Pressure Relaxation of the Equilibrium of the Pig Heart Lactate Dehydrogenase System

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The relaxation behaviour of a system of reactants equilibrated in the presence of pig heart lactate dehydrogenase was studied after pressure perturbation. Two relaxations were observed when protein fluorescence was recorded, but only the slower relaxation was apparent in observations of A_{340} . The faster relaxation therefore involves transfer between free and enzyme-bound NADH, whereas the slower relaxation represents the reduction of NAD^+ . Both relaxations were observed in Tris buffer, where there is little effect of pressure on pH, and in phosphate buffer, where pH changes are significant; however, the amplitudes depended on the buffer used. The slower reciprocal relaxation time increases with increasing total enzyme concentration and decreases slightly with increasing NAD^+ concentration. Computer simulations, based on a proposed mechanism, were compared with the experimentally determined amplitudes and relaxation times as a test of the mechanism.

Preliminary observations (Davis & Gutfreund, 1976) of the pressure perturbation of a system of reactants equilibrated in the presence of pig heart lactate dehydrogenase showed the existence of relaxation phenomena. Transient kinetic analysis of stopped-flow experiments in this laboratory (Whitaker *et al.*, 1974; Boland & Gutfreund, 1975; Holbrook *et al.*, 1976) has resulted in considerable elucidation of our understanding of the reaction of lactate dehydrogenase with its substrates and allows pressure-relaxation effects to be analysed in

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terms of the mechanism in Scheme 1 [based on the conclusions of Boland & Gutfreund (1975) and Holbrook *et al.* (1976)]. The analytical solution of the relaxation equations describing this mechanism, in terms of nine relaxation times, can be provided in principle, but would not be of significant practical use.

In this paper we describe a detailed analysis of the pressure-relaxation behaviour of lactate dehydrogenase. As a test of the mechanism, computer simulations were used to compare experimentally observed relaxations with those predicted by the model.

Experimental

Materials

Pig heart lactate dehydrogenase was obtained from Boehringer (Mannheim) G.m.b.H., Mannheim,

Scheme 1. Reaction of lactate dehydrogenase with its substrates
Abbreviations: Lac, lactate; Pyr, pyruvate.

Germany (batch 1046318). Stock solutions were prepared by desalting on a column of Sephadex G-25 and protein concentrations were determined as described by Whitaker *et al.* (1974). The active-site concentration of enzyme solutions, determined by titration with NADH, was 80% of the protein concentration as described by Whitaker *et al.* (1974). All molarities of enzyme given in the present paper refer to the concentration of active sites.

NAD⁺ (grade III), NADH (grade III) and lithium L-(+)-lactate (grade L-X) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and used without further purification. Phosphate and Tris buffers (both pH 8.0, *I* 0.1) were prepared by standard procedures (Long, 1961). All solutions, other than buffers, were freshly prepared on the day used.

Instrumentation

Pressure-relaxation experiments were carried out by using the equipment described by Davis & Gutfreund (1976); pressure release was effected with a mechanical valve. Changes in NADH absorbance were followed at 340 nm by using a tungsten-filament lamp and Farrand monochromator; the path length was 1 cm. Fluorescence was excited with a mercury discharge lamp (100 W) and Farrand monochromator with 10 mm or 20 mm slits. Protein fluorescence was excited at 300 nm and observed through Schott W.G. 335 and U.G.11 filters. Nucleotide fluorescence was excited at 340 nm and observed through a Schott KV393 filter (Bagshaw *et al.*, 1974).

Data analysis and simulation experiments were carried out on a PDP8/e computer with 12K store by using the 8K FOCAL language.

Experimental methods, data collection and analysis

Mixtures of known concentrations of NAD⁺, lactate and enzyme were prepared, allowed to equilibrate at atmospheric pressure and introduced into the pressure-relaxation apparatus. The pressure was increased to 15–20 MPa (150–200 atm) and then rapidly released [within about 80 μ s (Davis & Gutfreund, 1976)]. All experiments were carried out at 25°C, pH 8.0 and *I* 0.1.

The photomultiplier signals (*Y*) from each individual pressure-jump experiment were stored as 1024 8-bit data points by means of a Data Laboratories DL905 transient recorder and were then transferred to magnetic tape for subsequent data reduction and analysis. The data points were subsequently read from the tape into the core of the PDP8/e computer under the control of a machine-language program. The data are then accessible to FOCAL through a machine-language function FCOM.

In some cases single experiments were treated by using a linear least-squares analysis of the logarithm

of the absolute value of ($Y - Y_{\infty}$) versus time. Usually computation of an average transient (CAT) by accumulation of data from several identical experiments was carried out. To decrease the noise and the space required for data storage to within the limits allowed by the FOCAL language, the 1024 data points for each experiment were averaged in groups. Points were averaged in groups of three for the first 42 points, of five for the next 100, of ten for the next 400 and of 20 for the remainder. The resulting data points were added to the stored sums from previous runs. After accumulation of data from a batch of experiments the average transient was plotted and the relaxation times were evaluated by one of the following methods. Usually a first-order plot, as described above, was used to determine the longer relaxation time; this was followed, where necessary, by determination of the shorter relaxation time by logarithmic extrapolation and a logarithmic plot of the resulting differences (Gutfreund, 1975, p. 126). Where the two relaxation times were sufficiently separated to allow the slower relaxation to appear as a straight line on the experimental record, this line (extrapolated to zero time) was subtracted from the experimental points for the early part of the reaction and a logarithmic plot used to determine the shorter relaxation time. Errors quoted for reciprocal relaxation times are standard deviations.

Results and Discussion

Measurements of changes in A_{340} showed single relaxations for perturbation of equilibrium mixtures in both phosphate (Fig. 1) and Tris buffers. In contrast, measurements of changes in protein fluorescence (as illustrated in Fig. 2) showed a slow relaxation corresponding to the single relaxation observed by absorbance measurements, together with a more rapid relaxation. The relative amplitudes of the two relaxations depend on whether the ionization of the buffer used is pressure-dependent, as in phosphate, or not, as in Tris.

The plot in Fig. 2(a) was obtained from a single pressure relaxation. Considerable improvement in signal-to-noise ratio was obtained by averaging the transients from four relaxations carried out under identical conditions; the result of such averaging is shown in Fig. 2(b). All rate constants reported in this paper from experiments on protein or nucleotide fluorescence are from the average transients of at least four experiments. In some cases computation of an average transient was also used for experiments measuring A_{340} .

Experiments in Tris buffer

Experiments carried out in Tris buffer (pH 8.0, *I* 0.1) at pressures up to 20 MPa involve a pH change

Fig. 1. Observation of change in total NADH after pressure perturbation in phosphate buffer

Initial concentrations were: enzyme, $4.2\ \mu\text{M}$; NAD^+ , 2.0mM ; lactate, 2.0mM . The pressure change (arrow) was 17MPa . The rapid initial decrease in A_{340} was shown by control experiments to be due to the effect of pressure perturbation on free NAD^+ . The slower increase in A_{340} is described by a single relaxation with a reciprocal relaxation time ($1/\tau_2$) of $8.01 \pm 0.35\text{s}^{-1}$.

of less than 0.005 and are interpreted here as pressure effects at constant pH. The overall equilibrium of $[\text{NADH}][\text{pyruvate}]/[\text{NAD}^+][\text{lactate}]$ was examined at relatively low enzyme concentration ($0.06\ \mu\text{M}$ -enzyme, 2mM - NAD^+ , 2mM -lactate) at pressures up to 20MPa . No change was observed in the equilibrium concentration of NADH by measurement of A_{340} in an experiment in which a change of 0.1% in NADH concentration would have been clearly discernible. This shows that the product of all the intermediate equilibrium constants is essentially unaffected by pressure.

At enzyme concentrations above $1\ \mu\text{M}$ significant amounts of enzyme-bound intermediates are present at equilibrium, and pressure relaxations are observable. Fig. 3 shows the effect of a rapid pressure decrease on the protein fluorescence of an equilibrium mixture. The faster relaxation contributes about 70% of the total amplitude of the change in Tris buffer (Fig. 3a). The reciprocal relaxation time for the faster relaxation ($1/\tau_1$), evaluated by subtraction of a straight line representing the slower relaxation (Fig. 3b), is $299 \pm 47\text{s}^{-1}$. The slower reciprocal relaxation time ($1/\tau_2$) is $15 \pm 9\text{s}^{-1}$ (Fig. 3a).

From observation of changes in A_{340} , a single slow relaxation with $1/\tau_2 = 21 \pm 10\text{s}^{-1}$ was observed, corresponding to the slower relaxation observed by protein fluorescence.

The decrease in protein fluorescence observed after a rapid pressure decrease (Fig. 3) represents an increase in the concentration of NADH-containing

Fig. 2. Observation of change in bound NADH after pressure perturbation in phosphate buffer

Initial concentrations were: enzyme, $5.1\ \mu\text{M}$; NAD^+ , $20\ \mu\text{M}$; lactate, 2.0mM . The protein fluorescence of the enzyme was followed after a pressure change of 18.5MPa (arrow). (a) A single pressure-relaxation experiment. The slower relaxation has a reciprocal relaxation time ($1/\tau_2$), derived from the log plot, of $18.0 \pm 9.3\text{s}^{-1}$. (b) The average of four consecutive experiments, including that shown in (a). The two relaxations have reciprocal relaxation times, determined as described in the text, of $220 \pm 20\text{s}^{-1}$ ($1/\tau_1$) and $15.6 \pm 2.9\text{s}^{-1}$ ($1/\tau_2$). The exponential curve through the original plot was generated by the computer to correspond to the amplitude and $1/\tau_2$ for the slower relaxation.

enzyme complexes (Holbrook & Gutfreund, 1973), whereas the increase in A_{340} reflects an increase in the total concentration of NADH, free and bound. Since the faster relaxation τ_1 involves only bound NADH and not total NADH, it must characterize the transfer between free and bound NADH. The slow relaxation τ_2 , which involves both bound and total NADH, must represent the reduction of NAD^+ .

The amplitudes of the observed changes in protein fluorescence, determined as a fraction of the total fluorescence at atmospheric pressure, were converted

Fig. 3. Observation of change in bound NADH after pressure perturbation in Tris buffer

Initial concentrations were: enzyme, $8.3 \mu\text{M}$; NAD^+ , $20 \mu\text{M}$; lactate, 2.0 mM . The protein fluorescence was recorded after a pressure change at zero time of 18 MPa . (a) Amplitudes of the two relaxations. (b) Evaluation of $1/\tau_1$ by using an expanded time-scale. For procedure see the text. The value of $1/\tau_1$ is $299 \pm 47 \text{ s}^{-1}$. The curve through the experimental record was generated for the sum of an exponential with $1/\tau$ of 299 s^{-1} and the straight line shown.

into changes in the degree of saturation of lactate dehydrogenase with NADH (Holbrook, 1972). The calculated changes in concentration of enzyme-bound NADH are given in Table 1, together with the change in total NADH concentration determined from measurements of A_{340} .

The faster relaxation shows a slight dependence of $1/\tau_1$ on enzyme concentration (Table 2); the variation of $1/\tau_1$ with NAD^+ concentration (20 – $400 \mu\text{M}$) is within the experimental error of the observations. The slow relaxation has a small amplitude at constant pH and was studied more extensively in the 'pH-jump' experiments in phosphate buffer.

The amplitudes of the relaxation observed in nucleotide fluorescence were too small for accurate determination of relaxation times. However, two relaxations were observed, with relaxation times consistent with those observed by protein fluorescence and absorbance. The observation of the faster relaxation in nucleotide fluorescence supports the assignment of this relaxation to transfer between free and bound NADH.

Table 2. Dependence of $1/\tau_1$ on lactate dehydrogenase concentration

Mixtures of NAD^+ ($20 \mu\text{M}$), lactate (2 mM) and enzyme were allowed to equilibrate and subjected to a rapid decrease in pressure. Reciprocal relaxation times were determined as described in the text. Tris buffer ($\text{pH} 8.0$, $I 0.1$) was used. The simulated values of $1/\tau_1$ were obtained by interpolation from values calculated at enzyme concentrations of 2.0 , 4.0 , 6.0 and $8.0 \mu\text{M}$.

Lactate dehydrogenase (μM)	$1/\tau_1$ (s^{-1})	
	Experimental	Simulated
1.8	203 ± 25	159
3.5	209 ± 38	171
5.3	228 ± 46	182
7.9	252 ± 50	199

Table 1. Amplitudes of relaxations determined from pressure perturbations (15 MPa) and simulation experiments

The concentration changes from pressure perturbations were determined at $8 \mu\text{M}$ -enzyme, $20 \mu\text{M}$ - NAD^+ and 2 mM -lactate as described in the text. The amplitudes of simulated relaxations are the computer-calculated responses to 10% perturbations in k_{-6} , k_{-7} and k_{+9} , with in addition a pH change of 0.05 for the simulation of pressure plus pH change, for the same concentrations.

	Change in bound NADH (μM)			Change in total NADH (μM)
	Total change	Fast phase	Slow phase	
Experimental				
Tris buffers	0.122	0.092	0.030	0.10 ± 0.01
Phosphate buffers	0.241	0.048	0.193	0.26 ± 0.02
Simulated				
Pressure only	0.117	0.093	0.024	0.073
Pressure+pH	0.227	0.110	0.117	0.298

Experiments in phosphate buffer

Experiments carried out in phosphate buffer (pH 8.0, $I 0.1$) involve an increase in pH of about 0.07 for a decrease in pressure of 20 MPa (Davis & Gutfreund, 1976). This increase leads to a perturbation of the overall equilibrium of the reaction towards increasing NADH concentration. At low enzyme concentration ($0.06 \mu\text{M}$ -enzyme, 2 mM - NAD^+) a pressure decrease of 20 MPa produced an increase in absorbance corresponding to a 4% increase in the NADH concentration.

Under these conditions we observed two relaxations from measurements of protein-fluorescence changes (Fig. 2). The values of $1/\tau_1$ ($220 \pm 20 \text{ s}^{-1}$ at $5.1 \mu\text{M}$ -enzyme) and $1/\tau_2$ ($23.6 \pm 3.5 \text{ s}^{-1}$ at $7.9 \mu\text{M}$ -enzyme) agree with those determined at constant pH in Tris buffer. Observations of changes in A_{340} show a single relaxation (Fig. 1) at all enzyme and NAD^+ concentrations used. The reciprocal relaxation time is similar to the value of $1/\tau_2$ determined from protein-fluorescence measurements under the same con-

ditions and that determined from absorbance measurements in Tris buffer.

The amplitudes in phosphate buffer, however, are different from those in Tris buffer. In phosphate the total observed change in NADH concentration, monitored by either protein fluorescence (bound NADH) or absorbance (total NADH) is about twice as large as in Tris (Table 1). The faster relaxation contributes about 30% of the observed change in protein fluorescence in phosphate buffer (Fig. 2), compared with about 70% in Tris.

Fig. 4. Dependence of $1/\tau_2$ on initial concentration of lactate dehydrogenase

Initial concentrations were: NAD^+ , $20 \mu\text{M}$; lactate, 2.0 mM . The experimental values (solid symbols) were determined in phosphate buffer. Results from recording of absorbance ($\bullet$) and protein fluorescence ($\blacksquare$) are shown. The error bars represent $\pm \text{s.d.}$ Simulated values (open circles) were determined as described in the text, being a combined pressure and pH perturbation.

Fig. 5. Dependence of $1/\tau_2$ on initial NAD^+ concentration

The initial concentrations of enzyme and lactate were $4.2 \mu\text{M}$ and 2.0 mM respectively, in phosphate buffer. Relaxation times were determined as described in the text. The error bars represent $\pm \text{s.d.}$; where error bars are not shown, they are less than the radius of the circle.

Fig. 6. Observation of change in nucleotide fluorescence after pressure perturbation in phosphate buffer

Initial concentrations were: enzyme, $1.7 \mu\text{M}$; NAD^+ , $20 \mu\text{M}$; lactate, 2.0 mM . The decrease in NADH fluorescence was recorded after a pressure change at zero time of 15 MPa . The trace shown is the average of four consecutive records and has a reciprocal relaxation time ($1/\tau_2$) of $5.6 \pm 1.1 \text{ s}^{-1}$.

The increase in $1/\tau_2$ with increasing total enzyme concentration is shown in Fig. 4. The value of $1/\tau_2$ decreases slightly with increasing NAD^+ concentration (Fig. 5), which is in agreement with the behaviour of the simulated model (see below).

From measurement of nucleotide fluorescence, a slow relaxation was observed (Fig. 6), with $1/\tau_2$ values ($24.5 \pm 4.6 \text{ s}^{-1}$ at $7.9 \mu\text{M}$ -enzyme, $5.6 \pm 1.1 \text{ s}^{-1}$ at $1.7 \mu\text{M}$ -enzyme) consistent with those determined from measurement of protein fluorescence or absorbance. The fast relaxation observed by protein fluorescence was assigned to the binding of NADH. This implies that a fast relaxation should be present in measurements of nucleotide fluorescence, which reflects mainly the concentration of the enzyme-NADH binary complex (Holbrook & Gutfreund, 1973). In fact, a fast relaxation appears to be present in records of nucleotide-fluorescence changes at $1.7 \mu\text{M}$ -enzyme (Fig. 6), but the small amplitude and relatively noisy signals prevented accurate determination of relaxation times and comparison with those obtained from protein-fluorescence measurements.

Simulation experiments

Simulations were carried out by numerical integration (Wiberg, 1974) of the differential equations describing the kinetic model given in Scheme 1. The final set of rate constants used to obtain the simulated results quoted below are given in Table 3.

Table 3. *Individual rate constants for the lactate dehydrogenase reaction*

Values of the rate constants of Scheme 1 used in the simulation experiments are given. All values are at pH 8.0 and 20–25°C. References: a, Schwert *et al.* (1967); b, see the text; c, $k_{-5} [\text{H}^+] = 200 \text{ s}^{-1}$ at pH 8.0 (Holbrook *et al.*, 1976); d, from Holbrook *et al.* (1976); e, from Whitaker *et al.* (1974).

Rate constant	Value	Unit	Reference
k_{+1}	3.0×10^6	$\text{M}^{-1} \cdot \text{s}^{-1}$	a
k_{-1}	340	s^{-1}	a
k_{+2}	9×10^5	$\text{M}^{-1} \cdot \text{s}^{-1}$	a
k_{-2}	1100	s^{-1}	b
k_{+3}	3500	s^{-1}	b
k_{-3}	10000	s^{-1}	b
k_{+4}	900	s^{-1}	b
k_{-4}	800	s^{-1}	b
k_{+5}	400	s^{-1}	b
k_{-5}	2.0×10^{10}	$\text{M}^{-1} \cdot \text{s}^{-1}$	c
k_{+6}	150	s^{-1}	d
k_{-6}	3.5×10^6	$\text{M}^{-1} \cdot \text{s}^{-1}$	d
k_{+7}	12850	s^{-1}	b
k_{-7}	3.0×10^9	$\text{M}^{-1} \cdot \text{s}^{-1}$	b
k_{+8}	500	s^{-1}	b
k_{-8}	2.5×10^9	$\text{M}^{-1} \cdot \text{s}^{-1}$	b
k_{+9}	50	s^{-1}	e
k_{-9}	3.0×10^7	$\text{M}^{-1} \cdot \text{s}^{-1}$	e

The initial concentrations of NAD^+ , lactate and enzyme were set, the simulation was run to equilibrium and the equilibrium concentrations of the intermediates were noted. To simulate pressure-relaxation experiments, the system was perturbed to a new (high-pressure) equilibrium, the concentrations were recorded and then the variable corresponding to the desired optical signal was plotted against time after restoration of the original (atmospheric-pressure) parameters (Table 3). The reciprocal relaxation times were obtained from logarithmic plots of the simulated data. To simulate the effect of pressure perturbation at constant pH (corresponding to experiments in Tris buffer), k_{-6} , k_{-7} and k_{+9} were increased by 10% at the high pressure, and the overall equilibrium constant remaining unchanged (K 4.0 pM). This particular approach was chosen because we concluded from our experimental data that the fast relaxation involved transfer between free and bound NADH. Preliminary experiments with a simpler model (Coates *et al.*, 1977) showed unsatisfactory agreement between observed and simulated amplitudes when the rate constants changed did not include those of NADH binding or dissociation. The simulation of the combined effects of pH change and direct pressure perturbation was obtained by simultaneously changing k_{-6} , k_{-7} , k_{+9} and the H^+ concentration; the pH was decreased by 0.05 unit for a 15 MPa pressure increase (Davis & Gutfreund, 1976).

Many of the rate constants in Table 3 were taken from the results of steady-state and transient kinetic experiments. The value of k_{+4} given by Whitaker *et al.* (1974) is 1200 s^{-1} ; however, allowing for the contribution of the steady-state rate to the burst rate constant (Gutfreund, 1975, p. 200) decreases k_{+4} to about 900 s^{-1} . The rate-limiting step for single-turnover pyruvate reduction at low pH is taken to be k_{-4} , and the observed pH-dependence (Boland & Gutfreund, 1975) suggests that k_{-4} is about 800 s^{-1} . The rate constants k_{+3} and k_{-3} are unknown, but their ratio is defined by the proportion of hidden transient (phase 1) in lactate oxidation (0.3 at pH 8.0; Whitaker *et al.*, 1974). Both k_{+3} and k_{-3} are taken to be faster than 1000 s^{-1} , since k_{+3} , the slower one, controls the hidden phase in the stopped-flow experiments (Whitaker *et al.*, 1974).

The ratio k_{+8}/k_{-8} represents the ionization of the enzyme-NADH binary complex ($\text{p}K_a$ 6.7; Holbrook & Gutfreund, 1973). Because pyruvate binding at pH 8.0 occurs before proton uptake (Boland & Gutfreund, 1975), k_{-8} was made sufficiently small to give a negligible flux through the protonated binary complex at pH 8.0. The ratio k_{+5}/k_{-5} represents the ionization of the enzyme-NADH-pyruvate ternary complex; the $\text{p}K_a$ is probably higher than 6.7 (Holbrook & Gutfreund, 1973; Boland & Gutfreund, 1975), but the exact value has not been determined.

Therefore the ratio, and hence k_{+5} , was varied when we attempted to find a set of parameters that gave simulated behaviour in reasonable agreement with the transient and relaxation experiments. The value of k_{-2} is defined for each k_{+5} value by the relationship:

$$\frac{k_{+1}k_{+2}k_{+3}k_{+4}k_{+5}k_{+6}k_{+9}}{k_{-1}k_{-2}k_{-3}k_{-4}k_{-5}k_{-6}k_{-9}} = K_{\text{eq.}} = 4.0 \mu\text{M}$$

The ratio k_{+7}/k_{-7} is also defined once k_{+5} is set. The value of k_{+7} must be much greater than k_{-4} to give the full bursts observed in lactate oxidation (Whitaker *et al.*, 1974). Also, the second-order rate constant for pyruvate binding, k_{-7} , must be set at a value that is not unreasonably fast.

In simulations of the transient kinetic experiments of Whitaker *et al.* (1974) at pH 8.0, the correct hidden phase amplitude was obtained with $k_{+3}/k_{-3} = 0.35$. The total transient amplitude (phases 1 and 2) was effectively equal to 1 mol of NADH per mol of enzyme sites, after taking into account the steady-state rate (Gutfreund, 1975, p. 201), when k_{+7} was 10 times k_{-4} , or greater. Because the pK_a of the enzyme-NADH-pyruvate complex is unknown, simulations of pressure-jump experiments were carried out with pK_a values in the range 7.2–8.7 (and k_{+5} values between 1260 and 40s^{-1}) for standard initial concentrations of enzyme of $8\mu\text{M}$, of NAD^+ $20\mu\text{M}$ and of lactate of 2 mM.

The simulations, with both perturbations, showed only two relaxations of significant amplitude. Both relaxations were present when the sum of NADH-containing complexes (bound NADH) was recorded (Figs. 7 and 8), corresponding to the behaviour observed by protein fluorescence. The slower relaxation generally predominated when total NADH (free and bound) was recorded. Although the fast relaxation was present in the simulations for each pK_a , the amplitude was usually so small that it would not have been observed by A_{340} in the experiments. However, for a ternary-complex pK_a of 8.7, the faster relaxation contributed 60% of the total amplitude in the simulation of a constant-pH experiment, and this is clearly inconsistent with our failure to observe a fast relaxation in the corresponding (absorbance) experiment.

Since, in our experiments, we were able to measure relaxation times more precisely than amplitudes, the former were regarded as the main criteria for correspondence between simulations and experiments. The simulated slower reciprocal relaxation time increases from 13.3s^{-1} for a ternary-complex pK_a of 7.2 to 18.9s^{-1} for a pK_a of 7.7 or 8.2; the higher $1/\tau_2$ value is in reasonable agreement with the observed value of $1/\tau_2$, $23.6 \pm 3.5\text{s}^{-1}$ at $7.9\mu\text{M}$ -enzyme. The simulated $1/\tau_2$ for a pK_a of 8.7 is lower than that for a pK_a of 7.7 or 8.2. The faster reciprocal relaxation times ($1/\tau_1$) obtained from the simulations

Fig. 7. Simulation of change in bound NADH after pressure perturbation at constant pH: for comparison with experiments in Tris buffer at pH 8.0

Initial concentrations were: enzyme, $8.0\mu\text{M}$; NAD^+ , $20\mu\text{M}$; lactate, 2.0 mM. The model given in Scheme 1 was used, with the rate constants in Table 3. The effect of a 15 MPa change in pressure (at zero time) was simulated by 10% changes in k_{-6} , k_{-7} and k_{+9} (see the text for details). The log plot is shown to 95% of the total change in bound NADH. Two relaxations are visible, with reciprocal relaxation times of 199s^{-1} ($1/\tau_1$) and 19.0s^{-1} ($1/\tau_2$).

Fig. 8. Simulation of change in bound NADH after combined pressure and pH perturbation: for comparison with experiments in phosphate buffer at pH 8.0

Initial concentrations were: enzyme, $8.0\mu\text{M}$; NAD^+ , $20\mu\text{M}$; lactate, 2 mM. The model given in Scheme 1 was used, with the rate constants in Table 3. The effect of a 15 MPa change in pressure (at zero time) was simulated by 10% changes in k_{-6} , k_{-7} and k_{+9} and a 0.05 unit pH change (see the text). The log plot is shown to 95% of the total change in bound NADH. The $1/\tau$ values are as given in the legend to Fig. 7.

were not greatly dependent on the pK_a chosen. Since the relative amplitudes of the two relaxations in the simulations were a little closer to those observed experimentally when the ternary-complex pK_a was set at 7.7, the corresponding set of rate constants (Table 3) was used for all simulations discussed below.

The amplitudes of the relaxations recorded for total NADH and for bound NADH in the simulation experiments with the rate constants in Table 3 are given in Table 1, together with the amplitudes calculated from observed changes in absorbance and protein fluorescence. The magnitude of the change in k_{-6} , k_{-7} and k_{+9} , used to simulate a pressure perturbation at constant pH, was chosen to give satisfactory agreement between the simulated total amplitude for bound NADH and the total amplitude calculated from the protein-fluorescence experiments. A change of 10% in k_{-6} , k_{-7} and k_{+9} (for a pressure change of 15 MPa) corresponds to a volume of reaction of $-16\text{ cm}^3\cdot\text{mol}^{-1}$, which is not unreasonable (Davis & Gutfreund, 1976). Table 1 shows that the simulated total amplitudes for effects involving simultaneous pressure perturbation and pH change are approximately double the amplitudes for pressure change alone, in agreement with the observed difference between experiments in phosphate and Tris buffer. Furthermore, the proportions of the total change in bound NADH attributable to the two relaxations are significantly different in simulations of pressure perturbation at constant pH and of simultaneous pressure and pH change (Figs. 7 and 8 and Table 1). This is in agreement with the observation that the proportion of the protein-fluorescence change contributed by the faster relaxation is considerably greater in Tris buffer. However, the contribution of the faster relaxation to the change in bound NADH is higher in the simulation of a combined pressure and pH perturbation than in the experiments in phosphate buffer. Also the amplitude (μM) of the fast phase for bound NADH is considerably less in phosphate than in Tris buffer, whereas in the simulations the inclusion of a pH perturbation increases the amplitude slightly. This indicates that the effect of pH must be rather more complex than we have assumed in the simulations and therefore in the proposed mechanism.

The simulated relaxation times for the rate constants in Table 3 show pleasing agreement with the experimental values, in view of the number of rate constants used in the simulation and the uncertainties in their values. For example, at an enzyme concentration of $8.0\text{ }\mu\text{M}$ simulation gives $1/\tau$ values of 197 s^{-1} and 18.9 s^{-1} compared with experimental values of $252 \pm 50\text{ s}^{-1}$ and $23.6 \pm 3.5\text{ s}^{-1}$. Furthermore, the effects of enzyme and NAD^+ concentration on the relaxation times are consistent with those observed experimentally. The simulations predict a positive dependence of $1/\tau_2$ on enzyme concentration, in agreement with

the experimental results (Fig. 4); also predicted is a decrease in $1/\tau_2$ with increasing total NAD^+ concentration, for which the corresponding experimental behaviour is shown in Fig. 5. Although the experimentally determined values of $1/\tau_1$ (Table 2) have large standard deviations that do not allow firm conclusions with regard to concentration-dependence, the apparent trend in $1/\tau_1$ values is clearly consistent with the positive dependence on enzyme concentration predicted by the simulations (Table 2).

Conclusions

Holbrook & Gutfreund (1973) presented kinetic and chemical evidence for a mechanism of lactate oxidation with three essential steps after substrate binding. The catalytic step is observed as a rapid equilibration between enzyme-bound substrates and products, with the proton from the hydroxy group of lactate transferred to the imidazole group of histidine-195 of the enzyme. Dissociation of the three products occurs in only two distinguishable steps, first the appearance of free H^+ and pyruvate and secondly the appearance of free NADH.

During the last few years further work has been concentrated on gaining more information about the steps involved in the dissociation and association of the three products of lactate oxidation. This involved studies in both directions of the processes on the right-hand side of the hydride-transfer reaction in Scheme 1 (Whitaker *et al.*, 1974; Boland & Gutfreund, 1975; Holbrook *et al.*, 1976).

The pressure-relaxation and computer-simulation studies reported in the present paper were designed to test and refine the proposed mechanisms. The experimental data reported above are recorded in Scheme 1 and Table 3, which are derived from the transient kinetic studies in this laboratory.

The agreement of simulated reaction profiles with both transient and relaxation kinetics is reasonable. It does not seem worth while to refine the rate constants by extensive curve fitting unless more accurate experimental values are obtained by improved methods.

Some general lessons have been learned from the analysis of the relaxation experiments on this reversible enzyme-catalysed reaction. The reaction of lactate dehydrogenase is particularly suitable for such an investigation because of the number of different signals available for the concentrations of intermediates and products. Yet it is quite clear that the results can only be interpreted faithfully because of the extensive previous studies by transient and steady-state kinetics as well as chemical investigations.

Bernasconi's (1976) treatment of relaxations of enzyme reactions points to the difficulties in obtaining

any detailed information about the mechanism of the reaction caused by the coupled nature of the slow relaxation τ_2 . This is a general feature of complete enzymic systems where regeneration of free enzyme and cyclic redistribution of intermediates, in a turnover-like process, occur during the establishment of the new equilibrium concentrations after perturbation.

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