Mechanistic Studies on Equine Liver Alcohol Dehydrogenase. I. The Stoichiometry Relationship of the Coenzyme Binding Sites to the Catalytic Sites Active in the Reduction of Aromatic Aldehydes in the Transient State*

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ABSTRACT: The course of the transient kinetics for the equine liver alcohol dehydrogenase catalyzed reduction of chromophoric aromatic aldehydes by reduced nicotinamide–adenine dinucleotide has been studied in the pH region 8–10 using rapid-mixing stopped-flow spectrophotometric instrumentation. Two kinetic processes, well separated in rate, are observed for the conversion of reactants into products under conditions of excess enzyme. The amplitude of the optical-density change accompanying the rapid initial step corresponds to the conversion of *exactly* one-half of the limiting substrate (aldehyde or reduced nicotinamide–adenine dinucleotide) to product (alcohol or oxidized nicotinamide–adenine dinucleotide). The slower second process accounts for the conversion of the remaining material into product and is found to have a first-order rate numerically similar to the steady-state turnover number. When reduced nicotinamide–adenine dinucleotide and aldehyde are present in excess of enzyme, there occurs a rapid initial process, prior to the attainment of the steady state, with an optical-density change corresponding to the conversion of substrates (aldehyde and reduced nicotinamide–adenine dinucleotide) into products in an amount equal to exactly *one-half* of the catalytically functional enzyme sites as determined by coenzyme binding titrations. These observations obtain for the three aromatic aldehydes used in this study, benzaldehyde, β-naphthaldehyde, and the chromophore 4-(2′-imidazolylazo)benzaldehyde. In addition, these observations are independent of the origin of the optical density changes (*i.e.*, aldehyde or reduced nicotinamide–adenine dinucleotide). The observation of two distinct kinetic processes of equal amplitude when $E_0 \gg$ aldehyde or reduced nicotinamide–adenine dinucleotide, and the unequal but integral numerical relationship between substrate (or coenzyme) binding sites (*two* per enzyme) and those active sites titrated by the initial rapid process (*one-half* of the enzyme–coenzyme binding sites), place drastic restrictions on possible kinetic models. Models based on nonequivalent, but interconvertible, states of the two sites, as well as models involving two thermodynamic states of the protein exhibiting different catalytic activities, are considered.

The nicotinamide–adenine dinucleotide requiring enzyme horse liver alcohol dehydrogenase (EC 1.1.1.1) catalyzes the interconversion of aldehydes and alcohols (eq 1) with equilibrium thermodynamically favored in the direction shown for most substrates at accessible pH (Sund and Theorell, 1962).

$$RCHO + NADH + H^+ \rightleftharpoons RCH_2OH + NAD^+ \quad (1)$$

Good evidence exists for two coenzyme binding sites per molecule of enzyme of molecular weight 84,000 (Ehrenberg and Dalziel, 1958; Theorell and Winer, 1959; Theorell and Bonnichsen, 1961; Theorell and Yonetani, 1963; Geraci and Gibson, 1967). The enzyme exhibits a rather broad specificity for aldehydes of widely varying structure; however, specificity for coenzyme analogs is somewhat more restrictive (Sund and Theorell, 1962). The kinetic sequence for the binding of coenzyme and substrate prior to reaction has been demonstrated to be ordered with the compulsory binding of coenzyme as the initial step under steady-state conditions (Theorell and Chance, 1951). Wratten and Cleland (1963, 1965) have demonstrated the presence of ternary complexes among enzyme–coenzyme–substrate under steady-state conditions, and they have concluded that for certain aromatic aldehydes the dissociation rate of the aryl alcohol product may determine the steady-state rate. The mechanism of coenzyme binding has been the subject of recent rapid kinetic studies (Geraci and Gibson, 1967; Shore, 1969). These findings together with equilibrium binding studies (Taniguchi, *et al.*, 1967; Sigman, 1967) have failed to provide evidence for the cooperative binding of either substrate or coenzyme to the two sites per molecule.

In choosing substrates for this study we have taken advan-

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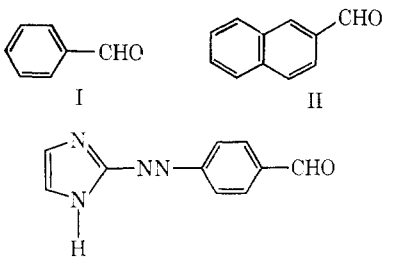
tage of the broad specificity of LADH.[1] For transient studies aromatic aldehydes have the particular advantage of a high affinity for the enzyme site. In addition, the particular aldehydes chosen have spectral properties which readily allow for the observation of either the aldehyde → alcohol or the NADH → NAD^+ transformation during catalysis. Thus, benzaldehyde (I) allows the unperturbed observation of the NADH spectral change. β-Naphthaldehyde (II) offers the advantage of a relatively large value of $\Delta\epsilon$ for reaction at 330 mμ from the overlapping of its spectrum with the spectrum of NADH. The long wavelength π,π^* transition of the azoaldehyde, 4-(2′-imidazolylazo)benzaldehyde (III), allows spectral observations at wavelengths remote from the enzyme and the NADH absorption bands.



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The use of chromophoric and fluorophoric substrate analogs in the study of the transient approach to the steady state has led to important stoichiometry relationships between the interactions of subunits, active sites, and substrates (Malhotra and Bernhard, 1967; Fernley and Walker, 1966; Trentham and Gutfreund, 1968; O'Leary and Westheimer, 1968).

In the pH region 8–10, two kinetic processes have been observed in the transient state when the signal observed is derived from either the aldehyde or NADH spectral changes accompanying reaction. The stoichiometry relationship of the moles of substrate converted to product in the rapid initial kinetic process to the normality of enzyme active sites has been investigated. The stoichiometric results are invariant over this pH range although the velocities of reaction are pH dependent. For consistency, all illustrative results presented herein are at a single pH (8.75).

Experimental Section

Enzyme Preparation. The LADH used in this study was the commercially available preparation from C. F. Boehringer and Soehne. In general, further purification to remove the ethanol already present was carried out by decanting the clear supernatant from the precipitated enzyme. The precipitate was dissolved in 0.05 M pyrophosphate buffer (pH 8.75; 3–5 ml/100 mg of enzyme) and incubated with 1,4-dithioerythritol (Cleland, 1964), 200 μl of 10^{-2} M/100 mg of enzyme, for a period of 18–20 hr. The resulting enzyme solution was then passed through a P-30 Bio-Gel column (40 × 15 mm) equilibrated with 0.05 M pyrophosphate buffer (pH 8.75). The main enzyme component eluted with the solvent front, and was followed by a lower molecular weight component (λ_{max} 280 mμ) possessing no LADH activity. This material appeared to be present in greater amounts in the decanted supernatant. The above procedure resulted in a preparation having a low ethanol content according to the fluorometric assay of Taniguchi (1967). However, incubation of the enzyme with 100 μM NAD^+ for long periods of time (2–3 hr) resulted in the formation of NADH in amounts comparable with the enzyme active site concentration. This slow formation of NADH persisted after dialysis against 0.05 M sodium pyrophosphate buffer (pH 7.3) (four–five changes of 1.5 l. over a 10-hr period) prior to the P-30 Bio-Gel treatment, before or after 1,4-dithioerythritol incubation. The omission of 1,4-dithioerythritol from the procedure gave enzyme preparations having a substantially lower activity and aging stability. However, the transient-state behavior was unaffected.

Activity and Site Stoichiometry Measurement. The activity of our enzyme preparations, as measured by the Dalziel procedure (Dalziel, 1957), ranged between 90 and 130%. Active site concentrations were routinely determined by the spectrophotometric titration of LADH coenzyme binding sites with NAD^+ in the presence of excess pyrazole (Theorell and Yonetani, 1963). These values are expressed throughout this paper as N, the normality of the enzyme-coenzyme binding capacity. The coenzyme binding capacity of our preparations ranged from 85 to 95% based on a molar extinction, $\epsilon_{280} = 3.53 \times 10^4\ M^{-1}\ cm^{-1}$ (Dalziel, 1957). The normality, N, and per cent binding activity, % *A*, were calculated in the following way.

$$N = \frac{\Delta OD_{300}}{\Delta\epsilon_{300}};\ \Delta\epsilon_{300} = 7.2 \times 10^3\ N^{-1}\ cm^{-1}$$

$$\%\ A = \frac{N(100)}{2(OD_{280})/(\epsilon_{280})}$$

A linear correlation between the Dalziel assay and the pyrazole-NAD^+ titration was found (0.75 × % Dalziel ≃ % *A* coenzyme binding capacity), and the same site normalities were obtained from either the pyrazole-NAD^+ titration or the isobutyramide-NADH titration of Theorell and McKinley-McKee (1961). The purification procedure gave enzyme preparations having a 280:260 optical density ratio of 1.40–1.44.

Substrates. Reagent grade benzaldehyde was freshly distilled before use. β-Naphthaldehyde (Aldrich Chemical Co.) was purified by sublimation [25° (0.05 mm)] and stored in amber bottles.

4-(2′-Imidazolylazo)benzaldehyde was prepared by the diazotization of *p*-aminobenzaldehyde with HCl and $NaNO_2$ in the usual fashion after incubating *p*-aminobenzaldehyde polymer in 1.5 N HCl for about 1 hr. The resulting 4-benzenediazonium carboxaldehyde ion was coupled *in situ* with a slight molar excess of imidazole by carefully adjusting the pH to neutrality. On completion of reaction, acidification with HCl gave the product as a finely divided reddish-yellow precipitate. Recrystallization from acetonitrile gave red needles, dec >200°.

Anal. Calcd for $C_{10}H_8N_4$: C, 60.00; H, 4.03; N, 28.02. Found: C, 60.33; H, 4.39; N, 27.69.

[1] Abbreviations used are: LADH, horse liver alcohol dehydrogenase; rabbit GPD, rabbit muscle glyceraldehyde phosphate dehydrogenase; MDH, pig heart malic dehydrogenase.

The alcohols β-hydroxymethylnaphthalene and 4-(2′-imidazolylazo)benzyl alcohol were prepared for spectral measurements by the reduction of the corresponding aldehydes with sodium borohydride in (pH 8.75) pyrophosphate buffer.

Coenzymes. The highest grade nicotinamide–adenine dinucleotide preparations were purchased from C. F. Boehringer and Soehne and used without further purification.

Kinetic Measurements. Experiments concerning the effect of aldehyde concentration on the transient approach to the steady state were carried out by maintaining enzyme and NADH concentrations constant, and varying the substrate concentration. The range of conditions employed was subject to limitations imposed by substrate solubilities and total optical densities. The enzyme concentrations used were 5 to 20 μN. Aldehyde concentrations were varied from 2 to 50 μM for the azoaldehyde, and from 2 to 500 μM for benzaldehyde and β-naphthaldehyde. The NADH concentrations used were 70 to 200 μM.

Experiments on the effect of NADH concentration in the transient state were carried out at constant enzyme and aldehyde concentrations, and variable NADH concentrations. The NADH concentration was varied from 3 to 100 μM, with enzyme concentrations of 5 to 20 μN, and aldehyde saturating with respect to the steady-state dissociation parameter, K_m[8].

In a typical experiment, pyrophosphate buffered enzyme stock solutions were prepared and stored at 4° until just prior to a run. The order of mixing in the stopped flow, *i.e.*, enzyme–NADH solutions mixed with aldehyde solutions, enzyme–aldehyde solutions mixed with NADH solutions,[2] or enzyme solutions mixed with aldehyde–NADH solutions, was found to make no difference, as all combinations gave identical experimental observations. Enzyme assays (pyrazole–NAD^+ titration) were made prior to each experiment. Initial and final optical densities were measured independently for each set of concentrations with the Cary Model 16.

In those experiments involving β-naphthaldehyde and benzaldehyde, the isosbestic point, 330 mμ, for free and enzyme-bound NADH was chosen as the wavelength of observation. The Δε values used were respectively 7.48×10^3 M^{-1} cm^{-1} (β-naphthaldehyde plus NADH changes) and 5.85×10^3 M^{-1} cm^{-1} (NADH changes). The maximum in the difference spectrum for the conversion of the azoaldehyde to the corresponding alcohol occurs at 395 mμ. However, to avoid any contributions from NADH, observations were made at the wavelength 410 mμ ($\Delta\epsilon = 7.00 \times 10^3$ M^{-1} cm^{-1}). Since the azoaldehyde and the corresponding alcohol have an appreciable overlapping of spectra at this wavelength, the Δε value for aldehyde reduction by NADH was determined by careful titration with NADH in the presence of catalytic amounts of enzyme to provide a consistent set of extinction coefficients based on the NADH coefficient, $\epsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1} (Horecker and Kornberg, 1948).

All steady-state kinetic runs were carried out on either a Beckman DB or on a Cary Model 14 spectrophotometer, each equipped with thermostated cell compartments at 25 ± 0.5°. Small optical density measurements were made on a Beckman DB equipped with a Sargent recorder (Model SRL) modified

[2] Provided that the concentrations of E and NADH are high enough to prevent the binding step from becoming the rate-determining step for the rapid initial process.

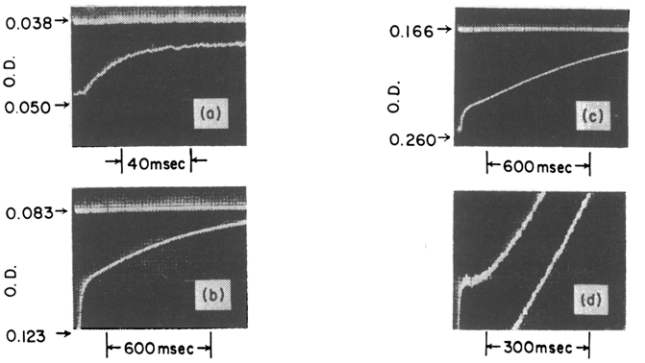

FIGURE 1: Oscilloscope traces of the time course of the change in transmission (*mv*) at 410 mμ as a function of the azoaldehyde concentration for the LADH-catalyzed reduction of aldehyde by NADH at 25 ± 1° in 0.05 M pyrophosphate buffer, pH 8.75. The different azoaldehyde concentrations employed were: trace a, 1.72×10^{-6} M; trace b 5.72×10^{-6} M; trace c, 1.34×10^{-5} M; and trace d, 4.02×10^{-5} M. $[NADH]_0$ 1.10×10^{-4} M and E_0 8.75×10^{-6} N were employed throughout. The initial (t_0) and final (t_∞) optical densities, calculated from the corresponding transmission values (*mv*), are given for traces a–c. The time scales (msec) are indicated for each trace. In traces a–c, the upper line corresponds to completion of reaction (t_∞). The initial optical density value (t_0) is corrected for the "instrument dead-time" (~3 msec; see Experimental Section). The first trace in d is illustrative of the lag process observed under conditions of NADH and azoaldehyde in large excess of E_0 during the transient approach to the steady-state rate. The second trace in d is the rate of change in transmission after the attainment of the steady-state rate.

to allow the expansion of 0.02 to 0.10 optical density changes to full scale.

Stopped-Flow Instrumentation. All rapid kinetic experiments reported in this paper were carried out with a stopped-flow rapid-mixing apparatus designed and constructed in the laboratory of H. Gutfreund. This apparatus which has been described in detail (Gutfreund, 1967, 1968) has an eight-jet mixer filling a 1 × 0.2-cm observation chamber, and is designed for a flow rate of about 5 m/sec. In our assembly, a 150-W tungsten Atlas projector lamp, a Schoeffel monochromator (Model F-11-10), and a EMI 9529B photomultiplier tube were used. The time course of transmittance change was recorded with a Tektronix storage oscilloscope (Model 564) equipped with 2A63 and 3B3 plug-in time base units. Permanent traces were recorded with a Polaroid camera. The instrument "dead-time" was found to be approximately 3 msec as measured by the reaction of ascorbic acid with 2,6-dichloroindophenol (Gutfreund, 1968). Measurements were carried out at 25 ± 1° in a thermostated room.

Data Treatment. The optical density changes for the initial transient process (see Figures 1 and 2) were obtained in the following way. The time zero (t_0) transmittance value (*mv*) for each stopped-flow trace was estimated by extrapolation back along the trace to a point corresponding to ~3 msec before the flow stopped (the mixing dead time). The amplitude of the initial step was then measured as the difference in transmission (Δ*mv*) between the t_0 point and the t_∞ point. The $t_\infty mv$ value is defined as the point at which deviation is observed from a fit of the second step to a single exponential expression when two steps are observed (Figures 1a,b and 2a,b), or as the point which gives the maximum Δ*mv* value prior to the lag process and the attainment of the

TABLE I: Steady-State Parameters Measured at 25 ± 0.1° in Pyrophosphate Buffer, pH 8.75.

Substrate	K_{eq}[a] × 10^{12} (M)	V[b] $\left(\frac{[\text{Aldehyde}]}{N \text{ sec}}\right)$	K_m^S[c] × 10^6 M	K_m^{NADH}[d] × 10^6 M
Benzaldehyde	~40	4.0 ± 0.3	20 ± 7	2.1 ± 0.1
Azoaldehyde	~2	2.0 ± 0.2	4 ± 0.3	~2
2-Naphthaldehyde	~10	0.4 ± 0.2	1.5 ± 0.3	~2
Acetaldehyde	9.72[e]	10 ± 1	210[e]	2.1[f]

[a] Defined as $K_{eq} = \frac{[NADH][RCHO][H^+]}{[NAD^+][RCH_2OH]}$. [b] The steady-state turnover number calculated on the basis of the enzyme normality (pyrazole assay see Experimental Section). [c] The Michaelis–Menten constant for substrate measured in the presence of a large (saturating) excess of NADH (~1×10^{-4} M). [d] The Michaelis–Menten constant for NADH measured in the presence of saturating amounts of substrate (~1×10^{-4} M), except for benzaldehyde (~5×10^{-4} M) and acetaldehyde (~5×10^{-3} M). [e] Values taken from Sund and Theorell (1962). [f] Values taken from Wratten and Cleland (1965).

steady state (Figures 1c,d and 2c,d). The large differences between the apparent rate of the initial process and subsequent processes generally allowed these amplitudes to be measured directly on the stopped-flow traces. The actual optical density changes were calculated by the relationship Δoptical density = log $(mv)_{t_\infty}/(mv)_{t_0}$. Total optical density changes for completion of reaction calculated in this way were in good agreement with direct measurements on the Cary 16. The instrument RC time constant was 3.3×10^{-4} sec throughout.

Results

Figure 3 compares the spectrum of NADH with the spectra of each aromatic aldehyde and the corresponding alcohol (prepared either *via* the enzyme–NADH catalyzed reduction or *via* $NaBH_4$ reduction). These spectra are pH independent over the range 6–10.

Table I summarizes the steady-state and equilibrium parameters for the substrates employed in this study, measured under conditions of saturating NADH or aldehyde.

The experimental approach to the study of the initial transients during the establishment of the steady-state rate

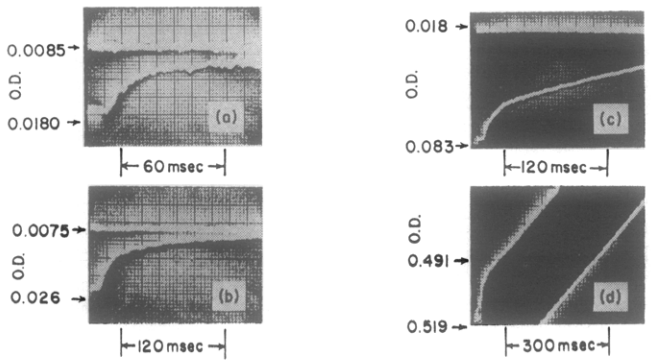

FIGURE 2: Oscilloscope traces of the time course of the change in transmission (mv) at 330 mμ as a function of $[NADH]_0$ for the LADH-catalyzed reduction of benzaldehyde at 25 ± 1° in 0.05 M pyrophosphate buffer, pH 8.75. The different NADH concentrations employed were: trace a, 1.62×10^{-6} M; trace b, 3.14×10^{-6} M; trace c, 1.11×10^{-5} M; and trace d, 9.90×10^{-5} M. Benzaldehyde 1.0×10^{-4} M and E_0 9.15×10^{-6} N were employed throughout. The initial (t_0) and final (t_∞) optical densities, calculated from the corresponding transmission values (mv), are given for traces a–c as in Figure 1. The first trace in d is illustrative of the lag process (only just discernible) observed under conditions of NADH and benzaldehyde in large excess of E_0 during the transient approach to the steady-state rate. The initial (t_0) and final (t_∞) optical density values corresponding to completion of the rapid initial step are given. The second trace in d is the rate of change in transmission after the attainment of the steady-state rate.

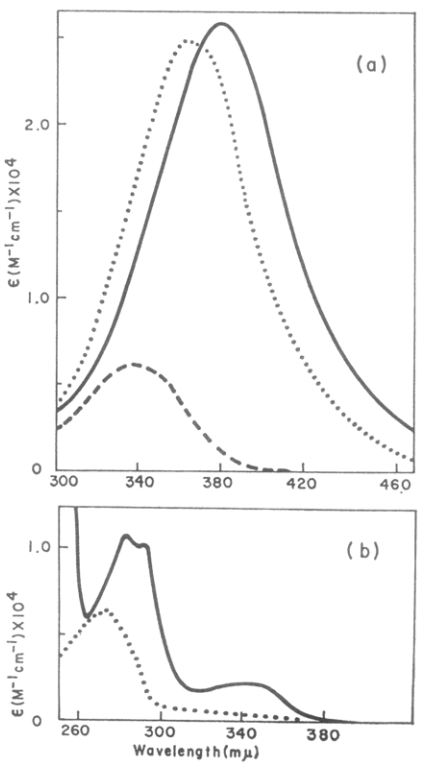

FIGURE 3: (a) The comparison of the spectrum of the azoaldehyde III (——) with the spectrum of the corresponding alcohol (. . . .) and with the spectrum of NADH (– – – –). (b) The comparison of the spectrum of β-naphthaldehyde (II) (——) with the spectrum of the corresponding alcohol (. . . .). These spectra were measured in 0.05 M pyrophosphate buffer, pH 8.75, at 25 ± 0.1°.

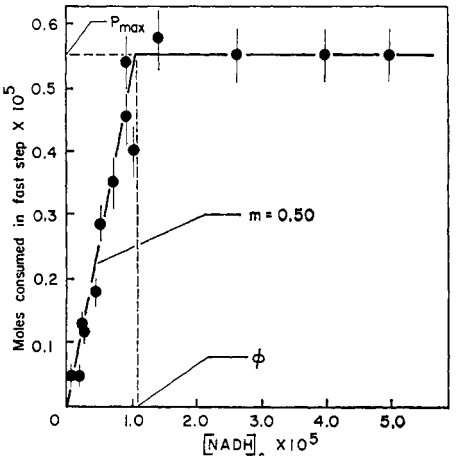

FIGURE 4: The variation in the moles of reactant consumed in the rapid initial step as a function of the concentration of $[NADH]_0$ for the system equine LADH–benzaldehyde–NADH. Observations were made with a rapid-mixing stopped-flow spectrophotometer at 330 mμ. Conditions: E_0, 1.16×10^{-5} N; benzaldehyde, 4.45×10^{-5} M; NADH varied from 9.5×10^{-7} M to 5.0×10^{-5} M in 0.05 M (pH 8.75) pyrophosphate buffer and 25 ± 1°. The initial solid line is theoretical for a slope (m) of 0.05. The ordinate and abscissa intercepts of this line with the horizontal end-point line, P_{max} and ϕ, respectively, are indicated (see text).

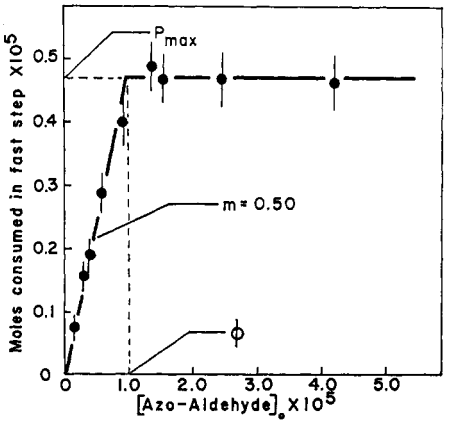

FIGURE 5: The variation in the moles of reactant consumed in the rapid initial step as a function of the concentration of the azo-aldehyde for the system equine LADH–azoaldehyde–[NADH]. Observations were made with a rapid-mixing stopped-flow spectrophotometer at 410 mμ. Conditions: E_0, 8.75×10^{-6} N; $NADH_0$, 1.10×10^{-4} M; azoaldehyde varied from 1.72×10^{-6} M to 4.20×10^{-5} M in 0.5 M pyrophosphate buffer, pH 8.75, and 25 ± 1°. The slope of the initial solid line and the intercepts, P_{max} and ϕ, are as in Figure 4.

was to vary the concentration of one substrate (either the aldehyde or NADH) from conditions of excess enyzme to conditions of excess substrate, while maintaining the concentrations of the other two components (enzyme and the other substrate) constant.

The traces in Figures 1 and 2, taken directly from the storage oscilloscope, are illustrative of typical data obtained from the stopped-flow apparatus for these aldehydes.

The conversion of reactants into products occurs *via* distinct kinetic processes when the enzyme concentration exceeds that of either NADH or aldehyde (Figures 1a and 2a). The rapid initial optical density changes have the form of a single exponential process that is well separated in rate from the second process (by at least an order of magnitude), and can be observed above pH 8 for benzaldehyde, the azo-aldehyde, and β-naphthaldehyde. The slower second transient process also is single exponential in form. *The first-order rate of this process is numerically similar to the steady-state turnover number.* The mechanistic relationship of these two transient processes to the overall catalytic sequence is the subject of a subsequent paper.

The qualitative results of the transient experiments described in this paper are as follows. (1) Two distinct kinetic processes occur under the conditions of E_0 (the total concentration of enzyme sites, N, as defined by pyrazole–NAD^+ titration; see Experimental Section) in excess of the limiting substrate concentration with the second substrate present in large excess. (2) The rapid initial kinetic process persists even when both substrate concentrations are in large excess over E_0, *i.e.*, conditions for the steady state (see Figures 1d and 2d). (3) Under the conditions of aldehyde and NADH both in excess of E_0, there is a lag in the approach to the steady state occurring after the rapid initial kinetic process (see Figures 1d and 2d). (4) The amplitude of the optical density change accompanying the rapid initial step increases with increases in the concentration of the varied substrate (aldehyde or NADH) below E_0 and saturates at concentrations in excess of the enzyme normality. (5) When E_0 is greater than $[NADH]_0$ (Figure 1a,b) the rapid initial step accounts for exactly one-half of the *total* optical density change. When E_0 is greater than the aldehyde concentration (Figure 2a,b), the amplitude of the initial step approaches one-half of the total optical density change. (6) When both aldehyde and NADH are in excess of E_0, the amplitude of the optical density change accompanying the rapid initial step is directly proportional to the enzyme concentration. (7) In the pH range which has been investigated (pH 8–10), all these observations are independent of the signal source (*i.e.*, aldehyde or NADH),

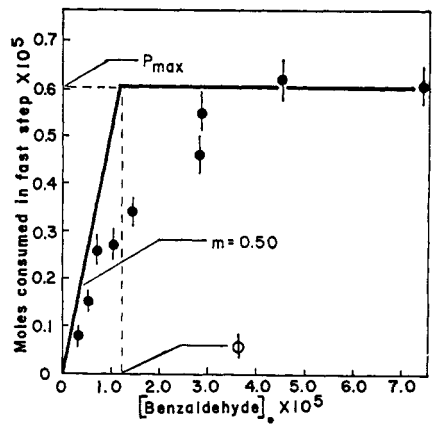

FIGURE 6: The variation in the moles of the reactant consumed in the rapid initial step as a function of the concentration of benzaldehyde for the system equine LADH–benzaldehyde–NADH. Observations were made with a rapid-mixing stopped-flow spectrophotometer at 330 mμ. Condictions: E_0, 1.10×10^{-5} N; NADH, 5×10^{-5} M; benzaldehyde varied from 3.10×10^{-6} M to 7.5×10^{-5} M in 0.05 M pyrophosphate buffer, pH 8.75, and 25 ± 1°. The slope of the solid line and the intercepts, P_{max} and ϕ, are as in Figure 4.

TABLE II: A Summary[a] of the Parameters Derived from the Titration Plots Illustrated by Figures 4–6.

Aldehyde (Wavelength Utilized in the Absorption Kinetics and $\Delta\epsilon$ in Parentheses)[b]	$N \times 10^6$[c]	P_{max}[d] $\times 10^6$ (M^{-1})	P_{max}/N[e]	Initial Slope[f]	Binding Stoichiometry[g] (ϕ/N)
	NADH as the Variable Reactant				
Benzaldehyde (330 mμ, 5.85×10^3)	11.0	5.5 ± 0.4	0.50 ± 0.08	0.52 ± 0.10	1.0 ± 0.4
β-Naphthaldehyde (330 mμ, 7.65×10^3)	7.0	4.0 ± 0.3	0.57 ± 0.08	0.60 ± 0.10	0.94 ± 0.2
Azoaldehyde (410 mμ, 7.0×10^3)	10.5	5.3 ± 0.5	0.50 ± 0.10	0.50 ± 0.10	1.0 ± 0.2
	8.75	4.6 ± 0.5	0.53 ± 0.12	0.48 ± 0.10	0.97 ± 0.2
	5.32	2.7 ± 0.3	0.51 ± 0.06	0.49 ± 0.08	1.0 ± 0.2
	Aldehyde as the Variable Reactant				
Benzaldehyde (330 mμ, 5.85×10^3)	11.0	6.10 ± 0.7	0.55 ± 0.14	< 0.5	1 ± 1.0
	48.0	24.0 ± 2	0.50 ± 0.08	0.45 ± 0.12	1.1 ± 0.4
β-Naphthaldehyde (330 mμ, 7.65×10^3)	7.70	3.70 ± 0.4	0.48 ± 0.10	0.58 ± 0.14	0.95 ± 0.2
Azoaldehyde (410 mμ, 7.0×10^3)	8.70	4.63 ± 0.4	0.53 ± 0.10	0.50 ± 0.16	1.1 ± 0.4

[a] These values were obtained at pH 8.75, 0.05 M pyrophosphate buffer at 25 ± 1°. [b] The values of the wavelength (λ) and the extinction coefficient ($\Delta\epsilon$) used for the amplitude measurements in the stopped-flow apparatus. [c] The normality of coenzyme binding sites determined by the pyrazole–NAD^+ method of titration to a precision of ±10%. [d] These values are expressed as moles/l. calculated from the relationship Δ optical density/$\Delta\epsilon$, where Δ optical density is the amplitude of the initial step, and $\Delta\epsilon$ is the appropriate extinction coefficient. [e] The ratio of the maximum number of moles consumed in the initial step to the enzyme normality, P_{max}/N. [f] The initial slope, m, expressed as moles/l. consumed in the rapid step per mole of the variable reactant. [g] The ratio of the abscissa intercept, ϕ, to the enzyme normality, N.

and independent of the order in which the three components were mixed (see Experimental Section).

Figures 4, 5, and 6 are representative plots of the data for the variation in the amplitude of the optical density change accompanying the initial rapid step (expressed as moles of reactant consumed in the initial step) *vs.* the concentration of the varied reactant. Figure 4 is typical of the data obtained for all three aldehydes when NADH is the concentration variable. Figure 5 is typical of the data obtained when β-naphthaldehyde or the azoaldehyde is varied. The high affinities of NADH and the azoaldehyde for the enzyme binding sites are reflected in the nearly linear increase in the rapid ("burst") yield of product in the initial step with increasing NADH and aldehyde concentration as is illustrated in Figures 4 and 5. These plots are analogous to stoichiometric titration curves in which the ordinate intercept at the titration end point (P_{max}) is a measure of the stoichiometry relationship between the moles of reactant consumed in the initial step (when all sites are saturated in all ligands) and the enzyme normality. The abscissa intercept (ϕ) gives the stoichiometry relationship between the number of equivalents of NADH (or aldehyde) bound and the number of equivalents of enzyme–NADH (or enzyme–aldehyde) binding sites. The experimental values for these stoichiometry relationships are given in Table II for each aldehyde–NADH system. *Of particular note in Table II are the initial slope value (m) of ~0.5 for the number of equivalents of reactant consumed per ligand-saturated active site (P_{max}/N) and the value of unity for ϕ/N.*

In Figure 6, where benzaldehyde is the varied reactant, the variation in the moles of reactant consumed in the rapid step is seen to increase as a hyperbolic function with increasing aldehyde concentration, with an apparent hyperbolic constant of 1×10^{-5} M. *Again, extrapolation of the moles consumed in the initial step to infinite benzaldehyde concentration gives a value (P_{max}) equal to one-half the enzyme normality (N).* The best fit value of these data to a hyperbola indicates that the stoichiometry relationship (ϕ/N) between aldehyde and the enzyme–aldehyde binding sites is 1:1 (see Table II). Data obtained under conditions of a fivefold increase in the enzyme concentration (~5×10^{-5} M; Table II) gives a plot with an initial slope (m) of ~0.5 mole of reactant consumed in the burst per mole of benzaldehyde (the varied reactant).

Discussion

The observation that conversion of aldehyde and NADH into products takes place *via two* kinetically distinct processes of *equal* (concentration change) amplitude when $E_0 \gg$ [aldehyde] or [NADH] (Figures 1 and 2) places drastic constraints on the possible kinetic models. Furthermore, under conditions of excess substrate and coenzyme, the amplitude of the rapid process corresponds to *one-half* the number of active sites, and is insensitive to further increases in concentration.

We have been unable to envisage a *reasonable* mechanism that accommodates these transient data without invoking a nonequivalence among the enzyme–NADH–aldehyde sites. Any explanation based on active and inactive states of the enzyme, for example, the allosteric models of Monod *et al.* (1965), the induced fit models of Haber and Koshland (1967), or the occurrence of isozymes, must explain why only (and precisely) one-half of the substrate or coenzyme molecules react rapidly when the enzyme is in large excess.

In order for any proposed model to be consistent with the

data presented herein, it must be postulated that the dissociation rates of ligands are slow relative to the time required for chemical transformation. In the presence of a large excess of enzyme, these dissociation rates must be slow since two distinct time constants can be observed. Otherwise, rapid reequilibration of active enzyme with the unreacted NADH and aldehyde would provide a pathway for the total conversion of the limiting reactant at the (fast) rate of the initial burst. The demand for slow dissociation rates seems reasonable for β-naphthaldehyde and the azoaldehyde in view of the relatively small values of K_m (see Table I). Previously reported data on coenzyme dissociation rates (Geraci and Gibson, 1967; Shore, 1969) together with conclusions drawn from steady-state studies (Theorell and Chance, 1951; Dalziel, 1963; Wratten and Cleland, 1963) indicate that coenzyme dissociation is a slow process. In this regard it is interesting to note that the *maximal* velocities for the fast redox reaction reported here (in particular, Figure 2a–c) are nearly as rapid as the estimated (bimolecular) rate of NADH binding. It should be noted however that at high NADH concentration the redox rate saturates and that for a given aldehyde substrate the same saturating velocities obtain with either aldehyde or coenzyme concentration limiting. In any case, were the fast process limited by the rate of coenzyme (or perhaps substrate) association, this would not help to explain the "burst" stoichiometry of one-half in the fast redox reaction.

A number of mechanisms are capable of satisfying these data.

One mechanism provides for the stoichiometric limitation by invoking catalytically nonequivalent (but interconvertible) states of the two subunits (sites) making up each enzyme molecule (mol wt 84,000). The applicability of this asymmetric protomer mechanism to LADH will no doubt be decided by the three-dimensional X-ray structure presently in progress (Zeppezauer, *et al.*, 1967). Preliminary information (Branden, 1969) indicates that such asymmetry may be induced by coenzyme binding.

The stoichiometric limitation of one-half on the number of catalytically functional sites is also satisfied by invoking two thermodynamic states of the protein, differing in catalytic activity. This mechanism seems to us to involve too fortuitous a happenstance, since the equilibrium constant for the state transition must be very close to unity and must be unperturbable by either of the bound ligands (aldehyde or coenzyme). For the reasons cited above, the rate of the state transition must be slow relative to the slowest observed transient.

Alternatively, in order to allow for a distinct two-step transient sequence of transformation in which substrates are converted to products in each step (under conditions of excessive enzyme concentration), a model involving a high degree of cooperativity between the binding of NADH and of aldehyde to the two sites per molecule must be postulated. In this subunit model, cooperativity in ligand binding must be such as to overwhelmingly favor the species E(NADH,-aldehyde)$_2$. Two steps will then be observed if the species E-(NADH, aldehyde)$_2$ reacts *via* the rapid step to give the species E(NADH,aldehyde)(NAD$^+$,alcohol) which can then only slowly be converted into final products. The slow process for this conversion may be either the rate-limiting dissociation of any of the components, or the slow reaction of E(NADH,-aldehyde)(NAD$^+$,alcohol) to give E(NAD$^+$,alcohol)$_2$. This subunit model has the advantage of providing a structural relationship between the stoichiometric limitation of one-half and the number of functioning sites (one out of two per enzyme molecule).

A seeming paradox in any model for LADH based on cooperative subunit interactions is the conspicuous lack of evidence for nonMichaelian behavior in steady-state ligand binding studies (Theorell and Winer, 1959; Theorell and Bonnichsen; 1951, Geraci and Gibson, 1967; Shore, 1969). One of the most frequently encountered characteristics of cooperative subunit interaction is the sigmoidal shape of the ligand saturation curve at low ligand concentrations. However, lack of sigmoidal character in the initial rise of a binding curve is not a sufficient criterion for ruling out cooperative phenomena (Frieden, 1964, 1967; Monod *et al.*, 1965; Bethell *et al.*, 1968).

The transient phenomena in this paper are not unique to LADH. Studies on rabbit muscle glyceraldehyde phosphate dehydrogenase (Malhotra and Bernhard, 1967; MacQuarrie and Bernhard, 1969), *Escherichia coli* alkaline phosphatase (Fernley and Walker, 1966; Trentham and Gutfreund, 1968), acetoacetate decarboxylase (O'Leary and Westheimer, 1968) demonstrate in each case that only one-half of the sites are active toward substrates in the transient state. Harada and Wolfe (1968) have recently proposed a "reciprocating subunit" mechanism for pig heart malic dehydrogenase which supposes that the two identical subunits alternate in the role of catalyst and negative effector, with reaction taking place at one site only when the second site is unoccupied. Product dissociation in this mechanism takes place *via* subunit interactions mediated by substrate and coenzyme binding at the second site. According to their mechanism, the transient behavior for MDH is expected to be similar to that of rabbit GPD, alkaline phosphatase, and LADH.

Perhaps these integral but unequal stoichiometric relationships between "binding sites" and "functioning sites" will prove to be a general phenomenon of multisited (subunit) enzymes. If the phenomenon is general, then the understanding of its biological relevance, and the teleology for the evolution of organisms with enzymes exhibiting these properties, are of considerable interest.

Conclusions

These transient-state studies on the catalytic action of equine LADH demonstrate the following. (1) The conversion of reactants to products occurs *via* two kinetic processes of equal amplitude when $E_0 \gg$ [aldehyde] or [NADH]. Under these conditions, precisely one-half of the limiting substrate (or coenzyme) is transformed to product during the course of the initial transient. (2) When both of the presumed sites per enzyme molecule of mol wt 84,000 (as titrated by coenzyme binding) are saturated ($S_0 > E_0 < [\text{NADH}]_0$), the observed rapid presteady-state rate of reaction accounts for the conversion of aldehyde and coenzyme to products in amounts equal to one-half of the limiting number of enzyme sites. (3) The second transient process is single exponential in form, and has a first-order rate numerically similar to the steady-state turnover number.

In order that these transient data be accommodated within a framework of *reasonable* mechanistic considerations, we conclude that a nonequivalence among the enzyme–NADH–

aldehyde sites must be invoked so as to allow exactly one-half of the reaction to proceed *via* the rapid initial step, while the remainder is obligated to proceed *via* the slower second process.

Possible kinetic models that allow the accommodation of these severe restrictions include an asymmetric protomer model, a model based on two thermodynamic states of the protein, and an interacting subunit model involving a high degree of cooperativity in ligand binding.

Since these observed but unequal integral stoichiometric relationships between "binding sites" and "functioning sites" are not unique to LADH (*viz.* rabbit GPD, alkaline phosphatase, acetoacetate decarboxylase, and pyridoxamine pyruvate transaminase) we suggest that perhaps this phenomenon will prove to be general for multisited (subunit) enzymes.

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