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Biphasic Transient Kinetics Are a Property of a Single Site in Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The transient kinetics under single turnover conditions of the dimeric enzyme horse liver alcohol dehydrogenase (LADH) are often biphasic with nearly equal amplitudes in the fast and slow components of the reaction. These observations have been the basis for the suggestion that this enzyme displays half of the sites reactivity or kinetic site–site interactions in its catalytic cycle [Bernhard, S. A., Dunn, M. F., Luisi, P. L., & Shack, P. (1970) *Biochemistry 9*, 185]. Alternatively, the biphasic kinetics could be the result of a complex mechanistic path at a single site with no kinetic interactions between the active sites [Kvassman, J., & Pettersson, G. (1976) *Eur. J. Biochem. 62*, 279; Kvassman, J., & Pettersson, G. (1978) *Eur. J. Biochem. 87*, 417]. To resolve this question, we have prepared LADH in which one of the two active sites is occupied by the strongly bound, slowly exchanging ternary complex inhibitors NAD with trifluoroethanol or NAD with pyrazole. These half-inhibited dimers show essentially the same biphasic transient kinetics as the native enzyme. These results strongly support mechanisms in which the observed kinetics are a property of each independently functioning site of the dimer. The possibility of half of the sites reactivity or kinetic site–site interaction appears to be ruled out.

The dimeric alcohol dehydrogenase from horse liver (LADH) is one of the simplest oligomeric enzymes thought to possess half-site reactivity (Bernhard et al., 1970; Luisi & Favilla, 1972; Luisi & Bignetti, 1974; Baici & Luisi, 1977). It is one of several dimers thought to be half-sited, including porcine heart malate dehydrogenase (Harada & Wolfe, 1968) and *Escherichia coli* alkaline phosphatase (Lazdunski, 1973). Half-site reactivity in alkaline phosphatase has been strongly challenged (Bloch & Schlesinger, 1973) as has half-site reactivity in a variety of membrane-associated transport proteins (Kyte, 1981). Challenges to the existence of a half-site reactivity in LADH have also been reported (Kvassman & Pettersson, 1976, 1978; Weidig et al., 1977; Kordal & Parsons, 1979; Tatemoto, 1975a,b).

Although a myriad of other enzymes have been reported to display half-sited behavior (Lazdunski, 1973; Levitzki & Koshland, 1974; Seydoux et al., 1974), it is of interest to carefully examine this phenomenon for the simple (in terms of quaternary structure) enzymes. In view of the long controversy involving LADH and its reported kinetic half-sited behavior, we have decided to determine the transient kinetic properties of individual half-inhibited dimers of LADH in solution. Such transient kinetic properties of the native enzyme are the main evidence for the initial suggestion of half-sitedness (Bernhard et al., 1970) or subunit interactions in this system (McFarland & Bernhard, 1972; Dunn et al., 1979).

Our approach to exploring the interpretation of these transient kinetic properties is to obtain an enzyme preparation in which one active site has been blocked and one is normal. We have employed two tightly bound, but not covalently attached, specific inhibitors of LADH to inhibit a given site. These are NAD·pyrazole and NAD·trifluoroethanol (TFE). We are then able to determine the kinetic properties of this enzyme for comparison with the uninhibited enzyme. The distribution of uninhibited (E_2), half-inhibited (E_2I) (where I = inhibitor), and fully inhibited (E_2I_2) dimers can be uniquely determined by using the equilibrium binding isotherms of the inhibitors. We have measured the binding curves for the above

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two inhibitors and their lifetime when bound to LADH in the preceding paper (Anderson & Dahlquist, 1982). For both sets of inhibitors, the coenzyme site and the separate substrate site are occupied, thus preventing binding or catalysis of real substrates at inhibited subunits [Plapp et al., 1978; C.-I. Brändén, quoted in Dahlbom et al. (1974)].

The substrate used here is *p*-(trifluoromethyl)benzaldehyde, which is typical of the many aromatic aldehydes which have been used to study LADH. It has been the subject of previous stopped-flow investigation as well as ^{19}F NMR study (Anderson & Dahlquist, 1980). Our results strongly suggest that the biphasic kinetics are a property of single independent sites on the enzyme, and strongly support mechanisms explaining the biphasic transient kinetics in terms of a complex reaction pathway at a single site such as those proposed by Kvassman & Pettersson (1976) or Weidig et al. (1977). Explanations for the biphasic kinetics which suggest half of the sites reactivity or functional subunit interactions for this dimeric enzyme appear to be incorrect.

Materials and Methods

The enzyme used in this paper was prepared and stored according to Anderson & Dahlquist (1979). Our preparative procedure yields pure EE isozyme of very high specific activity, which is suitable for use in experiments potentially sensitive to impurities, isozymes, or inactivated subunits. The substrate *p*-(trifluoromethyl)benzaldehyde was obtained from PCR Research Chemicals, Inc. (Gainesville, FL), vacuum distilled over sodium bicarbonate, and stored frozen at −20 °C. Aqueous stocks were made up as necessary and stored tightly stoppered at 4 °C. ^{19}F NMR was used to assay aldehyde purity (Anderson & Dahlquist, 1980). Trifluoroethanol and pyrazole were from Aldrich; reagent grade sodium pyrophosphate decahydrate was from J. T. Baker. β-NADD was a generous gift of Dr. Steve Koerber.

Stopped-flow experiments were performed in the laboratory of Dr. Michael Dunn of the Department of Biochemistry, University of California at Riverside. The Durrum stopped-flow spectrophotometer, nonlinear least-squares data analysis program, and associated computer interfacing are described in Dunn et al. (1979). All kinetic traces were obtained at 25 °C and monitored at 328 nm, the isosbestic point of free and binary complex NADH. The transient kinetics of the LADH-catalyzed oxidation of NADH with aromatic aldehydes are generally characterized by a rapid exponential phase with an apparent rate constant of ca. 100–200 s^{-1} and a 25–200-fold slower exponential. NADD is used in place of NADH to slow the rate of the fast step by approximately 3-fold, reflecting a primary kinetic isotope effect for this step (McFarland & Bernhard, 1972). Due to the disparity of apparent rate constants, separate traces were collected on different time scales and independently analyzed. Use of a single trace to fit both phases results in a poor estimation of the amplitude and rate of one of the phases, depending on the time used for data collection.

The final concentrations of NADD and *p*-(trifluoromethyl)benzaldehyde (*p*-$CF_3C_6H_4CHO$) after being mixed in the stopped-flow spectrophotometer, 100 and 200 μM, were essentially saturating. In all cases involving analysis of the slow phase of the reaction, the stopped flow was shown to be free of drift on the same time scale.

In situations where a variety of partially inhibited enzyme species are present, it is necessary to be able to treat the transient kinetics of substrate reduction in a quantitative fashion without necessarily knowing the distribution of enzyme species or the activity of each form.

For this purpose, we have employed an average rate constant, $\langle k \rangle_{obsd}$, which is defined below. Consider a reaction consisting of i exponentials

$$A(t) = \sum_i A_i e^{-k_i t} \tag{1}$$

where A represents amplitude and k represents a first-order rate constant. Thus

$$\frac{d \ln A(t)}{dt} = \frac{1}{\sum_i A_i e^{-k_i t}} \frac{d \sum_i A_i e^{-k_i t}}{dt} = \frac{1}{\sum_i A_i e^{-k_i t}} \left(-\sum_i A_i k_i e^{-k_i t}\right) \tag{2}$$

For data obtained at early times, $k_i t \ll 1$, and this reduces to

$$-\frac{d \ln A(t)}{dt} \simeq \frac{\sum_i A_i k_i}{\sum_i A_i} = \langle k \rangle_{obsd} \tag{3}$$

The latter ratio is defined as $\langle k \rangle_{obsd}$. Thus, the values of the initial slope of a logarithm of the amplitude of reactant remaining vs. time plot give directly the aggregate kinetic parameter $\langle k \rangle_{obsd}$ which will be a population-weighted average of $\langle k \rangle$ for E_2 ($\langle k \rangle_{E_2}$) and $\langle k \rangle$ for E_2I ($\langle k \rangle_{E_2I}$):

$$\langle k \rangle_{obsd} = f_{E_2} \langle k \rangle_{E_2} + f_{E_2I} \langle k \rangle_{E_2I} \tag{4}$$

Here $\langle k \rangle_{E_2}$ and $\langle k \rangle_{E_2I}$ refer to the average rate constants for the native and half-inhibited enzyme, respectively, where f_{E_2} is the fraction of active enzyme unliganded by inhibitor and f_{E_2I} is the fraction monobound with inhibitor. These latter parameters have been determined directly from the known NAD binding isotherms (see Results). Since $\langle k \rangle_{E_2}$ of eq 4 can be determined from control experiments, $\langle k \rangle_{E_2I}$ can be calculated from the average value.

In practice, we fit the observed time course to two exponentials. This treatment gives a very good fit to the total observed time course. However, other more complicated sums of exponentials would fit the data equally well. To remove the ambiguity of exactly which sum of exponentials is most appropriate for a given experiment, we have calculated the value of $\langle k \rangle_{obsd}$ from the double exponential fit, which is the same as would be obtained from using a fitting function with more exponentials or from extrapolating the slope of a logarithm of amplitude vs. time plot to very early times.

Results

Transient Kinetic Properties of LADH Partially Inhibited by NAD·Pyrazole. Figure 1A shows two representative stopped-flow traces of 60 μN LADH, preincubated with 40 mM pyrazole, reacted with 200 μM NADD + 400 μM *p*-$CF_3C_6H_4CHO$ at pH 8.7, 25 °C. The top trace shows an expanded time scale (50-ms full width) used to fit the fast phase of the reaction to a single exponential, while the bottom trace (5-s full width) is used to fit only the slow phase. Analysis of seven experiments gives a fast phase rate constant of 197 ± 30 s^{-1}, a slow phase rate constant of 0.9 ± 0.1 s^{-1}, and percentage of the total reaction amplitude occurring in the fast phase of 73 ± 10%. With the above rate constant for the fast phase, the half-time for the reaction is 3.4 ms. Thus, a little less than half of the total amplitude is lost in the dead time of the stopped flow, but the trace is fit over all of the remaining reaction.

Figure 1B shows stopped-flow traces obtained for enzyme partially inhibited with NAD·pyrazole. A solution of 60 μN LADH was preincubated with 42 μN NAD and 40 mM py-

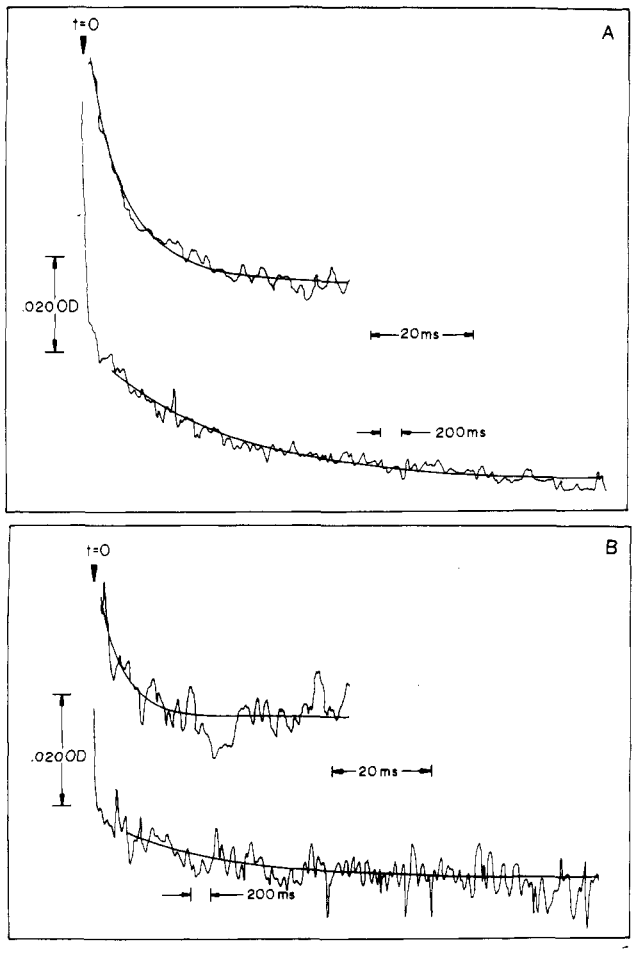


FIGURE 1: (A) Transient kinetic properties of uninhibited LADH, preincubated with pyrazole. Stopped-flow kinetic traces shown result from reaction of 60 μN LADH plus 40 mM pyrazole in pH 8.7 50 mM sodium pyrophosphate buffer in one syringe, with 400 μM *p*-$CF_3C_6H_4CHO$ and 200 μM NADD in the other syringe. Reaction was observed at 328 nm, at 25 °C. Best-fit single exponential decays are overlaid on the data, with k_{fast} = 197 s^{-1}, k_{slow} = 0.9 s^{-1}, and 73% of the reaction occurring in the fast phase after correction for instrument dead time of 3.0 ms. The time span (in milliseconds) and absorbance scale for each trace are indicated. Since NADD is being oxidized, the absorbance is decreasing with time. The start of the reaction is indicated by t = 0. (B) Transient kinetic properties of LADH partially inhibited by NAD·pyrazole. In this experiment, 60 μM LADH was preincubated with 45 μM NAD and 40 mM pyrazole. Other concentrations are as in Figure 1A. NAD binding isotherms suggest that the ratio of half-NAD·pyrazole-inhibited dimers to uninhibited dimers is 8.3. The best-fit single exponential decays overlay each separate trace, giving k_{fast} = 221 s^{-1} and k_{slow} = 0.84 s^{-1}, with 73% of the reaction occurring in the fast phase after correction for the instrument's dead time. The start of the reaction is indicated at t = 0. Reaction is in pH 8.7 50 mM sodium pyrophosphate buffer at 25 °C and is monitored at 328 nm. Changes in absorbance at 328 nm are scaled as shown with the data.

razole. The second syringe was loaded with 200 μM NADD and 400 μM *p*-$CF_3C_6H_4CHO$. The reaction is still clearly biphasic, although with less amplitude in each phase since the final concentration of uninhibited enzyme sites is 9 μN instead of 30 μN. Analysis of five experiments over the first 40 ms of reaction gave a fast phase rate constant of 221 ± 139 s^{-1}. For six experiments, an average slow phase rate constant of 0.84 ± 0.11 s^{-1} was obtained; 73 ± 19% of the reaction occurred in the fast phase. The smooth curves represent the calculated time courses of the fast and slow phases.

Figure 2 shows the dependence of the fast phase rate constant, slow phase rate constant, and the fraction of the total amplitude of the reaction occurring in the fast step as a

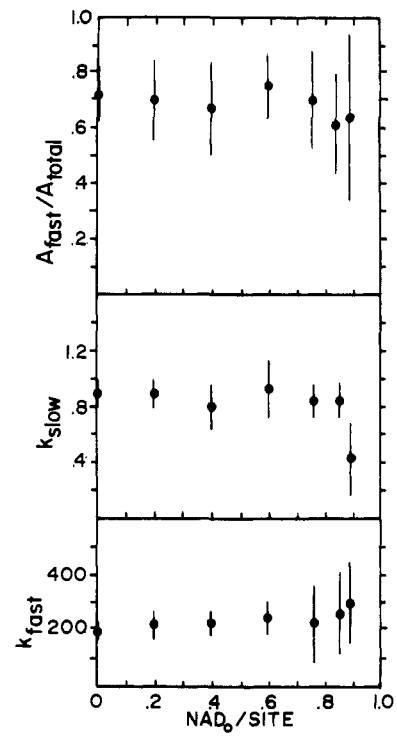


FIGURE 2: Dependence of transient kinetic parameters on NAD·pyrazole inhibition of LADH. This figure shows the variation of k_{fast} (s^{-1}), k_{slow} (s^{-1}), and the percentage of reaction amplitude occurring in the fast phase (A_{fast}/A_{total}) with NAD preincubated with LADH and pyrazole. All reaction conditions are as in Figure 1A,B except for variable NAD concentrations. Kinetic parameters were derived from single exponential fits to the data as in Figure 1A,B; the error bars shown represent one standard deviation on either side of the average value calculated for (generally) six experiments for each point. See text for analysis.

Table I: Kinetic Properties of E_2pyz_2·NAD

$[NAD]_0/E_0$	E_2pyz_2·NAD/E_2pyz_2	$\langle k\rangle_{obsd}$[a]	$\langle k\rangle_{E_2pyz_2\cdot NAD}$	$\langle k\rangle_{E_2pyz_2}$
0.40	1.5	152	157	144
0.60	3.7	183	193	144
0.76	8.3	162	164	144
0.84	14.1	154	155	144
0.89	22.1	187	189	144

[a] $\langle k\rangle_{obsd} = [A_{fast}/(A_{fast} + A_{slow})]k_{fast} + [A_{slow}/(A_{fast} + A_{slow})]k_{slow}$; $\langle k\rangle_{E_2pyz_2\cdot NAD} = (\langle k\rangle_{obsd} - f_{E_2\cdot pyz_2})/f_{E_2pyz_2\cdot NAD}$.

function of $[NAD]_0/E_0$ in the presence of saturating pyrazole. The error bars represent one standard deviation on either side of the mean for five to seven experiments for each phase of the reaction. For incubation of E_2pyz_2 with five different levels of NAD, the distribution of active forms favors the half-inhibited dimer, E_2I, which in this case is composed of two sites, two bound pyrazole (pyz) molecules, and one bound NAD molecule. The average value of $\langle k\rangle_{E_2I}$ of 172, with a range from 155 to 193, is only a little higher than the value of $\langle k\rangle_{E_2}$ of 144. A more quantitative treatment which involves comparison of $\langle k\rangle_{E_2}$ with derived values for $\langle k\rangle_{E_2I}$ is shown in Table I.

Inactivation of LADH sites, measured by the decrease in summed fast and slow phase reaction amplitudes in the presence of preincubated NAD·pyrazole, was found to be linear with $[NAD]_0$/site with complete inhibition at 1.0 NAD/site.

Transient Kinetic Properties of LADH Partially Inhibited by NAD·TFE. Figure 3A shows representative stopped-flow

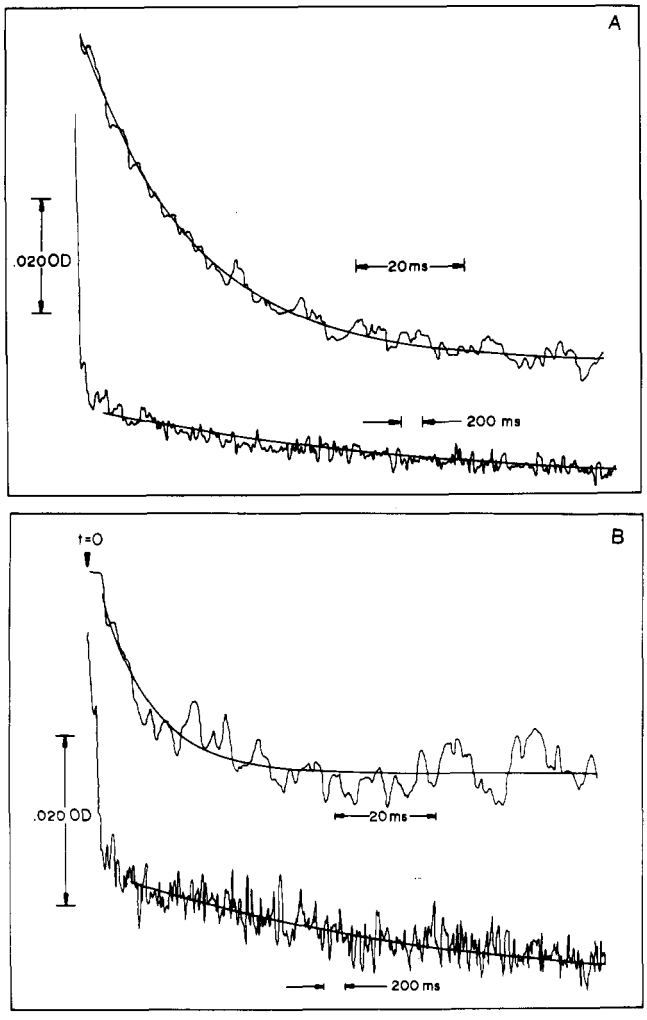


FIGURE 3: (A) Transient kinetic properties of uninhibited LADH, preincubated with TFE. Stopped-flow kinetic traces shown result from reaction of 70 μM LADH and 100 mM TFE, in one syringe, with 400 μM p-$CF_3C_6H_4CHO$ and 200 μM NADD, in the other syringe. Reaction is at pH 8.7, 25 °C, and is monitored at 328 nm. The best-fit single exponentials are overlaid on each individual trace; the results were k_{fast} = 56 s^{-1} and k_{slow} = 0.4 s^{-1}, with 83% of the reaction occurring in the fast phase, after dead-time correction. The time span and amplitude of each trace are as indicated. (B) Transient kinetic properties of LADH partially inhibited by NAD·TFE. Other conditions are as in Figure 3A. Analysis in the text suggests that the ratio of half-inhibited dimers to uninhibited dimers under these conditions is 4.9. Parameters derived from the best-fit single exponentials shown overlaying the data are k_{fast} = 82 s^{-1}, k_{slow} = 0.3 s^{-1}, and A_{fast}/A_{total} = 0.68. The start of the reaction is at t = 0.

traces of 70 μN LADH and 100 mM TFE, mixed with 400 μN p-$CF_3C_6H_4CHO$ plus 200 μM NADD at pH 8.7, 25 °C. In the top trace, the burst phase is fit to a single exponential with an average rate constant of 56 ± 4 s^{-1} for six experiments. The bottom trace shows the slow phase of the reaction also fit to a single exponential. An average $\langle k \rangle_{slow}$ of 0.41 ± 0.10 s^{-1} for four experiments was found, while 83 ± 4% of the reaction occurred in the fast phase, after correction for a 3-ms dead time.

Figure 3B shows transients resulting when enzyme is inhibited with NAD·TFE. As is the case with the control experiment of Figure 3A, the reaction is clearly biphasic. In one syringe, 70 μN LADH was mixed with 56 μN NAD and 100 mM TFE. The other syringe contained 200 μM NADD and 400 μM p-$CF_3C_6H_4CHO$. The top trace shows a single exponential fit to the fast phase of the reaction, with an average $\langle k \rangle_{fast}$ of 82 ± 16 s^{-1} for six experiments. The bottom trace shows a single exponential fit to the slow phase of the reaction. $\langle k \rangle_{slow}$ was 0.28 ± 0.17 s^{-1} for four trials. The fast phase of the reaction accounted for 68 ± 16% of the reaction. Since the final concentration of uninhibited enzyme sites is approximately 7 μN, there is more noise apparent in the traces, and thus a larger uncertainty in best-fit parameters.

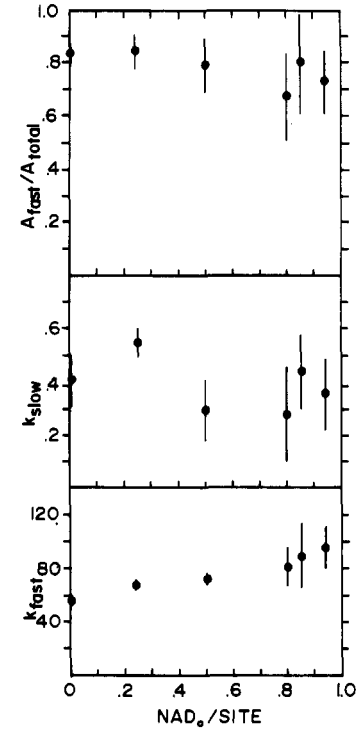


FIGURE 4: Dependence of transient kinetic parameters on NAD·TFE inhibition of LADH. The values of the kinetic parameters k_{fast} (s^{-1}) and k_{slow} (s^{-1}) and the percentage of reaction occurring in the fast phase of the reaction, A_{fast}/A_{total}, are plotted against the ratio of preincubated NAD per enzyme site. In the presence of saturating TFE, this is equivalent to the fractional inhibition of the enzyme with NAD·TFE. Data for each point were derived from experiments like those shown in Figure 3A,B, under identical conditions except for the concentration of NAD, which was varied for each point. The error bars represent one standard deviation on either side of the average values calculated from the data, which generally consisted of six experiments for each point. See text for analysis.

Table II: Kinetic Properties of E_2T_2·NAD

$[NAD]_0/E_0$	E_2T_2NAD/E_2T_2	$\langle k \rangle_{obsd}$	$\langle k \rangle_{E_2T_2NAD}$[a]	$\langle k \rangle_{E_2T_2}$
0.50	1.5	57	64	47
0.80	4.9	56	57	47
0.85	6.7	72	75	47
0.94	17.3	70	71	47

[a] $\langle k \rangle_{obsd}$ and $\langle k \rangle_{E_2T_2NAD}$ calculated as in Table I for pyrazole.

Figure 4 shows the dependence of the fast and slow phase apparent rate constants, and percentage reaction in the fast phase, on the ratio of preincubated NAD to enzyme sites in the presence of saturating TFE. The data were derived from experiments like those presented in Figure 3 for other ratios of $[NAD]_0/E_0$. A more quantitative treatment involves comparison of $\langle k \rangle_{E_2T_2}$ with the derived values for $\langle k \rangle_{E_2T_2NAD}$ (Table II). For incubation of E_2T_2 with four different levels of NAD, $\langle k \rangle_{E_2T_2NAD}$ can be derived since the distribution of active enzyme forms favors the half-inhibited enzyme. The average value of $\langle k \rangle_{E_2T_2NAD}$ obtained, 67, with a range from 57 to 75, is only slightly higher than the value obtained for $\langle k \rangle_{E_2T_2}$ of 47. As before in the case of NAD·pyrazole, site inhibition with preincubated NAD·TFE was linear with

$[NAD]_0$/site with essentially complete inhibition at 1.0 NAD/site.

It should be noted that these are single turnover experiments, making use of trifluoroethanol not only as a partner with NAD in inactivating enzyme sites in one reactant syringe but also as a reagent [see Anderson & Dahlquist (1982)] limiting reaction to a single turnover. Due to the high final concentration of TFE (50 mM) relative to its K_d from E·NAD·TFE of 6 μM, the E·NAD formed after one turnover of E·NADH·p-$CF_3C_6H_4CHO$, followed by alcohol desorption from E·NAD·p-$CF_3C_6H_4CH_2OH$, will be rapidly inactivated by formation of E·NAD·TFE.

Discussion

The biphasic transient kinetic properties of single turnover experiments of substrates and coenzymes with LADH have formed the basis for the argument that this enzyme shows half of the sites reactivity. The argument suggests that the fast and slow components of the biphasic time course reflect the kinetic properties of the two active sites of this protein, one showing the rapid time course and one the slow.

The approach used here is to obtain the kinetic properties of LADH dimers in which one of the two active sites is blocked by an essentially irreversibly bound inhibitor. We have employed the inhibitors trifluoroethanol and pyrazole for this purpose. These inhibitors bind rather weakly to the enzyme alone but bind quite tightly to form ternary complexes with the enzyme and NAD. In addition, the bound lifetimes of these ternary complexes are quite long [see Anderson & Dahlquist (1982)] so that on the time scale of a single enzyme turnover the inhibitor ternary complexes are essentially irreversibly bound. Importantly, however, the binary complexes of these inhibitors are both weak and short lived (we have determined the bound lifetime to be less than 1 ms in the case of trifluoroethanol). Thus, the inhibitors themselves have relatively little effect on the transient kinetics observed by using an aromatic alcohol such as p-(trifluoromethyl)benzaldehyde and NADD as reactants. As noted above, the inhibitors will trap any NAD formed as the reaction proceeds and thus limit the reaction to a single turnover. To produce the inhibited forms of the enzyme, it is preincubated with various amounts of NAD in the presence of pyrazole or TFE. In the preceding paper, we have carefully determined the binding isotherm of NAD to the enzyme in the presence of high concentrations of these inhibitors. This binding shows essentially no cooperativity. However, rather than assume this to be true, we have used the experimentally determined values of the Adair constants ψ_1 and ψ_2 which are used to describe the general case of ligand binding to a protein with two sites. If $\bar{Y}$ is the fraction of binding sites occupied by NAD, and N is the NAD concentration, the binding of NAD to the enzyme saturated with the substrate analogue pyrazole or trifluoroethanol is given by

$$\bar{Y} = \frac{1}{2}\frac{\psi_1 N + 2\psi_2 N^2}{1 + \psi_1 N + \psi_2 N^2} \quad (5)$$

This formalism also allows the fractions of enzyme present with one NAD bound, f_{E_2I}, and with two NAD molecules bound, $f_{E_2I_2}$, to be determined directly as

$$f_{E_2I} = \frac{\psi_1 N}{1 + \psi_1 N + \psi_2 N^2} \quad (6)$$

and

$$f_{E_2I_2} = \frac{\psi_2 N^2}{1 + \psi_1 N + \psi_2 N^2} \quad (7)$$

Table III: Parameters Used in Calculating the Equilibrium Distribution of E_2I_2 and E_2I_2·NAD

I	parameter	value[a]
TFE	ψ_1	46.5 μM^{-1}
TFE	ψ_2	1029 μM^{-2}
pyrazole	ψ_1	241 μM^{-1}
pyrazole	ψ_2	10279 μM^{-2}

[a] From preceding paper (Anderson & Dahlquist, 1982). Values are averages from four separate experiments.

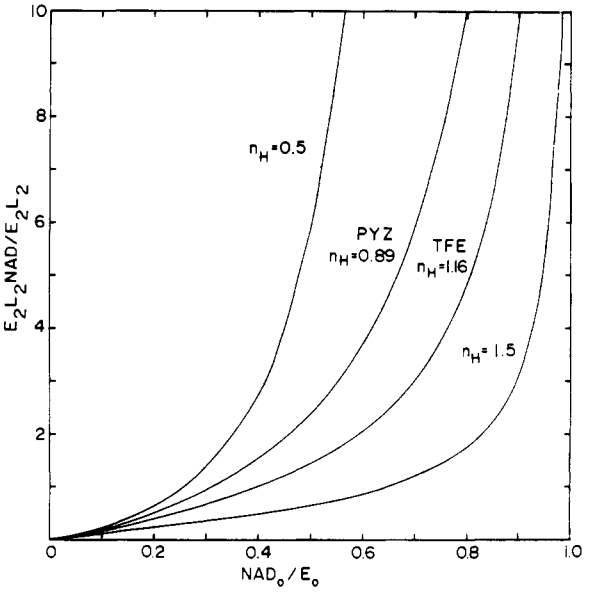


FIGURE 5: Distribution of the two types of active LADH, E_2 and E_2I, under varying conditions of partial inhibition with saturating pyrazole or trifluoroethanol concentrations and limited amounts of NAD. The different curves represent cases of varying cooperativity in NAD binding ranging from negative cooperativity (n_H = 0.5 is used) to positive cooperativity (n_H = 1.5 is used). The curve with n_H = 0.89 is that calculated for saturating trifluoroethanol concentration. See text for details of the calculations.

Thus, for determination of the distribution of bound enzyme forms, values of ψ_1, ψ_2, and free [NAD] are necessary.

The best-fit values for ψ_1 and ψ_2 are derived from binding isotherms of NAD to enzyme in the presence of trifluoroethanol, or in the presence of pyrazole (Table III). The free [NAD] values are calculated in the following manner. Since the binding constants for NAD in the presence of saturating amounts of trifluoroethanol ($K_d \sim 33$ nM) or pyrazole ($K_d \sim 10$ nM) are very small, binding is nearly stoichiometric to enzyme 1000-fold in concentration above these binding constants. Thus, the fractional saturation of enzyme with NAD can be closely approximated by the ratio of total (substoichiometric) [NAD] to total enzyme sites. With the known values for ψ_1 and ψ_2 (Table I) and $\bar{Y} = N_0/E_0$ (ranging from 0 to 1), the resultant quadratic equation in free NAD can be solved for the conditions of preincubation used in stopped-flow experiments.

Figure 5 presents the E_2I:E_2 ratio as a function of the fractional saturation of enzyme sites with NAD for different combinations of ψ_1 and ψ_2. These combinations are reflected in the Hill coefficient for NAD binding to LADH of $4/[(\psi_1/\psi_2^{1/2}) + 2]$. Results are presented by using known Hill coefficients for NAD binding in the presence of pyrazole and trifluoroethanol, as well as Hill coefficients reflecting marked positive cooperativity in binding (n_H = 1.5) and marked negative cooperativity in binding (n_H = 0.5) for reference. Fully inhibited enzyme E_2I_2 is completely inactive and

therefore cannot contribute to any observed stopped-flow transients. As a result, only the distribution of E_2 and E_2I is of interest.

It should be noted that the isotherms for NAD binding to E·pyrazole were collected at pH 7.5, 25 °C, and not at higher pH, since NAD binding at lower $[H^+]$ becomes too tight to quantitate the cooperativity of binding. The stopped-flow experiments with pyrazole were at pH 8.75, 25 °C, so predictions of the distribution of unbound to mono NAD bound LADH at this higher pH involve an assumption of similar cooperativity of NAD binding at both pH values.

Bound Lifetimes of Inhibitory Complexes. Under the conditions of the stopped-flow experiments reported in this paper, the bound lifetimes of trifluoroethanol and pyrazole have been measured. Using data from displacement experiments presented in the preceding paper, we determined the desorption rate, k_{off}, for trifluoroethanol (pH 8.75, 25 °C) from E·NAD·TFE to be 0.025 s^{-1}, giving a bound lifetime of $1/k_{off}$ = 40 s. For pyrazole, $k'_{off} \sim 0.0025$ s^{-1}, giving a bound lifetime at pH 8.75, 25 °C, of $1/k'_{off}$ = 400 s. Since the slowest observed rate constant is 0.4 s^{-1}, inhibited sites will stay occupied during the time course of *both* kinetic phases, with no reordering of inhibitor.

Kinetic Properties of Half-Inhibited Dimer. The decrease in the amplitude of the reaction in single turnover experiments under inhibited conditions provides information concerning the activity of the half-inhibited forms of the enzyme. The total amplitude of the biphasic time course was seen to linearly decrease as NAD was added to solutions of LADH in the presence of saturating amounts of TFE or pyrazole, passing through zero activity when two NAD molecules per enzyme dimer were added. This loss of activity parallels the fractional saturation of the enzyme with ternary complexes of NAD and pyrazole or TFE. Combined with the lack of any substantial positive cooperativity in NAD binding to these ternary complexes, this implies that the half-inhibited forms are capable of catalytic activity on a time scale comparable to that of the native enzyme. The main goal of this paper is to estimate the transient properties of half-inhibited dimeric LADH. The first inhibitor pair used is NAD·trifluoroethanol. Since trifluoroethanol is competitive with ethanol (Sigman, 1967; Dubied & von Wartburg, 1976) and since strong structural similarities of trifluoroethanol with the active substrate p-$BrC_6H_4CH_2OH$ in a ternary complex with NAD have been observed by X-ray diffraction (Plapp et al., 1978), this complex can be viewed as analogous to an NAD·alcohol reactant pair.

As seen in Figure 5 and Table II, the half-inhibited enzyme E_2T_2·NAD begins to predominate over uninhibited enzyme (E_2T_2) when $[NAD]_0/E_0$ goes above 0.4–0.5. Thus at four values of $[NAD]_0/E_0$ above 0.4, the observed kinetics predominately reflect those of the half-inhibited form. When $[NAD]/E_0$ reaches 0.8 or 0.94, this form predominates over E_2T_2 by 4.9-fold and 17.3-fold, respectively. As the data show, even under these extreme conditions, the enzyme shows nearly unchanged biphasic kinetics. As can be seen from Figure 4, both rate constants are similar to control enzyme (NAD/site = 0) as is the percent of reaction occurring in the fast phase. Deriving the average properties of E_2T_2NAD in Table II for values of $[NAD]_0/E_0$ above 0.40 suggests that they are similar to those of E_2T_2, with k_{fast} (which dominates the calculated $\langle k \rangle$ since $k_{fast} \gg k_{slow}$ and $A_{fast} > A_{slow}$) on the average being slightly larger than that for E_2T_2.

Inhibiting subunits with NAD·pyrazole, thought to form a 1,4-dihydronicotinamide adduct on the enzyme surface (Theorell & Yonetani, 1963), was also employed. The bond lifetime of ternary complex pyrazole is 400 s under the experimental conditions, so that NAD·pyrazole-occupied sites have no chance to react with substrates. Since pyrazole is also competitive with ethanol, NAD·pyrazole inhibition prevents any coenzyme or substrate binding.

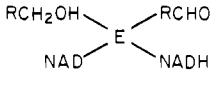
From the curve in Figure 5, E_2pyz_2·NAD is the main active enzyme form in solution when NAD/site goes above 0.3–0.4, reaching a 22-fold excess over E_2pyz_2 when NAD/site reaches 0.89. For five values of $[NAD]_0/E_0$, the kinetic properties of half-inhibited enzyme can be calculated (Table I). The average value of 172 is not appreciably higher than $\langle k \rangle_{obsd}$ for E_2pyz_2 of 144. The average value of 172 is not appreciably higher than $\langle k \rangle_{obsd}$ for E_2pyz_2 of 144. The values for k_{fast}, k_{slow}, and A_{fast}/A_{total} in Figure 2, for NAD/site of 0.4 and above, also clearly suggest that the half-inhibited dimer retains the same (biphasic) kinetic properties as the uninhibited enzyme E_2·pyz_2.

The distribution of inhibitor-bound species in the case of NAD·pyrazole is somewhat uncertain in view of the fact that the NAD-binding isotherms required for its derivation were available only at pH 7.5, while the stopped-flow experiments were performed at pH 8.75. However, even if NAD binding became much more positively cooperative at higher pH so that a Hill coefficient of 1.5 described the binding, at $[NAD]_0/E_0$ = 0.89 the ratio of uninhibited sites on E_2·pyz_2·NAD to those on E_2·pyz_2 would still be 2.8:1, and the same inference about half-inhibited enzyme properties would be derived from the data.

The biphasic kinetic properties of both sets of control enzyme, E_2T_2 (Figure 3A) and E_2·pyz_2 (Figure 1A), are readily explained. TFE and pyrazole by themselves are poor inhibitors, with dissociation constants of 1.3 mM and 9 mM at pH 8.75 and 7.5, respectively [see Anderson & Dahlquist (1982)]. Studies of trifluoroethanol interaction with LADH suggest a bound lifetime of 10^{-4}–10^{-5} s. This is likely to be true of pyrazole as well. Thus, the inhibitors above are not expected to greatly affect the biphasic nature of the reaction with substrates because they exchange rapidly relative to the time courses measured.

Importance of Kinetic Properties of Half-Inhibited Dimer. Since biphasic transient kinetics originating with similar para-substituted benzaldehydes have been used to suggest half-site reactivity (Bernhard et al., 1970; Seydoux et al., 1974) or subunit interaction (McFarland & Bernhard, 1972; Dunn et al., 1979; McFarland et al., 1977), our finding that half-inhibited LADH also possesses these kinetics with p-(trifluoromethyl)benzaldehyde has profound implications for these theories. Our findings, under conditions where subunit interaction mediated by ligand binding or catalysis at the subunit adjacent to the active subunit is impossible due to the specific blockage of *both* substrate and coenzyme sites on this adjacent subunit, rule out the above use of biphasic kinetics in inferring half-site reactivity or subunit interactions in this enzyme.

Furthermore, we have essentially constructed LADH with the products of NADH·aldehyde reduction, NAD·alcohol (alcohol = TFE here) on one subunit. This constitutes a test of the hypothesis presented in McFarland et al. (1977) that this species should be inactive until product desorbs from the NAD·alcohol-bound subunit. That is



should be inactive. Our finding that it possesses biphasic transient kinetics is inconsistent with this and other models which invoke (NAD·alcohol) desorption from an LADH

subunit as a trigger for reaction at the adjacent subunits (Bernhard et al., 1970).

Our results also suggest that the observed kinetics are the property of a single site on the enzyme, and therefore in uninhibited enzyme are due to equivalently functioning sites. Our results support mechanisms of the type proposed by Kvassman & Pettersson (1976) and Weidig et al. (1977), based on earlier suggestions by Holbrook & Gutfreund (1973) and Hijazi & Laidler (1973). They propose that biphasic kinetics can result at a single catalytic site when several equilibria are coupled to chromophore appearance or disappearance, without the need for subunit interactions. Indeed, as Kvassman & Pettersson (1976) have shown, this is a solid prediction of an ordered ternary complex reaction mechanism, with the observed kinetics depending on values of various rate constants. Weidig et al. (1977) have determined nearly all microscopic rate constants for the benzyl alcohol–benzaldehyde substrate pair in an assumed ordered ternary complex mechanism for this enzyme and have quantitatively predicted the observed biphasic kinetics for this substrate pair.

The amplitudes of reaction occurring in the fast and slow phases of reaction are also important to note. First, data in Figure 3A,B represent the use of trifluoroethanol as a suicide reagent, as suggested in the preceding paper (Anderson & Dahlquist, 1982). Its ternary complex with NAD has no UV absorption bands near 300 nm, and so fast and slow phase amplitudes require no correction due to the formation of this ternary complex. Under these conditions, ca. 80% of the total reaction occurs in the fast phase. While the reaction remains clearly biphasic, 50% of the NADH does *not* disappear in each step, as postulated by Bernhard et al. (1970). Indeed, our finding of a fast step fractional reaction considerably larger than 50% agrees with most recent results (Dunn et al., 1979; Kordal & Parsons, 1979; Weidig et al., 1977; Kvassman & Pettersson, 1976) as well as some early data [cf. Figure 5 of McFarland & Bernhard (1972)]. Such results are consistent with a complex reaction pathway at a single site involving at least two intermediates. Such a mechanism will accommodate virtually any ratio of fast and slow step amplitudes. It is difficult to reconcile any of these results with a half-sited function for LADH.

Results in the literature also suggest that purified half-carboxymethylated dimers are active, in ethanol oxidation or benzaldehyde reduction (Andersson & Mosbach, 1979), although McFarland et al. (1977) qualitatively suggested the inactivity of these species. W. Bloch and C. Stiefbold (unpublished results) have also prepared by covalent modification a different half-inhibited dimer, which, in agreement with results here, exhibits biphasic transient kinetics with several aromatic aldehydes. Kordal & Parsons (1979) also showed that there is no change in transient kinetic properties when reaction occurs at one site of an individual native dimer. McFarland & Bernhard (1972) preincubated LADH with 0.55 NAD/site in the presence of excess pyrazole. Their observation of unchanged biphasicity fully agrees with our results. Their prediction of total positive cooperativity in NAD·pyrazole binding is not, however, borne out in the binding isotherms presented in our preceding paper (Anderson & Dahlquist, 1982). Based on our isotherm, they were probably looking at a ratio of 3 E_2·pyz_2·NAD to 1 E_2·pyz_2. Thus, their data also lead to our conclusion that half-inhibited enzyme is biphasic.

We feel the results presented here, as well as the weight of results in the literature, strongly suggest an independent function for each active site of the LADH dimer.

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