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Catalytic Steps during the Single-Turnover Reduction of Aldehydes by Alcohol Dehydrogenase†

James T. McFarland‡ and Sidney A. Bernhard*

ABSTRACT: The reaction of pyrazole with liver alcohol dehydrogenase·nicotinamide–adenine dinucleotide complex has been studied kinetically. The pseudo-first-order rate constant for reaction in 0.02 M pyrazole at 25° and pH 8.75 is 105 sec^{-1}, a specific rate far in excess of that for the slowest step in the transient reaction of aromatic aldehydes with the enzyme–reduced coenzyme (NADH) complex at this temperature and pH. The reverse dissociation of ternary complex to E·NAD^+ complex has a first-order specific rate too slow to be of significance to the transient course of conversion of free aldehyde to free alcohol. Pyrazole does not inhibit any reaction or interaction involving NADH or aldehyde. Therefore it can be utilized as a rapid and specific poison of enzyme sites once NAD^+ has been produced by reduction of aldehyde. This "catalytic-site suicide" can be utilized to follow a single transient reduction of aldehyde substrate in an amount equivalent to the total number of *sites*, under conditions of excessive substrate and coenzyme. The biphasic rate behavior for the formation of products without any detectable intermediates noted previously under conditions of excessive enzyme sites (Bernhard, S. A., Dunn, M. F., Luisi, P.-L., and Shack, P. (1970), *Biochemistry 9*, 185) persists under these newly accessible conditions. Particularly at high-substrate concentrations, the fractional reaction associated with each of the two steps are not necessarily equal, although the biphasic character is always apparent by casual inspection of the kinetic profiles for reduction of aromatic aldehydes. The faster of the two steps is 2.5-fold slower for the transfer of deuterium from NAD-D to substrate, independent of the nature or concentration of substrate. The slow step shows no deuterium isotope effect. These results substantiate previous conclusions that the rate of the slow transient (but not the fast) is regulated by desorption rather than chemical transformation. In correspondence, no fast rate of formation of the ternary complex, E·NAD^+·pyrazole, is observable. The pseudo-first-order rate constant for this complex formation under aromatic aldehyde reaction conditions is the same as that for the slow step of substrate disappearance. This result suggests that the rate of the slow transient, and the turnover number, is limited by alcohol desorption from the ternary product complex. The present results strongly affirm previous hypotheses (Bernhard, S. A., Dunn, M. F., Luisi, P.-L., and Schack, P. (1970), *Biochemistry 9*, 185; Harada, K., and Wolfe, R. G. (1968), *J. Biol. Chem. 243*, 4123, 4131) that the state of liganding at one subunit in oligomeric dehydrogenases regulates the activity at the other(s).

Horse liver alcohol dehydrogenase (EC 1.1.1.1) catalyzes the reduction of aldehyde by NADH (eq 1). The enzyme is a dimer (mol wt 84,000) with two apparently independent coenzyme binding sites (Ehrenberg and Dalziel, 1958; Theorell and Bonnischen, 1951; Theorell and Winer, 1959). The amino acid sequence of the ethanol-active isozyme indicates that the two polypeptide chains making up the dimer are identical (Jörnvall, 1970). The steady-state mechanism of ethanol–acetaldehyde catalysis is apparently compulsory ordered (eq 2) with coenzyme dissociation rate limiting (Theorell and Chance, 1951; Wratten and Cleland, 1963).

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

$$E + NAD^+ \rightleftharpoons E(NAD^+) \rightleftharpoons E(NAD^+)(C_2H_5OH) \rightleftharpoons E(NADH)(CH_3CHO) \rightleftharpoons E(NADH) \quad (2)$$

† From the Institute of Molecular Biology and the Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received November 3, 1971.* This work was supported by research grants from the National Science Foundation (GB 6173X) and the Public Health Service (2 R01 GM 10451-08).

‡ Present address: Department of Chemistry, University of Wisconsin, Milwaukee, Wis. 53201. Recipient of Public Health Service Postdoctoral Fellowship No. 5 F02 GM 41050-02.

* To whom correspondence should be addressed.

Preliminary X-ray investigation of horse liver alcohol dehydrogenase crystals indicates that native coenzyme-free dimer contains a crystallographic twofold symmetry axis (Bränden *et al.*, 1969). This symmetry is destroyed on binding coenzyme (Zeppezauer *et al.*, 1967; Bränden, 1965). Recent transient studies of the reduction of aromatic aldehydes at pH 8.75 show that the rate of reduction of substrate or concommitant oxidation of coenzyme is biphasic, *i.e.*, the formation of 1 equiv of alcohol occurs *via* the rapid formation of 0.5 equiv followed by a slower formation of a second 0.5 equiv under conditions of an excess of enzyme sites over substrate. This observation has been interpreted to be the result of substrate-effected subunit interactions in the dimer (Bernhard *et al.*, 1970). Although the restriction of half of the chemical transformation occurring at a slower rate than the other half is strongly suggestive of cooperative and ordered function at the two sites per dimeric enzyme, alternative trivial kinetic models might lead to the same biphasic rate observations. For example, an independent-site model in which there is partial "transient burst" of product due to an initial preequilibrium distribution of reactive and unreactive enzyme–substrate complexes, followed by a steady-state "turnover" mechanism until substrate is exhausted.

Preliminary experiments on a hybrid alcohol dehydrogenase dimer involving distinctly different constituent specificities indicated that each half-molecule site functioned independently of the other (Theorell, 1969). This report has been seriously disputed recently, and indeed still other hybrid alcohol dehydrogenases have been reported to have properties different from that predicted for the sum of the enzymic constituents (Lutstorf *et al.*, 1970).

We now wish to report on a method for following the transient single turnover of substrate at a horse liver alcohol dehydrogenase site under conditions where both substrate and coenzyme are in large excess over enzyme sites. The ability to carry out such an experiment (under varying excessive substrate concentrations) would permit a direct distinction between function *via* coordinated activity at two sites and any biphasic rate data which might arise from a mixture of transient burst phenomena with substrate turnover.

Our procedure for studying enzymic transients in the presence of excessive aldehyde and NADH substrates involves utilizing the reaction of pyrazole with the enzyme·NAD^+ complex (Theorell and Yonetoni, 1963). Since the rate of the bimolecular reaction of pyrazole addition to $E(NAD^+)$ can be made very rapid and the dissociation of the ternary complex is very slow (see Experimental Section), it is possible to transiently inactivate the enzyme site in the presence of NADH and pyrazole following the stoichiometric reduction and desorption of aldehyde *via* formation of the E·NAD·pyrazole ternary complex (eq 3). Thus a specific site

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

is inactivated during aldehyde reduction by NADH in the presence of a high concentration of pyrazole, only and immediately after alcohol product desorption. Details of this useful experimental procedure for the study of transients in dehydrogenase reactions, and of the specific results with horse liver alcohol dehydrogenase and aromatic aldehydes are described below.

Materials

Enzyme Preparation and Active-Site Determination. The enzyme preparation has been described previously (Bernhard *et al.*, 1970). The enzyme was supplied as a solid suspension from C. F. Boehringer and Soehne. Ethyl alcohol was removed by decanting the liquid and dissolving the solid in pH 8.75 pyrophosphate buffer (0.05 M). This solution was then incubated for 18–20 hr with 1,4-dithiothreitol (200 μg of 0.1 M aqueous reagent/100 mg of enzyme) (Cleland, 1964). The enzyme preparation was then chromatographed on a Bio-Gel P-30 column and assayed by specific titration with NAD^+·pyrazole (Theorell and Yonetoni, 1963). The assay procedure indicated that enzyme preparations were 90–100% active horse liver alcohol dehydrogenase when protein concentration was based on an extinction coefficient at 280 nm of 3.53×10^4 M^{-1} cm^{-1} (Dalziel, 1957). The active-site concentration is listed throughout this paper as N, the normality of enzyme sites as estimated by this procedure.

Substrates. Muscle glyceraldehyde-3-phosphate dehydrogenase was a gift of Dr. R. A. MacQuarrie. β-Naphthaldehyde (Aldrich Chemical Co.) was sublimed and stored in the cold. The preparation and purification of 4-(2′-imidazolylazo)-benzaldehyde (azoaldehyde) has been described previously (Bernhard *et al.*, 1970). Benzaldehyde (Mallinckrodt Chemical Co,, reagent grade) was distilled at reduced pressure under nitrogen and stored in the cold. *p*-Chloro- and *p*-methoxybenzaldehyde (Aldrich Chemical Co.) were distilled under reduced pressure and stored in the cold. Acetaldehyde (reagent grade) was used without purification. Pyrazole (Matheson Coleman & Bell) was used without further purification. Coenzymes of the highest purity available were purchased from C. F. Boehringer and Soehne and used without additional purification.

Preparation and Assay of DeuterioNADH. 4-DeuterioNADH was prepared from NAD^+ and 1,1′-dideuterioethyl alcohol (Merck of Canada) by the method of Rafter and Colowick (1957). In a typical preparation, NAD^+ (100 mg) was dissolved in Tris base (0.5 M) and incubated with 1,1′-dideuterioethyl alcohol (250 mg) in the presence of yeast alcohol dehydrogenase (2.0 mg) (Worthington Biochemical Co.). The mixture was incubated until the reaction was nearly complete (approximately 30 min). One milliliter of aqueous barium acetate solution (1.0 M) was added and the barium salt of the nucleotide was precipitated. The precipitate was washed with diethyl ether to remove ethyl alcohol, and it was dried *in vacuo* over calcium chloride. The ratio of optical densities at 340–260 nm was determined for NADH (commercial), NADH (prepared as outlined above), and deuterated NADH. Some optical properties of each of these samples is described in Table I. The three samples of nucleotide were then oxidized with benzaldehyde in the presence of horse liver alcohol dehydrogenase and by 1,3-diphosphoglycerate in the presence of glyceraldehyde-3-phosphate dehydrogenase. In both cases all material with absorption at 340 nm was oxidized.

Commercial and an enzymatically synthesized NADH control were compared by noting the steady-state rates of reaction with benzaldehyde in the presence of horse liver alcohol dehydrogenase. These controls (Table I) were run to investigate the possible presence of coenzyme and substrate inhibitors in the NADH. We conclude that NADH prepared enzymatically is as pure as commercially available NADH. The similarity of the kinetic data suggests that neither sample contains any appreciable enzyme inhibitors.

Kinetic Methods

Transient Experiments in the Presence of Pyrazole. Reacting solutions were mixed according to the various configurations

TABLE I: Optical and Kinetic Properties of DeuterioNADH.

Nucleotide Sample	OD_{340nm}/OD_{260nm}	Rate of LADH[d]-Catalyzed C_6H_5CHO Reduction (mM/min, μmoles of E) a	b	c
Commercial NADH	0.412	2.4	1.5	1.4
Control NADH	0.410	2.3	1.4	1.4
DeuterioNADH	0.416			

[a] $[C_6H_5CHO] = 1 \times 10^{-4}$ M, $[NADH] = 1 \times 10^{-5}$ M. [b] $[C_6H_5CHO] = 2 \times 10^{-5}$ M, $[NADH] = 1 \times 10^{-5}$ M. [c] $[C_6H_5CHO] = 2 \times 10^{-5}$ M, $[NADH] = 1 \times 10^{-4}$ M. [d] LADH, horse liver alcohol dehydrogenase.

illustrated in Figure 1. Over these configurations there was no variation in kinetic results indicating that no diffusion rate was limiting. Stopped-flow experiments were carried out as described previously. The final concentrations of reactants were in the following concentration ranges: $[E_0] \sim 5.5 \times 10^{-6}$ to 2.0×10^{-5} N; $[NADH] \sim 1.0 \times 10^{-5}$ to 4.0×10^{-4} N; [pyrazole] $\simeq$ 0.02 M. All experiments were run in 0.05 M pyrophosphate at pH 8.75, at 25.0 ± 0.5°. The stopped-flow reactions were usually run in configuration A of Figure 1.

The oxidation of NADH was observed at 330 nm, the isosbestic point for NADH bound to horse liver alcohol dehydrogenase and NADH in water. (The extinction coefficient of NADH at this wavelength is 5.8×10^3 M^{-1} cm^{-1}.) The reduction of azoaldehyde was observed at 410 nm where the difference extinction coefficient between azoaldehyde and alcohol is 7.0×10^3 M^{-1} cm^{-1} (Bernhard *et al.*, 1970). The rate of formation of enzyme·NAD^+·pyrazole complex was followed at 300 nm.

The concentrations of horse liver alcohol dehydrogenase, NADH, and aldehyde were determined prior to stopped-flow experiments on the Cary Model 16 spectrophotometer.

Transient Isotope Effects. Experiments involving deuterio-NADH were run in a manner identical with that with NADH, the reactants being mixed according to configuration A of Figure 1.

Kinetic and Stoichiometric Analysis of Transient Reactions. The stopped-flow instrumentation has been described previously (Gutfreund, 1967, 1968). This stopped-flow mixing chamber has a "dead time" of approximately 3 msec. Therefore, in experiments involving specific rates in excess of approximately 150 sec^{-1}, a substantial initial portion of reaction was not observable. The "true" optical density at zero time was obtained by extrapolation of the single exponential rate data at measurable early times back to the true zero time, as determined by the method suggested by Gutfreund (1967).

The kinetic behavior for the oxidation of coenzyme or reduction of aldehyde was biphasic (see Results section). It was, however, possible to separate the apparent biphasic behavior into two single exponential processes because of the greater than 20-fold difference in rate between the faster and slower process. All specific rates were calculated from the average of two or more determinations treated by linear regression analysis. The rates were assumed to be explicable on the basis of two sequential linear decay processes (see Results and Discussion sections).

Preincubation of Horse Liver Alcohol Dehydrogenase with NAD^+·Pyrazole. In one set of experiments, single turnover was measured as in configuration A of Figure 1, except that 0.5 equiv of NAD^+ was incubated with enzyme–pyrazole prior to mixing. In this configuration, the initial enzyme solution (before mixing) is already partially inactivated as is discussed in the Results.

Steady-State Experiment. In order to study the steady-state reaction of enzyme with substrates under conditions of high-enzyme concentration comparable to those employed in the single turnover experiments, the stopped-flow apparatus was required. Steady-state kinetic measurements are more usually determined at concentrations of enzyme approximately three to four orders of magnitude lower than typical enzyme concentrations employed herein (*ca.* 1.0×10^{-5} N). For comparison to previous results and conclusions based on conventional steady-state data, we repeated some of our steady-state experiments at more typical enzyme concentrations, *viz.*, 10^{-7}–10^{-8} N. These experiments were carried out on a Cary Model 14 spectrophotometer at 25 ± 0.5°. Both types of steady-state measurements were monitored at 330 nm for the disappearance of NADH and at 410 nm for the disappearance of azoaldehyde.

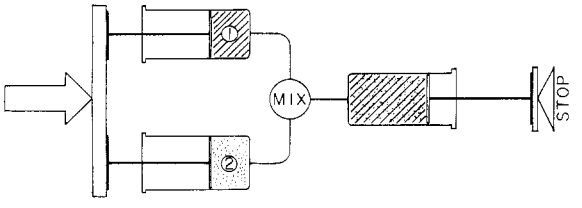

FIGURE 1: Configurations for the mixing of four reactants from two syringes in stopped-flow experiments.

	Contents of Syringe 1	Contents of Syringe 2
A	Aldehyde + pyrazole	E + NADH
B	NADH + pyrazole	E + aldehyde

Results

Single turnover experiments in the presence of pyrazole, in which addition of reagents was according to configuration A of Figure 1 are illustrated in Figure 2. For comparison, experiments performed in an excess of enzyme sites over limiting substrate are shown in Figure 3. Note in each case, the biphasic nature of the kinetic curve. Thus, the experiments performed in an excess of both substrates (but limited to a single turnover) confirm the earlier results (Bernhard *et al.*, 1970) regarding the biphasic nature of the reaction. The new "suicide" experiments for the inactivation of active sites once catalysis has taken place permits investigations over a wide range of substrate concentrations previously not accessible.

Transient Rates of Ternary Complex Formation and Dissociation. Previous estimates of the dissociation constant for the ternary enzyme·NAD^+·pyrazole complex (Theorell and Yonetani, 1963) coupled with measured diffusion rates for processes analogous to the association of NAD^+ to enzyme site (Geraci and Gibson, 1967; Shore, 1969) already suggest that the rate of dissociation of the ternary complex is exceedingly slow. In order to estimate this rate, the ternary complex was allowed to form, as described in the transient experiments with pyrazole, utilizing benzaldehyde as substrate (see Methods) and the subsequent slow reaction which occurred was monitored. The reaction was followed for a long time,

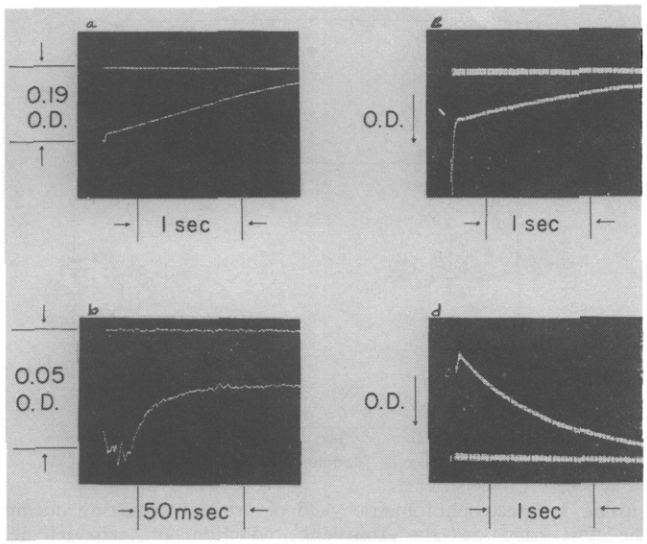

FIGURE 2: Stopped-flow oscilloscope traces of the reduction of azoaldehyde by NADH with and without added pyrazole. [Azoaldehyde] = 3.5×10^{-5} M, [NADH] = 9.5×10^{-5} M, [LADH] = 1.0×10^{-5} N. (a) At 410 nm. No Pyrazole. (b,c) At 410 nm. [Pyrazole] = 0.02 M. (d) At 300 nm. [Pyrazole] = 0.02 M.

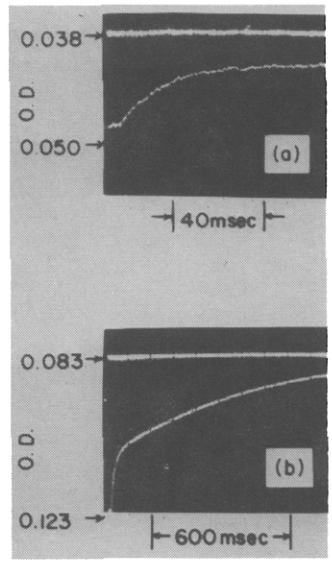

FIGURE 3: Stopped-flow oscilloscope traces of the reduction of a limiting concentration of azoaldehyde by an excess of enzyme sites at 410 nm. [NADH] = 1.10×10^{-4} M, [LADH] = 8.75×10^{-6} N. (a) [Azoaldehyde] = 1.17×10^{-6} M. (b) [Azoaldehyde] = 5.72×10^{-6} M. Data of Bernhard *et al.* (1970).

under saturating conditions for both substrates. Under these conditions the rate of reaction is totally limited by the rate at which new sites become available due to dissociation of the unreactive ternary complex. We compared this measured rate to the steady-state velocity under the same conditions but in the absence of pyrazole. The value, 1×10^{-3} sec^{-1}, for $V_{max}/(E_0)$ in the presence of pyrazole is enormously slower than the turnover number per site, 4.0 sec^{-1}, in its absence. The former rate constant is presumably the specific rate of dissociation of the ternary complex. Since the turnover number represents the *smallest* rate constant potentially measurable in the transient reaction, the formation of an enzyme·NAD$^+$·pyrazole complex is equivalent to the formation of an enzyme–irreversible inhibitor complex in such transient experiments. The ternary complex, once formed, is sufficiently stable to prevent turnover during the time course of rapid kinetic observation. Hence, the pyrazole method is suitable for the measurement of transient processes provided that the rate of formation of ternary complex from E·NAD$^+$ is sufficiently rapid relative to other transient processes. The rate of this reaction could be determined by mixing enzyme with an excess of both NAD$^+$ and pyrazole, and observing the characteristic optical density change due to formation of the ternary complex at 300 nm (see Methods section). The rate for the formation of the complex was found to be sufficiently slower than the estimated rates for diffusion of ligands to the site. The configuration of mixing (Figure 1) did not affect the kinetic results, although mixing NAD$^+$ with horse liver alcohol dehydrogenase before introduction into the stopped-flow apparatus leads to production of NADH which reduces the concentration of ternary complex, and hence the optical density change at 300 nm. This reduction of NAD$^+$ by alcohol in the purified enzyme preparations has been reported previously (Theorell and Winer, 1959). At the higher concentration of the pyrazole employed herein (0.02 M), a pseudo-first-order rate of ternary complex formation is observable. This first-order rate constant is dependent on NAD$^+$ concentration; following a linear reciprocal relationship between k^{-1} and NAD^{-1}. The intercept of a reciprocal plot (at infinite NAD$^+$ concentration) gives the desired specific rate, namely the rate of formation of ternary complex from E·NAD$^+$, since at this condition the enzyme sites are saturated with NAD$^+$. This rate constant (102 sec^{-1}) is substantially faster than the slower observable transient rate process described below. We are aware that higher concentrations of pyrazole lead to still more rapid specific rate constants. However, in the experiment described below, the transients of interest are never rate limited by ternary complex formation from E·NAD$^+$. At this concentration of pyrazole (0.02 M), the pyrazole method is suitable for measuring transient rate processes, provided that pyrazole, at the concentration employed, is not an inhibitor of substrate binding or chemical transformation at the site. Some evidence that pyrazole is inert prior to the chemical formation of E·NAD$^+$ is presented below.

These new results (Figure 2) confirm previous observations of biphasic kinetic behavior in the transient oxidation of coenzyme and in the reduction of substrate. Some experiments in an excess of enzyme sites in the absence of pyrazole, in which comparison may be made with concentration conditions in Figure 2, are illustrated in Figure 3. These earlier experiments, performed in an excess of enzyme sites, were limited by the cost, solubility and viscosity of the enzyme solution.

Mechanistic Studies on the Biphasic Rate Processes via the Deuterium Isotope Effect. As is evident from the illustrations (Figures 2 and 3), the chemical transformation of reactants to products proceeds *via* two kinetically distinct steps under a variety of conditions. The nature of the products of reaction, as evidenced by the absorption spectra, are the same in the faster and the slower processes. Both previous experiments (Bernhard *et al.*, 1970) and the present investigation suggest that the slower process is rate limited by something other than the transfer of hydrogen from coenzyme to substrate. To further investigate this hypothesis, deuterioNADH of the proper configuration for the transfer of deuterium from reduced coenzyme to aldehyde (α-deuterioNADH) was prepared, and utilized in exactly the same manner as the usual reduced coenzyme (see Materials section). The biphasic reaction was investigated at substrate and coenzyme concentra-

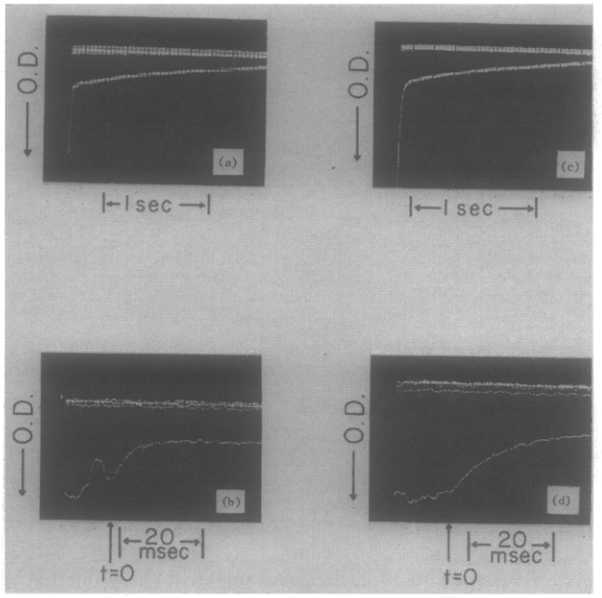

FIGURE 4: Stopped-flow oscilloscope traces of isotope effects during a single turnover reduction of β-naphthaldehyde. [β-Naphthaldehyde] = 2.3×10^{-5} M, [LADH] = 1.0×10^{-5} M, [pyrazole] = 0.02 M. (a,b) [NADH] = 9.5×10^{-5} M. (c,d) [NADD] = 9.7×10^{-5} M.

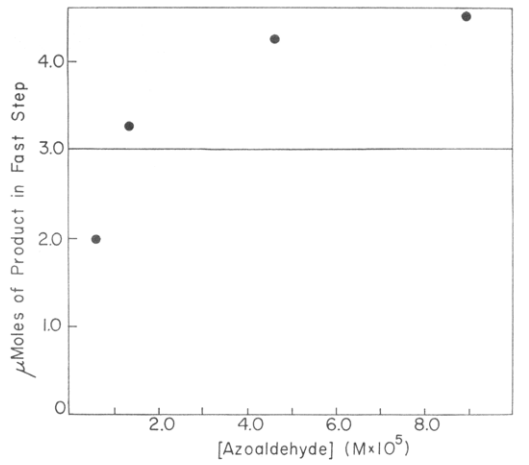

FIGURE 5: Spectrophotometric yield of product occurring during the fast step in the transient reduction of azoaldehyde. [LADH] = 6.0×10^{-6} N, [NADH] = 1.0×10^{-4} M, [pyrazole] = 0.02 M. The solid line represents chemical transformation at one-half of the limiting enzyme sites.

tions such that the effect of deuterium isotope on each of the two distinct transient processes could be determined. Typical experiments are illustrated in Figure 4 and the calculated specific rates for a variety of aldehydes are listed in Table II. Note that in every case a deuterium isotope effect of about a factor of 2.5 is observable in the fast component of the reaction profile but that no such deuterium isotope effect is observable in the slow step. Although, near saturation some of the rates are too fast to measure, it is clear that variations in concentration below this range (Table II) have equal effects on the hydrogen and the deuterium-transfer processes. These results lend further support to the conclusion that the biphasic nature of the kinetic profile is the result of two distinctly different processes, although the *chemical* transformations which occur in each step are identical.

Stoichiometry of the Biphasic Transient Processes. By making accessible a far greater range of concentration, the single turnover experiments in the presence of pyrazole permit a much wider investigation of the transient reaction conditions than was previously possible. In the presence of high concentrations and large excesses of substrates, the extent of chemical transformation which takes place by the rapid process increases and exceeds that which occurs by the slow process (Figure 5). Note that quite high concentrations of substrates are required to substantially increase the "yield" in the fast process over that in the slow. In all cases it should be noted, that the total yield (the sum of the fast and the slow step) is equivalent to at least 90% of the total enzyme normality as measured by NAD^+·pyrazole titration (Theorell and Yonetani, 1963). Thus, the apparent virtually total cooperativity noted previously for the isolation of chemical transformation into a rapid and a slow step of equal amplitude is apparent only over the much lower concentration range available when an excess of sites is required for transient observations (*i.e.*, in the absence of pyrazole).

TABLE II: Isotope Effects on k_1 at Single Turnover.[a]

Substrate	Substrate Concn	k_H[b]	k_D[b]	k_H/k_D[c]
C_6H_5CHO[a]	1.6×10^{-4}	142	56	2.5 ± 0.3
C_6H_5CHO[a]	1.1×10^{-4}	112	40	2.8 ± 0.4
p-ClC_6H_4CHO[a]	3.0×10^{-5}	148	53	2.8 ± 0.2
p-$OMeC_6H_4CHO$[a]	5.4×10^{-4}	148	45	3.3 ± 0.3
β-Naphthaldehyde[a]	2.3×10^{-5}	203	78	2.6 ± 0.2
Azoaldehyde[d,e]	4.2×10^{-5}	160	60	2.7 ± 0.3
Azoaldehyde[e]	6.1×10^{-6}	28	12	2.4 ± 0.4

[a] Horse liver alcohol dehydrogenase = 1.0×10^{-5} M; NADH = 1.0×10^{-4} M; pyrazole = 0.02 M. [b] In pseudo-first-order rate constant units of sec^{-1}. [c] Errors are calculated from ranges in rate. [d] 4-(2′-Imidazolylazo)benzaldehyde. [e] Horse liver alcohol dehydrogenase = 4.7×10^{-6} M; NADH = 8.8×10^{-5} M; pyrazole = 0.02 M.

Concentration Dependence of Transient Rate Processes. The rate of the fast step is substrate concentration dependent over the concentrations covered in our single turnover experiments with pyrazole. The dependence of the pseudo-first-order rate constant on substrate concentration is illustrated in Figure 6, utilizing azoaldehyde as substrate. This figure is typical of the results with the other substrates. The apparent hyperbolic nature of this plot suggests saturation of binding sites according to the model of eq 5. The solid line of Figure 7

$$E(NADH) + RCHO \overset{K_S}{\rightleftharpoons} E(NADH)(RCHO) \xrightarrow{k_1} \text{product} \quad (5)$$

is the theoretical plot according to this model with $K_{app} = (k_{-S} + k_1)/k_S = 5.3 \times 10^{-5}$ M, and $k_1 = 273\ sec^{-1}$.

Over the same range of substrate concentrations, the specific rate of the slower process is virtually independent of substrate concentration. The two rate constants describing the biphasic transient behavior were independent of whether coenzyme or β-naphthaldehyde disappearance was being monitored; a result consistent with that reported previously for the transient reduction of azoaldehyde (Bernhard *et al.*, 1970).

Qualitative Generalizations Regarding the Magnitudes of the Transient Rates and Stoichiometries. The following results

TABLE III: Comparisons of Transient and Steady-State Rate Parameters.

Substrate	Concn ($\times 10^5$ M)	k_{300}	k_2[a]	$V/(E_0)$[b]	$V_{max}/(E_0)$
Azoaldehyde	4.3[c]	1.0	1.1	1.2	2.0[d]
	3.5[e]	1.1	0.7	1.6	
β-Naphthaldehyde	5.4[c]	0.8	0.6	0.5	0.4[d]
	2.3[e]	0.5	0.6	0.7	
Benzaldehyde	20.0[c]	6.5	6.6	3.5	2.0[d]
	11.0[e]	5.1	3.9	3.1	
Acetaldehyde	200.0[c]	34.0	16.0	8.5	8.15[f]
	180.0[e]	46.0	32.0	9.5	

[a] Stopped-flow single turnover experiment, pyrazole = 0.02 M. [b] Steady-state measurement from stopped-flow experiment under identical conditions as for measurement of k_2, but with no added pyrazole. [c] Horse liver alcohol dehydrogenase = 1.0×10^{-5} N; NADH = 5.4×10^{-5} N. [d] Bernhard *et al.* (1970). [e] Horse liver alcohol dehydrogenase = 1.0×10^{-5} N; NADH = 1.0×10^{-4} N. [f] Theorell and McKinley-McKee (1961).

should be borne in mind regarding the magnitudes of various rate parameters. (1) Under conditions of very high substrate concentrations, such as are utilized in steady-state experiments, the slow rate constant (k_2), and *not* the fast rate constant, resembles the steady-state turnover rate. (2) The slow process still persists in the transient kinetic profile, *albeit* significantly reduced in amplitude, even at high substrate concentration. (3) The fast process persists *albeit* somewhat reduced in amplitude under conditions of very low substrate concentration. (4) The primary deuterium isotope effect is observable only in the fast step and its magnitude is independent of the extent of chemical transformation which occurs in that step.

Kinetics of E·NAD⁺·Pyrazole Complex Formation. The formation of the E·NAD⁺·pyrazole ternary complex is observable at 300 nm (Figure 2d). At this wavelength, observation of the ternary complex formation is virtually unperturbed by any transformation of reactants to product. Note, in sharp contrast to the results obtained for substrate disappearance, the monophasic kinetic behavior at 330 nm. *All* of the transformation at 300 nm occurs with the slow specific rate for substrate disappearance derivable from the biphasic transients illustrated in Figure 2. This is consistent with the assumption that the fast process involves reduction of the aldehyde but not desorption of the alcohol product. In the case of all substrates for which the kinetic processes illustrated in Figure 2 are separable into two distinctly different rates, the slower rate constant corresponds to the specific rate of ternary complex formation (Table III). Where the two rate constants are not readily separable, most notably in the case of acetaldehyde (P. L. Luisi, unpublished results), we are, of course, unable to make this comparison. However, even with this latter substrate, it is sufficiently clear that the rate of ternary complex formation is slower than the rate of the fastest component to the substrate–disappearance rate profile, but that the unimolecular (saturated) rate for complex formation exceeds the maximal turnover number for acetaldehyde (at this pH and temperature).

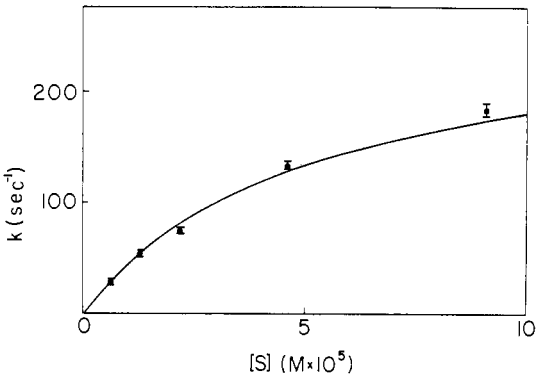

FIGURE 6: Pseudo-first-order rate of the fast transient in the reduction of azoaldehyde as a function of substrate concentration. [LADH] = 6.0×10^{-6} N, [NADH] = 1.0×10^{-4} M, [pyrazole] = 0.02 M. Solid line is the best fit to the hyperbolic equation $k = k_1[S]/([S] + K_{app})$ derivable from eq 4.

Effect of Ternary Complex Formation on the Transient Activity at Adjacent Sites. A variety of reaction mechanisms, some discussed previously (Harada and Wolfe, 1968; Bernhard *et al.*, 1970; Ladzunski *et al.*, 1971; Dunn and Bernhard, 1971), assume that reactivity at one of the two enzyme sites in the dimer is conditioned by the state of occupancy of the other site. For this reason, we attempted to fix occupancy of some sites by preincubating the enzyme with 0.5 equiv of NAD⁺ in the presence of an excess of pyrazole. Under these conditions, virtually all the added NAD⁺ is bound to the enzyme in the form of the ternary complex. We then tested this (partially) inactivated enzyme for transient reactivity. Results are shown in Table IV. As is quite obvious from the table, the *number* of available sites has been reduced (actually stoichiometrically according to the added NAD⁺) but the rates, biphasic character, and fraction of "sites of each type" has remained unchanged.

Comparison of Steady-State Results at High and Low Enzyme Concentrations. In the presence of pyrazole we can follow the transient reaction at substrate concentrations comparable to those employed under steady-state conditions but at a very much higher concentration of enzyme. It is hence of interest to compare the steady-state parameters, as conventionally determined at low enzyme concentration, with the

TABLE IV: Transient Kinetics of Horse Liver Alcohol Dehydrogenase Catalyzed Reduction of Azoaldehyde by Partially Inactivated NAD⁺·Pyrazole·Enzyme.[a]

Enzyme Sample	OD Total	Fraction OD in First Step	k_1	k_2
Horse liver alcohol dehydrogenase alone	$7.1 + 10^{-6}$	0.62	105	0.76
Horse liver alcohol dehydrogenase incubated with 4.3 μM NAD⁺	2.8×10^{-6}	0.61	129	0.77

[a] [LADH] = 7.8 μN; [NADH] = 75 μM; [pyrazole] = 0.02 M; [azoaldehyde] = 37 μM.

same steady-state parameters determined at high enzyme concentration utilizing the stopped-flow apparatus. As can be seen in the list of kinetic parameters given in Table III, there is no difference (within the precision of the experiments) in steady-state rate parameters when the enzyme concentration varies over many orders of magnitude.

Discussion

The results of the transient experiments reported above, in which substrates can be in great excess over enzyme sites, coupled with previous transient results in which enzyme rather than substrate was limiting, lead us to a variety of mechanistic conclusions. First, and perhaps most important, is the conclusion that these results are not explicable in terms of any model involving a static fraction of "active" and (partially) "inactive" sites. Under all conditions of concentration of enzymes, coenzymes, and substrates, two distinct transient processes are observable, and in every experiment the extent of chemical transformation occurring in the total of these two transient processes is equivalent to the stoichiometrically limiting participant (enzyme or substrate or coenzyme). The variety of concentration conditions covered are sufficient to exclude the possibility that reaction at two kinds of noninteracting sites in the dimeric molecule is ubiquitously demanded.

Furthermore, experiments with deuterioNADH and NADH (Figure 4, Table II) clearly indicate that the rate-limiting process in each of the two discernable transients is different. The rate-limiting process in the rapid transient involves, at least partially, the chemical transfer of hydrogen from coenzyme to product, since a primary isotope effect is observable only in the rapid transient. The fact that the slower transient shows no primary isotope effect is consistent with a prior postulate (Theorell and Chance, 1951; Wratten and Cleland, 1963) that this slower process is governed by the rate of desorption of coenzyme product from the enzyme. The transient data for the appearance of ternary complex (Figure 2, Table III) during the course of oxidation of NADH by aromatic aldehydes in the presence of pyrazole suggests, however, that in these cases the slower transient is due to the desorption of alcohol product. No desorption of oxidized coenzyme is required for the formation of ternary complex. Nevertheless, the rate of ternary complex formation with pyrazole is the same as the rate of the slower transient in the biphasic kinetic profile.

The marked dependence of the first transient rate on aldehyde concentration (Figure 6) suggests two possibilities. This dependence could result from the approach to saturation in aldehyde binding sites, or it could be the result of a change in rate-limiting step from hydrogen-transfer (at high aldehyde concentration) to complex formation between E·NADH and aldehyde (at low aldehyde concentration). If the latter were the case, the isotope effect at low aldehyde concentration would be smaller than at high aldehyde concentration because of the mixing of an association rate (no isotope effect) with the rate of hydrogen transfer (primary isotope effect). The concentration independence of the isotope effect (with azoaldehyde as substrate) (Table II) indicates that the concentration dependence of the fast transient (Figure 6) is a result of saturation of enzyme·NADH binding sites with aldehyde.

Ethanol is reported to be strictly competitive with pyrazole in binding to horse liver alcohol dehydrogenase (Theorell and Yonetani, 1963). From Table III it is clear that whatever limits the rate of reduction of substrate in the slow transient in the case of azoaldehyde and β-naphthaldehyde is also limiting for the true first-order rate of reaction of pyrazole with the horse liver alcohol dehydrogenase·NAD^+ complex, and for the steady-state turnover of enzyme. The most likely possibility is that alcohol desorption is rate limiting. This is in accord with results indicating that the rate of dissociation of E·NAD^+ binary complex (McFarland *et al.*, 1971)[1] is faster than the rate of steady-state turnover for aromatic substrates. It also is in accord with the indications that pyrazole can only add to the oxidized coenzyme after desorption of substrate (*i.e.*, pyrazole is competitive with alcohol substrate).

On the basis of the above, we are led to the conclusion that the biphasic kinetics, observable in two different kinds of experiments and over large variations of concentration, can only result from subunit interactions within the horse liver alcohol dehydrogenase dimer. These interactions are such that only a fraction of the stoichiometric equivalent reacts at the rate of chemical transformation (hydride transfer); the remainder of substrate must await isomerization of the enzyme triggered by desorbtion of the alcohol product. The interaction between adjacent sites in the dimeric enzyme molecule must, at least under some conditions, be quite strong. Elimination of one-half of the enzyme sites by preformation of the enzyme·NAD^+·pyrazole complex has no effect on the biphasic transient kinetics other than to reduce stoichiometrically the total transient yield of product (Table IV). It is difficult for us to conceive of any formal mechanism for these findings other than those involving the virtually total elimination of activity in one-half of the dimeric enzyme molecules, *i.e.*, mechanisms indicative of strong cooperative subunit interactions during the formation of ternary complex with NAD^+·pyrazole.

Our new results on the effect of excessive high substrate concentrations on the extent of chemical transformation at each step of the (biphasic) rapid transient modifies a previous report from this laboratory (based on a far more limited range of concentration); high substrate concentrations partially overcome the restriction of equal amplitudes of chemical transformation in each transient step. What significance the increased extent of reaction associated with the rapid transient at high substrate concentrations has is unclear. It is perhaps significant, albeit confusing to point out that the steady-state rate of turnover of substrate is comparable in magnitude to the rate of the slow transient process, even under concentration conditions in which this transient accounts for only a small fraction of the stoichiometric equivalent of reaction (Table III).

Since many dehydrogenases are known to readily form ternary complexes involving enzyme, coenzyme, and a substrate or product analog, the method we have described for following transients in the presence of large excesses of reactants may be far more generally applicable. The applicability depends, however, on the demonstration that such abortive complex formation is not itself rate limiting.

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Purification and Properties of α-D- and β-D-Mannosidases from Hen Oviduct†

Tadayoshi Sukeno, Anthony L. Tarentino, Thomas H. Plummer, Jr., and Frank Maley*

ABSTRACT: An α-D- and a β-D-mannosidase (EC 3.2.1.24 and 3.2.1.25) have been purified from hen oviduct approximately 1300- and 10,000-fold, respectively. Molecular weight determinations by the Sephadex gel exclusion procedure revealed α-D-mannosidase to be about 250,000 and β-D-mannosidase, about 100,000. The latter enzyme was stable to high pH and the former to heat, properties that were exploited to remove undesirable contaminating glycosidases from the respective mannosidases. The α-D-mannosidase was more labile to treatment with Ag^+ and guanidine hydrochloride than β-D-mannosidase. Almost complete inhibition of α-D-mannosidase could be effected by Ag^+, with no apparent effect on β-D-mannosidase. However, Hg^{2+} impaired both enzymes to almost the same degree. β-D-Mannosidase was apparently more susceptible to inhibition by mannono(1→5)lactone than α-D-mannosidase. The K_i of this inhibitor was 17 μM for the former enzyme and 110 μM for the latter. Of significance is the finding that Asn-$(GlcNAc)_2(Man)_1$, a widespread constituent of many glycoproteins, is a substrate for β-D-mannosidase, but not for α-D-mannosidase. The latter will, however, convert Asn-$(GlcNAc)_2(Man)_6$ to Asn-$(GlcNAc)_2$-$(Man)_1$. Evidence is presented to support the thesis that a single enzyme hydrolyzes both *p*-nitrophenyl β-D-mannopyranoside and Asn-$(GlcNAc)_2(Man)_1$. Further proof that the mannosyl residues in the latter compound is β linked to *N*-acetylglucosamine was obtained by optical rotatory dispersion and infrared analysis on the Man(→)GlcNAc disaccharide isolated from Asn-$(GlcNAc)_2(Man)_1$.

In the course of studies (Tarentino *et al.*, 1970) on the oligosaccharide sequence of RNase B glycopeptide, it was found that five of the six mannosyl residues associated with the glycopeptide could be removed by jack bean meal α-D-mannosidase. The proposal offered for the resistance of the last mannosyl residue to hydrolysis in the glycosyl-Asn sequence, Asn-$(GlcNAc)_2(Man)_1$,[1] isolated from RNase B, was that the Man(→)GlcNAc bond was possibly in the β configuration or that if α, it was resistant to cleavage for reasons still to be determined. Similar results were obtained with an apparently identical glycosyl-Asn derivative from ovalbumin.

Incubation of Asn-$(GlcNAc)_2(Man)_1$ with extracts from hen oviduct, under conditions that minimized the action of glycosyl asparaginase, revealed the presence of an enzyme that released the terminal mannosyl residue (Sukeno *et al.*,

† From the Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201. *Received December 2, 1971.*

[1] Abbreviations used are: Asn-GlcNAc, 2-acetamido-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; Asn-$(GlcNAc)_2(Man)_1$, 2-acetamido-4-*O*-[*O*-mannopyranosyl(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; *p*CMB, *p*-chloromercuribenzoate; DON, 5-diazo-4-oxo-L-norleucine.