosphate buffer, 0.05 M. Note that enzyme sites are limiting in D, while NADH is limiting in E. The inset to each trace shows the fast phase of the reaction on an expanded time scale.

spectively, corrected both for the loss of OD due to instrument dead time and for the OD contributed by the formation of the E(NAD–Pyr) complex. Note that, when the observed fast step amplitude is corrected for the OD changes estimated to occur in the dead time, the fast step amplitude is nearly independent of substrate concentration. These data demonstrate that the fast step amplitude for the *p*-chlorobenzaldehyde single-turnover time course accounts for 66 ± 10% of the total reaction; the fast step amplitude for the *p*-nitrobenzaldehyde single-turnover time course accounts for 50 ± 10% of the total reaction.

The LADH-catalyzed reduction of *p*-methylbenzaldehyde (data not shown) also yields a biphasic time course when reaction is limited to a single turnover due to the presence of 20 mM pyrazole. Our detailed studies demonstrate that the fast and slow step amplitudes depend on substrate concentration in a manner analogous to that found for benzaldehyde and anisaldehyde (viz., Figures 3A, 5A, and 7). At saturation, the fast amplitude accounts for 70 ± 10% of the total reaction.

As will be evident in the Discussion, the influence of the para substituent on the overall redox equilibrium constant (K_{eq}', viz., eq 4) for the LADH-catalyzed reduction of the benzaldehyde derivatives used in these studies is of interest to the discussion of the mechanism. Therefore, values of K_{eq}' (at

$$R\text{–}CHO + NADH + H^+ \overset{E}{\rightleftharpoons} RCH_2OH + NAD^+ \qquad (4)$$

$$K_{eq}' = K_{eq}[H^+] = \frac{[RCH_2OH][NAD^+]}{[RCHO][NADH]}$$

pH 8.75) were determined for the five benzaldehydes employed in the above kinetic studies. The values of K_{eq}' were found to be quite sensitive to the electronic nature of the para substituent, ranging from 7.80 for anisaldehyde to 926 for *p*-nitrobenzaldehyde. The strong interrelationship of K_{eq}' and the electron-withdrawing character of the substituent is made apparent in Figure 8 where log K_{eq}' for each aldehyde is plotted vs. the appropriate Hammet σ value for each substituent. This Hammet plot establishes the existence of a reasonably linear free energy relationship for the substituent effect. The rel-

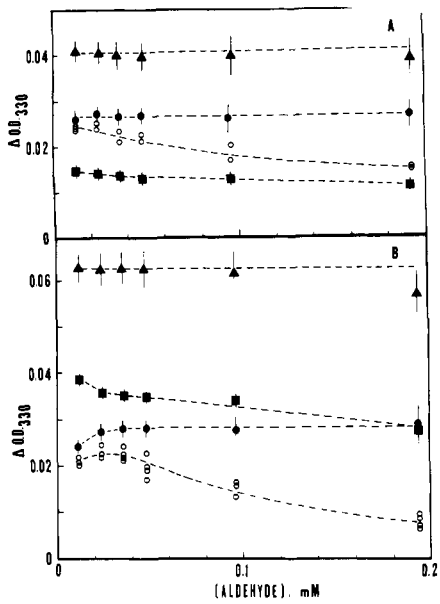


FIGURE 7: Dependence of the amplitudes of the fast and slow steps on the substrate concentration for the LADH-catalyzed reduction of *p*-chlorobenzaldehyde (A) and *p*-nitrobenzaldehyde (B) by NADH in the presence of pyrazole. (○) Uncorrected fast step amplitudes; (●) Fast step amplitudes corrected for the OD change occurring during the instrument dead time. (□) Slow step amplitudes corrected for the OD contribution due to the formation of the E(NAD$^+$–pyrazole) adduct. (▲) Total corrected amplitude for the biphasic time course. Experimental conditions were the same as described in the caption to Figure 6 (traces A–D). Note that the difference in total amplitudes in A and B is due to the use of different total concentrations of enzyme.

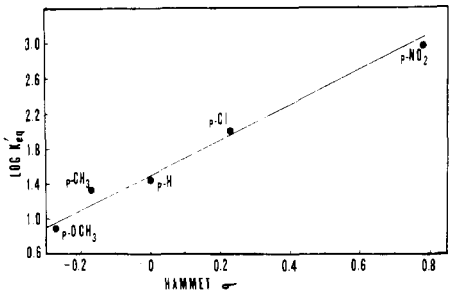


FIGURE 8: Hammet plot of the apparent redox equilibrium constant (K_{eq}') for the LADH-catalyzed reduction of para-substituted benzaldehydes by NADH at 25 °C in pH 8.75 buffer (0.05 M sodium pyrophosphate). Results were obtained utilizing low ($\sim 10^{-9}$ M) LADH concentrations by measuring NADH absorption in the presence of high (buffering) concentrations of alcohol and aldehyde. Klinman (1972) has obtained similar values.

atively large ρ value ($\rho = 1.96$) for the reaction (i.e., the slope of the best-fit straight line in Figure 8) reflects the high sensitivity of K_{eq}' to the electron-withdrawing nature of the substituent. As expected from chemical-bonding considerations, electron-withdrawing substituents destabilize the aldehyde ground state (relative to alcohol); therefore, the stronger the electron-withdrawing character of the para substituent, the more favorable the free energy change for aldehyde reduction.

Discussion and Conclusions

In this paper, as in our previous studies of the LADH single-turnover time course, our objective has been to construct a physicochemical description of the events which occur during catalysis. In the alkaline pH range (pH >8), the LADH single-turnover time course for a variety of aromatic aldehydes is biphasic (Bernhard et al., 1970; McFarland & Bernhard, 1972; Dunn, 1974; Kvassmann & Pettersson, 1976; Hadorn et al., 1975; Hardman & Blackwell, 1974; McFarland & Chu, 1975; Jacobs et al., 1974). The fast phase includes the chemical step of "hydride transfer" (McFarland & Bernhard, 1972; Jacobs et al., 1974). The slow step is unaffected by isotopic substitution, and hence the rate of this step must be limited by some other step in the mechanism.

McFarland & Bernhard (1972) and Kvassman & Pettersson (1976) have shown that the OD changes at 300 nm which result from the E(NAD–Pyr) adduct occur at a rate which is identical with the rate of the slow step and is limited by alcohol desorption from the site. Consequently, the increase in OD due to the formation of this chromophoric ternary complex reduces the amplitude of the OD decrease in the slow step but does not appreciably perturb the amplitude of the OD change in the fast step.

For all of the aromatic aldehydes studied, the rate of dissociation of alcohol product from the site limits the steady-state rate of turnover (Bernhard et al., 1970; McFarland & Bernhard, 1972; Dunn, 1974; Wratten & Cleland, 1963, 1965). It is significant to note that the apparent first-order rate constant of the slow step is numerically identical (within the limits of experimental error) with the value of k_{cat} calculated on the basis of site normality. Furthermore, all of the hydrogen ion uptake required by the overall reaction stoichiometry (1 mol of H^+ per mol of aldehyde reduced) occurs at the rate of the slow step (Dunn, 1974).

The work presented in this study (Figures 3, 5, and 7) shows that the rates (wherever measurable) and the amplitudes of both steps depend on substrate concentration. The hyperbolic nature of these isotherms (Figures 3, 5, and 7) indicates that saturation of binding sites is responsible for the observed dependencies. Note that in no instance does either the observed or the corrected amplitude of the fast step extrapolate to 100% of the enzyme sites as $[S]_0 \rightarrow \infty$. Indeed, the saturated corrected amplitudes range from ~50% (*p*-nitrobenzaldehyde) to 65–70% (benzaldehyde, *p*-chlorobenzaldehyde) of the total sites. In every case it has been possible to obtain measurements at substrate concentrations which are at least 20 × K_s [the kinetically determined apparent dissociation constant for substrate binding to the E(NADH) complex].

With the exception of the brief account published by Hadorn et al. (1975), there seems to be rather good quantitative agreement between our experimental results and those reported by several other groups (Kvassman & Pettersson, 1976; Hardman & Blackwell, 1974; McFarland & Chu, 1975; Jacobs et al., 1974). The excellent agreement between our results and the work of Kvassman & Pettersson (1976) is particularly striking. Kvassman & Pettersson have published a detailed kinetic analysis of the benzaldehyde single-turnover time course under conditions of limiting enzyme sites via the pyrazole–NAD$^+$ suicide technique using experimental conditions essentially identical with ours. Their data, uncorrected for the OD changes contributed by the formation of the E(NAD–Pyr) complex, appear identical in virtually every detail to the benzaldehyde data presented here. In their treatment of the data, Kvassman & Pettersson (1976) have employed analytical methods similar to our own for the determination of apparent rate constants and uncorrected amplitudes. A small difference arises in the corrections applied to the observed amplitudes. As already pointed out, in addition to dead-time corrections, the observed amplitude of the slow step (but not the fast step) is attenuated by the concomitant OD changes due to the formation of the E(NAD–Pyr) complex. Therefore, the amplitude of the slow step must be

corrected in order to accurately estimate the fraction of NADH consumed in either step. Kvassman & Pettersson did not make this correction.

Hadorn et al. (1975) also have published an investigation of the benzaldehyde time course using the pyrazole–NAD^+ suicide technique to limit reaction to a single turnover under conditions of limiting enzyme sites. The kinetic traces at pH 8.50 presented by Hadorn et al. (viz., Figure 1 in their paper) appear qualitatively similar to traces at pH 8.50 presented in this paper (Figure 1) and to those published by McFarland & Bernhard (1972) and by Kvassman & Pettersson (1976) at pH 8.75. However, the fast step rate constants and amplitudes which Hadorn et al. (1975) report are quite different from those reported by McFarland & Bernhard (1972) and by Kvassman & Pettersson (1976) or by ourselves in this study. Hadorn et al. (1975) report that virtually all of the NADH oxidation required for a single turnover occurs in the fast phase, the data of their Figure 1 showing a slow phase notwithstanding. Hadorn et al. (1975) correct the fast step amplitude rather than the slow step amplitude for the OD changes accompanying the formation of the E(NAD–pyrazole) adduct. We are unable to interpret the rate and amplitude analysis of Hadorn et al. (1975) other than to note that, under more favorable conditions for observation (i.e., the use of either NADD or aromatic aldehydes that give slower fast step rates), the assumptions used by Hadorn et al. in their estimation of rates and consequently of amplitudes are inconsistent with the markedly biphasic time courses we observe.

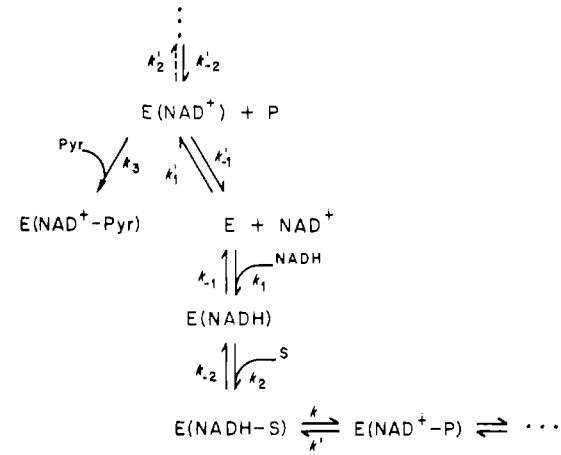
A variety of mechanistic proposals have been put forward to explain the biphasic rate behavior. These mechanisms are presented and discussed in the following paragraphs (in the order of increasing complexity) to ascertain which (if any) are adequate to account for the accumulated chemical and kinetic observations.

Absorbancies of the Ternary Complexes. Although the total change in OD at 330 nm can be experimentally corrected for the fractional change due to E(NAD–Pyr) formation, two other potential 330-nm chromophores require consideration before the total OD change can be related to the oxidation of the NADH chromophore. These are E(NADH–aldehyde) and $E(NAD^+$–alcohol); the spectral properties of which are less accessible to experiment. Kvassman & Pettersson (1976) have proposed that the persistent slow OD change observable experimentally arises from among the special absorbancies of the unaccounted for chromophores (the ternary ligand complexes) rather than from the oxidation of NADH. We maintain that this explanation is not correct for the following reasons.

(1) E(NAD–Pyr) complex formation decreases the *apparent* amplitude of the slow phase.

(2) At high enzyme concentration ($1–5 \times 10^{-4}$ M) and near stoichiometric NAD^+ concentration, there is no appreciable increase in OD upon addition of benzyl alcohol over and above that anticipated for an equilibrium constant of 30 in favor of the $E(NAD^+$–alcohol) ternary complex. (It is interesting to note that an equilibrium constant closer to unity would predict far more NADH at equilibrium in the above experiment). Since the *observed* OD change for the slow step amplitude is approximately 10–15% of the total change (for benzaldehyde), the value of $\epsilon_{(NAD^+-P)}^{330}$ would have to be of the order of 0.6×10^3 to 0.8×10^3 N^{-1} cm^{-1} to account for the observed amplitude of the slow step. The transient and/or pre-steady-state time course for benzyl alcohol oxidation has been studied in detail by several groups (McFarland & Chu, 1975; Hadorn et al., 1975; Weidig et al., 1977); the time courses reported do not exhibit rapid OD_{330} changes corresponding to the formation of $E(NAD^+$–P). Therefore, it appears unlikely that the slow step results from OD changes accompanying the conversion of $E(NAD^+$–P) to E(NAD–Pyr).

Scheme I



(3) In the case of *p*-trifluoromethylbenzaldehyde (Anderson, Dahlquist, & Bernhard, to be published) under conditions of very high enzyme concentration ($\geq 5 \times 10^{-4}$ M), the E-(NAD^+–alcohol) spectrum shows no significant 330-nm absorption. ^{19}F NMR indicates that, in this range of enzyme concentration and at substoichiometric levels of NAD^+ and substrate, virtually all substrate is bound (ternary complex) and that the bound substrate is virtually exclusively *p*-trifluoromethylbenzyl alcohol, as would be expected from the redox equilibrium constant in aqueous solution (see Figure 8). Hence, the ternary complex does not substantially contribute to the residual slow phase amplitude.

Partitioning of the E(NAD^+) Complex. Pettersson & Kvassman (private communication) also have suggested that the slow phase of the transient time course observed in the presence of pyrazole is due (at least in part) to the partitioning of the binary $E(NAD^+)$ complex as indicated in Scheme I.

Since $k_{-1}' \simeq 10\ s^{-1}$ at pH 8.75 and since k_3[pyrazole] $\simeq 100\ s^{-1}$ when [Pyr] = 20 mM, Pettersson & Kvassman argue that there should occur a slow step in which approximately 10% of the sites turn over a second time due to the partitioning of the intermediate, $E(NAD^+)$, as shown above. According to this argument, the slow phase of the biphasic time course includes the recycling of a significant fraction of the sites ($\sim$10%) through one or more additional turnovers and thereby generates a residual slow step. We believe that the above argument is oversimplified for the reason that the dissociation of $E(NAD^+)$ actually is a reversible process (as is the step k_{-2}'), and, under the conditions of the experiments in question, the rate of formation of E(NADH) from $E(NAD^+)$ should be sensitive to the free concentrations of NADH, alcohol, Pyr, and NAD^+. While it is difficult to predict how rapidly E-(NADH) forms via partitioning in these experiments, it is possible to test the validity of Kvassman & Pettersson's suggestion by direct experiment.

If the slow phase involves a significant recycling of enzyme sites via partitioning of the $E(NAD^+)$ complex, then under conditions where the concentration of NADH is limiting (i.e., $[NADH]_0 \leq [E]_0 \ll [S]_0$, and provided virtually all the NADH initially is present as the E(NADH) complex), a slow step should not be observed because all the NADH present would have been consumed in the fast step. Comparison of the traces presented in Figure 6D,E demonstrates that neither the rate nor the relative amplitude of the slow step observed

Scheme II

$$E + NADH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(NADH) \overset{S}{\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}} E(NADH\text{-}S) \underset{k'}{\overset{k}{\rightleftharpoons}} E(NAD^+\text{-}P) \xrightarrow{k_{-2}'} E(NAD^+) + P$$

$$E(NAD^+) \underset{k_{-3}'}{\overset{Pyr}{\longrightarrow}} E(NAD^+\text{-}Pyr)$$

in the LADH-catalyzed oxidation of NADH by *p*-nitrobenzaldehyde is altered when the concentration of NADH is limiting.

Furthermore, due to the reversibility of NAD^+ dissociation (step k_1', Scheme I), the amplitude of the slow step should be reduced or eliminated when reaction is carried out by mixing the E(NADH) complex with substrate, Pyr, and high concentrations of NAD^+. In contradiction to this prediction, the data presented in Figures 4 and 5 demonstrate that a high NAD^+ concentration (0.44 mM) has no effect on the biphasic LADH-catalyzed reaction of NADH and anisaldehyde. Similar results were found for benzaldehyde and *p*-nitrobenzaldehyde. These experiments make it seem unlikely that the partitioning of the $E(NAD^+)$ complex contributes significantly to the amplitude of the slow step.

The Noncooperative-Protomer Compulsory-Ordered Pathway. In a companion paper to the benzaldehyde studies of Kvassman & Pettersson (1976), Pettersson (1976) has derived transient-state analytical kinetic expressions for two-substrate enzyme reactions occurring by an ordered ternary-complex mechanism (i.e., the compulsory ordered pathway), Scheme II. (Here and in the following discussion we follow Pettersson's notation to avoid confusion.) In Scheme II it is assumed that the $E(NAD^+)$ complex is (rapidly) trapped via reaction with pyrazole, thus rendering steps k_{-2}' and k_{-3}' quasi-irreversible. Under the conditions $[E] \ll [NADH]$, [S] and neglecting the rate of dissociation of $E(NAD^+)$, then $[NADH] \simeq [NADH]_0$ and $[S]_0 \simeq [S]$. Solution of the set of differential rate equations for the total appearance of oxidized coenzyme leads to the prediction of marked biphasicity at substrate saturation only when $k \simeq k' + k_{-2}'$. According to Scheme II, if $k \gg k' + k_{-2}'$, then the amplitude of the fast phase [i.e., $A_{2max} = k/(k + k' + k_{-2}')$] approaches 1.0, and only a single phase reaction will be observed.

Kvassman & Pettersson (1976) conclude that for benzaldehyde $k \gg k' + k_{-2}'$, while Weidig et al. (1977) calculate that the value of k/k' is 25–50 at pH 8.75. The presteady-state reaction time course for the oxidation of benzyl alcohol consists of a fast phase that is followed by the steady state when $[NAD^+]_0$, $[S]_0 \gg [E]_0$. The fast phase is subject to a large primary kinetic isotope effect ($k_H/k_D \simeq 3$–4) when 1,1-dideuteriobenzyl alcohol is used. Hence, the fast phase includes the "hydride transfer" step. The saturated rate of the fast step, k_{obsd}, is reported to be 8.0 ± 0.2 s^{-1} at pH 8.75 by McFarland & Chu (1975), 18–20 s^{-1} by Weidig et al. (1977), and 21 ± 2 s^{-1} by Kvassman & Pettersson (1978). Therefore it follows that $k_{obsd} \geq k' \leq 21$ s^{-1} and $k \gg k' + k_{-2}'$ (Kvassman & Pettersson, 1978).

Furthermore, the data presented in Figure 8 demonstrate that the variations in the aqueous redox equilibrium constants measured for the series of para-substituted benzaldehydes arise from the electronic effects of the para substituents. It seems reasonable to assume that, to a first approximation, the variation in the equilibrium constants for the interconversion

Scheme III

$$E(NADH)(aldehyde) \underset{K^*}{\overset{fast}{\rightleftharpoons}} [E(NAD^+)(alcohol)]^* \overset{slow}{\rightleftharpoons} E(NAD^+)(alcohol) \xrightarrow{fast} E(NAD^+) + alcohol$$

of the ternary complexes is similarly determined by the same electronic effects. Note that a small substituent-dependent selectivity for alcohol vs. aldehyde might contribute to the ternary complex stability. Therefore as the electron-withdrawing character of the para substituent increases, the ratio k/k' should increase also (although a strict proportionality between k/k' and K_{eq}' need not hold). According to Scheme II, the amplitude of the rapid transient should tend toward 1.0 as K_{eq}' increases. Note that the observed trend in amplitudes for the substituted benzaldehydes studied *does not obey* this prediction. The K_{eq}' values (Figure 8) vary from 28 for benzaldehyde to 926 for *p*-nitrobenzaldehyde (a 33-fold change), while the fast step amplitude (A_{2max}) varies from ~0.70 to ~0.50. *Thus, the fast step amplitude appears to be relatively insensitive to electronic substituent effects while K_{eq}' is rather sensitive to electronic substituents effects (i.e., $\rho = 1.92$).* Note that in contradiction to the trend predicted by the Pettersson compulsory-ordered mechanism, A_{2max} approaches a value of 0.5 rather than 1.0 as the electron-withdrawing character of the para substituent increases. Indeed, the dependence of A_{2max} on substrate structure could reflect the affinity of the E(NADH) complex for substrate.

Since the observed characteristics of the biphasic time courses are in substantial quantitative and qualitative disagreement with the noncooperative-protomer compulsory-ordered pathway proposed by Kvassman & Pettersson (1976), we conclude that this mechanism does not satisfactorily describe the LADH single-turnover time course. (However, note that these data are in qualitative agreement with a compulsory-ordered mechanism for ligand binding and dissociation at the catalytic sites.)

An Equilibrium Constant Near Unity for "Hydride Transfer". Since kinetic biphasicity persists at substrate saturation, any plausible catalytic model must (unlike the model of Scheme II) allow for a substrate concentration independent mechanism for avoiding rapid saturation in the product ternary complex. There is no simpler (noncooperative) model than that given in Scheme III.[2] Although the kinetic data we present for each of the substrates can be accounted for by the pathway of Scheme III, the scheme seems unlikely to us.

Basically, in order for biphasicity to persist at substrate saturation according to Scheme III, the equilibrium constant, K^*, must be of the order of unity. Indeed, with most substrates, $k_{fast} \gg k_{slow}$, and hence according to Scheme III the ratio of amplitudes, A_{fast}/A_{slow}, reflects the value of the equilibrium constant K^*. The equilibrium constant, K_{eq}' (eq 4), is very much greater than unity both for benzaldehyde and for *p*-nitrobenzaldehyde (see above). These substrates are electronically near opposite extremes among the substrates we describe herein (see, for example, Figures 7 and 8). In spite of this situation, if Scheme III is correct then the equilibrium constant, K^*, must be of the order of unity (>0.2 to <1.3). Despite the large variation in electronic substituent effects among substrates, for Scheme III the experimentally derived constants, K^*, are insensitive to these effects. On the contrary,

[2] Note that [E(NAD)(alcohol)]* could be a chemical intermediate such as the alcoholate ternary complex.

the fast forward rate of oxidation of NADH is substituent dependent and shows a large kinetic deuterium isotope effect (McFarland & Bernhard, 1972; Dworschack & Plapp, 1977; and the data presented herein). Moreover, on the basis of Scheme III substitution of NADD for NADH should give a quasi-equilibrium deuterium isotope effect on the ratio, A_{fast}/A_{slow}. Our data (Figure 1) are not consistent with this prediction. In order to satisfy the rate dependent but (quasi-) equilibrium independent data, according to Scheme III, all electronic substituent and proton–deuteron dependent rapid kinetic phenomena would have to be compensated for by the same factor in rate modification in the reverse direction. Once hydrogen transfer to the carbonyl carbon has occurred, the effect of para substituents on the stability of the product (alkoxide?)[2] is virtually lost, whereas the effect of para substituents on the stability of conjugated carbonyls is large (as is apparent in the dissociation constants of carboxylic acids, the protonation of amides and esters, and in nucleophilic attack at the carbonyl by H_2O, CN^-, RS^-, etc.). It is conceivable that a slower rate of deuterium transfer (relative to hydrogen) in one direction might be compensated for by a corresponding deceleration factor in the opposite direction (if the transition state lies midway along the hydrogen-transfer coordinate). It seems most unlikely to us that the same substituent-dependent rate enhancement (or deceleration) factors could occur in both the forward and the reverse rates defining K^* for all the different aromatic aldehydes which we describe. An enzyme, particularly an "R" group nonspecific one like LADH, cannot provide a matched compensation for the effect of electronic structural variation on the reactivity of substrates; it may, however, provide a mechanism for switching rate-controlling events from electronic structure-dependent chemical transformation to protein conformational changes.

Therefore, we reiterate our prior conclusion (Bernhard et al., 1970) that more complicated mechanisms must be considered. The next simplest mechanism for the biphasic single-turnover time course assumes, in addition to a compulsory-ordered binding of ligands [as proposed by Theorell & Chance (1951) and Kvassman & Pettersson (1976)], ligand-dependent protomer–protomer interactions which critically influence protomer catalytic activity (Bernhard et al., 1970).

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