Pig Heart Lactate Dehydrogenase

BINDING OF PYRUVATE AND THE INTERCONVERSION OF PYRUVATE-CONTAINING TERNARY COMPLEXES

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1. Lactate oxidation catalysed by pig heart lactate dehydrogenase was studied in the presence of inhibitory concentrations of pyruvate. Experimental results show the presence of an intermediate which occurs immediately after the hydride transfer step, but before the dissociation of pyruvate and the H^+ produced by the reaction. The rate constant for pyruvate dissociation and the dissociation constant for pyruvate from the ternary complex differ from those obtained in pyruvate reduction experiments. 2. In single-turnover pyruvate reduction by pig heart lactate dehydrogenase at pH 8.0, pyruvate can bind to the enzyme before a H^+ is taken up, and the subsequent uptake of a H^+ is governed by a step that is also rate-limiting for single-turnover and steady-state NADH oxidation. 3. Observation of various intermediates in the single-turnover pyruvate reduction experiments has made it possible to determine separately the dissociation constant and K_m value for pyruvate at pH 8.0, and also the catalytic turnover rate and K_m for pyruvate under first-order conditions at different pH values. 4. Further studies on single-turnover pyruvate reduction carried out in 2H_2O , or in water at low temperature, show another step which, under these conditions, is slower than that controlling H^+ uptake and rate-limiting for NADH oxidation. A scheme is presented which explains these results.

Recent studies have resulted in considerable elucidation of our understanding of the reactions of lactate dehydrogenase with its substrates, in particular the pig heart enzyme. Studies on lactate oxidation by lactate dehydrogenase (Whitaker *et al.*, 1974) on the ms time-scale show three distinct phases of reaction. The first of these, the 'instantaneous' phase, is complete within the first ms of reaction and shows the formation of a complex containing NADH. The fraction of the enzyme in the NADH-containing complex formed during the first phase is pH-dependent and varies from 0.3 at pH 8.0 to 0.08 at pH 6.0. The second phase of the reaction is first-order and shows complete saturation of the enzyme with NADH, appearance of nucleotide fluorescence and release of a H^+ . This phase has been identified as showing the dissociation of pyruvate from the ternary complex, with a dissociation rate constant of about $1150s^{-1}$. The final phase involves release of

pyruvate binding to enzyme-NADH under conditions for pyruvate reduction show a dissociation rate constant that is much too slow to be compatible with that obtained by Whitaker *et al.* (1974), and it was decided to investigate the binding of pyruvate to enzyme-NADH under conditions of lactate oxidation.

Holbrook & Stinson (1973) have shown that the binding of oxamate and of *o*-nitrophenylpyruvate to pig heart lactate dehydrogenase depends on the protonation of a group with a pK_a of 6.8 ± 0.2 . The binding of oxamate to enzyme-NADH at high pH is known to cause the uptake of a H^+ at a rate too fast to be measured by stopped-flow methods (Holbrook & Gutfreund, 1973). These results have been interpreted to show that carbonyl compounds will only bind to the protonated form of enzyme-NADH (Holbrook & Stinson, 1973; Holbrook & Gutfreund, 1973):

protons and appearance of free NADH in a zero-order process due to steady-state turnover.

Experiments described in this paper investigating

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In either case the results can also be interpreted as showing a rapid H^+ -uptake step after binding, which can affect binding. However, the temperature-jump studies by Heck (1969) on the binding of oxamate to enzyme-NADH show that the relaxation time

Scheme 1. Formation of ternary enzyme-NADH-pyruvate complex
For details see the text.

extrapolated to zero concentration of enzyme and oxamate is pH-independent. This result is only compatible with a model in which the enzyme must be protonated before oxamate binds. Preliminary experiments in this laboratory using pH indicators seemed to show a result in conflict with the above mechanism (N. G. Bennett & H. Gutfreund, unpublished results).

The aim of the investigations described in the present paper was to investigate further the steps involved in the equilibria between the ternary enzyme-NADH-pyruvate complex and this free substrate pair. Scheme 1 shows a plausible kinetic mechanism that is compatible with all our results. The various starred forms of the enzyme indicate 'isomers', the interconversion of which is kinetically significant.

Experimental

Materials

Pig heart lactate dehydrogenase was obtained from Boehringer (Mannheim) G.m.b.H., Mannheim, Germany (batches 7324515, 7404315 and 7493115), and stock solutions were prepared as described by Whitaker *et al.* (1974). All molarities of enzyme expressed in the present paper refer to the concentration of active sites assuming 4 equivalent subunits per molecule.

NAD⁺ was obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. (lot 1208817), and solutions were prepared as described by Whitaker *et al.* (1974) except that the anion-exchange resin used was Sephadex A-25 (Pharmacia Ltd., Uppsala, Sweden) in a column of 1.5 cm × 10 cm.

NADH was obtained from Boehringer (Mannheim) G.m.b.H. (batches 7251179 and 7172114) and used without further purification. DL-Lithium lactate was used from the preparation of Whitaker *et al.* (1974). Assays showed that the L-lactate content was

unchanged since its preparation. Sodium pyruvate was obtained from L. Light and Co., Colnbrook, Bucks., U.K. Assays showed that it had a purity in excess of 95% and it was used without further purification. ²H₂O (99.7%) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and Phenol Red and Chlorophenol Red were obtained from the same supplier. All other reagents were of AnalaR or equivalent purity.

Phosphate buffers were made up by mixing the appropriate dilutions of 0.5M stock solutions of disodium hydrogen phosphate and potassium dihydrogen phosphate to give the required pH. All solutions other than buffers were made up fresh daily, or on the previous evening in the case of some NAD⁺ solutions, and stored refrigerated overnight.

Instrumentation

Rapid changes in extinction were recorded on a conventional single-beam stopped-flow apparatus, with a dead-time of 2.5 ms, constructed by Dr. D. W. Yates in this laboratory.

Rapid changes in nucleotide or protein fluorescence were recorded on a stopped-flow fluorimeter as described by Bagshaw *et al.* (1972). Nucleotide fluorescence was excited at 340 nm by using 10 nm-bandpass slits in the monochromator and observed through Wratten 47B and 2B filters. These filters have a maximum transmission in the range 430–480 nm. For protein fluorescence experiments the excitation wavelength was 300 nm, or 310 nm in cases where a high concentration of nucleotide was present, and Schott WG345 and UG11 filters were used in the observation path. The dead-time of this apparatus has been determined to be 1 ms (Whitaker *et al.*, 1974).

Spectra and slow extinction changes (such as in assays) were recorded on a Unicam SP.1800 spectrophotometer (Pye-Unicam Ltd., Cambridge, U.K.).

Methods and Results

In all experiments described the enzyme was kept in one syringe of the stopped-flow apparatus, and nucleotide, substrate and inhibitor (where used) were placed in the other syringe. This precludes any slow turnover of the enzyme due to minor contaminants. Plastic gloves were worn in all lactate oxidation and fluorescence experiments to avoid contamination of solutions with sweat (which contains appreciable amounts of lactate).

All experiments were carried out at room temperature (20°C) unless otherwise stated.

Pyruvate-binding experiments

In these experiments the binding of pyruvate to the enzyme-NADH binary complex is examined under various conditions.

(a) *Pyruvate inhibition of lactate oxidation monitored by nucleotide fluorescence.* When NADH is irradiated with light at about 340 nm it fluoresces with a maximum emission at 450 nm. When NADH binds to lactate dehydrogenase the intensity of this fluorescence is enhanced 3.5-fold (Velick, 1958), and when pyruvate binds to the enzyme-NADH complex the fluorescence is quenched to a value that is sufficiently low to be regarded as insignificant for these experiments (less than 7% of the enhanced value). When turnover occurs the fluorescence disappears entirely.

For lactate oxidation the rate-limiting step is the dissociation of NADH from the enzyme, and the highly fluorescent enzyme-NADH complex accounts for most of the enzyme in the steady state. When lactate dehydrogenase is mixed with NAD⁺ and lactate, a rapid increase in nucleotide fluorescence is seen due to the formation of enzyme-NADH as the steady state is established, and this is followed by a slower zero-order increase in nucleotide fluorescence due to the appearance of free NADH as turnover occurs. In the absence of added pyruvate, the rate of the rapid increase of nucleotide fluorescence is a measure of the rate constant for dissociation of pyruvate from the ternary complex.

Studies of the effect of added pyruvate on lactate oxidation were carried out in 0.1 M-phosphate buffers, with 2–4 μ M enzyme in one syringe of the stopped-flow fluorimeter, and 100 mM-lithium lactate and 2 mM-NAD⁺ in the other syringe. Various amounts of sodium pyruvate were added to the syringe containing the lactate, and experiments were carried out at pH 6.0, 7.0 and 8.0. Typical traces from experiments at pH 8.0 are shown in Fig. 1. The rate constant for the transient phase was obtained for each trace by the method described by Gutfreund (1972). Appropriate plots are shown in Fig. 1.

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When an appreciable amount of pyruvate is added, the main intermediate in the steady state will be an enzyme-NADH-pyruvate ternary complex instead of an enzyme-NADH binary complex. The transient observed as nucleotide fluorescence in these experiments therefore decreases in amplitude when pyruvate is added. The decrease in amplitude results in an apparent increase in the observed rate constant for the transient phase, and the dependence of this rate constant on pyruvate concentration is shown for each pH in Fig. 2. The line in Fig. 2 is described by the equation:

$$k_{\text{obs.}} = k_{\text{off}} + k_{\text{on}}[\text{Pyr}]$$

where $k_{\text{obs.}}$ is the rate constant of the observed transient, k_{off} is the apparent rate constant for dissociation of pyruvate from the ternary enzyme-NADH-pyruvate complex and k_{on} is the second-order rate constant for the binding of pyruvate to the newly formed enzyme-NADH complex. The ratio of the intercept to the slope will therefore give an apparent dissociation constant for pyruvate at each pH value. These values are shown in Table 1.

(b) *Pyruvate inhibition of lactate oxidation monitored by protein fluorescence.* Protein fluorescence is quenched by the presence of bound NADH, whether pyruvate is present or not. The quenching is not a linear function of saturation of the enzyme by NADH, and the mathematical function describing this quenching in terms of NADH saturation has been derived by Holbrook (1972). This function was used to convert observed fluorescence into fractional saturation of the enzyme with NADH.

Solutions used were identical with those described above for nucleotide fluorescence experiments. The apparatus was calibrated to give a signal of 8 V for the unquenched protein fluorescence. In the presence of NADH the fully quenched enzyme gave a signal of 2.8–3.2 V. Background fluorescence due to the buffer and substrates, plus stray light, gave a signal of 1.7–2.0 V, which was subtracted from the above signals to obtain the true fluorescence of the enzyme in each case. Typical traces are shown in Fig. 3.

The 'instantaneous' phase of protein fluorescence quenching described by Whitaker *et al.* (1974) was found to be present in all cases, but at low pH the amplitude of the observed phase decreased considerably in the presence of added pyruvate. At high pH values, however, the observed phase was apparently unaffected by added pyruvate. The final value reached by the observed phase shows the fraction of the enzyme that, in the steady-state reaction, contains bound NADH. This fraction is shown as a function of pyruvate concentration in Fig. 4, and the value reached at high pyruvate concentrations at each pH is given in Table 1.

(c) *Pyruvate inhibition of lactate oxidation monitored by Phenol Red.* The production of protons can be

Fig. 1. Reaction of lactate dehydrogenase with NAD^+ and lactate in the absence (a) and in the presence (b) of pyruvate, monitored by nucleotide fluorescence

(a) The reaction mixture contained $1 \mu\text{M}$ pig heart lactate dehydrogenase (final subunit concentration) from one syringe, and 50 mM -lithium lactate and 1 mM - NAD^+ from the other, in 0.1 M -phosphate buffer, $\text{pH } 8.0$, 20°C . Two identical traces are superimposed. (b) The reaction mixture was the same as for (a) but also contained 0.1 mM -sodium pyruvate from the syringe containing nucleotide and lactate. Two identical traces are superimposed. (c) Semi-logarithmic plots of the approach of the transient fluorescence ($F_{\text{obs.}}$) to that predicted by back extrapolation ($F_{\text{extrap.}}$) of the linear steady-state phase against time. ○, Data from (a); △, data from (b). Rate constants derived are 161 s^{-1} and 308 s^{-1} for (a) and (b) respectively.

Fig. 2. Dependence on pyruvate concentration of the rate constant for the pre-steady-state transient observed by nucleotide fluorescence when lactate dehydrogenase reacts with NAD^+ and lactate

Conditions are as described in Fig. 1 but with various amounts of sodium pyruvate added. Results are shown for $\text{pH } 6.0$ (□), 7.0 (△) and 8.0 (○).

monitored by using Phenol Red and carrying out the reaction in a very dilute buffer solution. In these experiments the buffer was 0.5 mM -phosphate containing 0.1 M - NaCl , and the pH was adjusted to 8.0 immediately before loading into the syringes of the stopped-flow apparatus. The solution in one syringe contained $20 \mu\text{M}$ enzyme and that in the other syringe contained 5.4 mM - NAD^+ and 100 mM -lithium lactate. Both solutions also contained $20 \mu\text{M}$ -Phenol Red. The reaction was monitored by the changes of

E_{560} . Subsequent reactions were then carried out with pyruvate added to the syringe containing lactate and NAD^+ , to give a final concentration of $250 \mu\text{M}$. Careful examination of the control experiment showed an exponential approach to a steady-state linear phase (cf. Fig. 2 of Whitaker *et al.*, 1974). The inhibited experiment, however, showed only a much decreased linear steady-state phase. The difficulty in observing the pre-steady-state phase in the control experiment is due to the relatively high steady-state rate, so, if a significant pre-steady-state step were to occur in the inhibited experiment, it would be expected to show itself more clearly than in the control experiment. This experiment therefore shows that the intermediate, which builds up in the steady-state reaction when it is inhibited by an excess of pyruvate, is still protonated.

(d) *Observation of pyruvate binding to enzyme-NADH by nucleotide fluorescence quenching.* When NADH at a concentration well in excess of the dissociation constant is pre-mixed with lactate dehydrogenase in a slight molar excess, the highly fluorescent enzyme-NADH complex is formed nearly quantitatively. When pyruvate binds to the binary complex the fluorescence is more than 90% quenched. At moderate pyruvate concentrations the dead-time of the stopped-flow fluorimeter (1 ms) is sufficiently small to observe the binding of pyruvate directly by quenching of nucleotide fluorescence at $\text{pH } 8.0$.

Fig. 3. Effect of pyruvate on the reaction of lactate dehydrogenase with NAD^+ and lactate, observed by protein fluorescence

All solutions contained 1–2 μM pig heart lactate dehydrogenase added from one syringe, and 50 mM-lithium lactate and 1 mM- NAD^+ from the other, in 0.1 M-phosphate buffer, 20°C. Sodium pyruvate, when used, was added from the syringe containing the nucleotide and lactate. Duplicate traces are superimposed in all photographs. Individual details are as follows: (a) pH 6.0, no pyruvate; (b) pH 6.0, 50 μM -pyruvate; (c) pH 7.0, no pyruvate; (d) pH 7.0, 100 μM -pyruvate; (e) pH 8.0, no pyruvate; (f) pH 8.0, 100 μM -pyruvate. The vertical scale on each left-hand trace also applies to the corresponding right-hand trace. The signal obtained from fully quenched enzyme was approx. 5 V lower than that from unquenched enzyme in all these experiments.

For these experiments 0.1 M-phosphate buffer, pH 8.0, was used. One syringe contained 13 μM -enzyme and 10 μM -NADH and the other contained various amounts of pyruvate. Under these conditions the NADH will be more than 90% bound to the enzyme before mixing. The observed trace, a typical example of which is shown in Fig. 5, is biphasic. The first phase represents binding of pyruvate to the enzyme–NADH complex; this step results in only limited quenching because the pyruvate concentration is near or below its K_m value. The slower phase therefore represents the oxidation

Fig. 4. Dependence on pyruvate concentration of the fraction of enzyme containing bound NADH in the steady state, calculated from the observed amplitude of protein fluorescence quenching when lactate dehydrogenase reacts with NAD^+ and lactate

Conditions are as described in Fig. 3 but with various pyruvate concentrations. Results are shown for pH 6.0 ($\square$), 7.0 (Δ) and 8.0 ($\circ$). Fractional saturation was calculated from fluorescence quenching by using the equation of Holbrook (1972).

of NADH. Because the concentration of pyruvate was kept high relative to that of NADH (and hence enzyme–NADH), both phases were treated as effectively first-order and the rate of the fast phase was obtained by subtraction of the contribution of the second phase, obtained by back extrapolation. The relevant semi-logarithmic plots are given in Fig. 5.

The dependence of the apparent rate constant for pyruvate binding on pyruvate concentration is shown in Fig. 6. The rate constants shown are not greatly different from those obtained at higher pH and lower temperature by Südi (1974). Our interpretation of the results is, however, slightly different. The equation for the disappearance of free enzyme (in this case enzyme–NADH) when a ligand (pyruvate) binds, and the complex subsequently turns over at a rate k_r , is:

$$k_{\text{obs.}} = k_{\text{on}}[\text{Pyr}] + k_{\text{off}} + k_r$$

provided the ligand concentration is well in excess of the enzyme concentration (Bagshaw *et al.*, 1974). Therefore the slope of the line in Fig. 6 will give the second-order rate constant for pyruvate binding, and the intercept will give the sum of the dissociation and turnover rate constants, not solely the dissociation rate constant (cf. Südi, 1974). Since the turnover rate constant at pH 8.0 is approximately 200 s^{-1} , it appears from Fig. 6 that the dissociation rate constant must be less than 100 s^{-1} and the dissociation constant for pyruvate must be in the range of 10–35 μM , considerably lower than the corresponding K_m value.

Table 1. Data obtained from pyruvate inhibition of lactate oxidation studies

pH	...	...	...	...	6.0	7.0	8.0
$10^{-6} \times$ Association rate constant for pyruvate ($M^{-1} \cdot s^{-1}$)					1.38	1.34	1.16
Apparent dissociation rate constant for pyruvate (s^{-1})					76	151	190
Apparent dissociation constant for pyruvate (μM)					51	113	200
Fractional saturation of lactate dehydrogenase with NADH in the presence of saturating pyruvate					0.32	0.75	≥ 0.9
Fractional saturation of lactate dehydrogenase with NADH in the 'instantaneous' phase*					0.08	0.22	0.30

* From Whitaker *et al.* (1974).

Fig. 5. Binding of pyruvate to the lactate dehydrogenase-NADH complex observed by nucleotide fluorescence quenching

The solution contained $6.5 \mu M$ pig heart lactate dehydrogenase and $5.0 \mu M$ -NADH from one syringe, and $105 \mu M$ -pyruvate from the other, in $0.1 M$ -phosphate buffer, pH 8.0, $20^\circ C$. (a) Duplicate oscilloscope traces superimposed. (b) Graph of logarithm (fluorescence) against time, showing the pseudo-first-order turnover and the pre-turnover transient. (c) Graph derived from (b) showing the first-order approach of the observed fluorescence (F_{obs}) to that predicted by back extrapolation of the slow phase (F_{ext}). A rate constant of $471 s^{-1}$ is obtained for this step.

The value of K_m for pyruvate is also implicit in Fig. 6, as the ratio of the intercept to the slope, which gives a value of approx. $95 \mu M$, is in good agreement with the value of $114 \mu M$ obtained in single-turnover experiments (Table 2).

Attempts to carry out similar experiments at pH 6.0 were unsuccessful because the rate of pyruvate binding was too fast to be properly observed even at very low pyruvate concentrations.

Single-turnover pyruvate reduction experiments

If NADH at a concentration well in excess of the dissociation constant is premixed with a slight molar excess of lactate dehydrogenase, the enzyme-NADH complex so formed is capable of only a single turnover as there is no further NADH available. The reaction of the binary complex with pyruvate can be followed in a number of ways. In the following experiments two methods are used. The E_{340} is a direct measurement of all the NADH present, bound or unbound, and the E_{560} , pH 8.0, of added

Phenol Red monitors the change in ionization of intermediates in the reaction by indicating small pH changes in weak buffers.

(a) *Observation of NADH oxidation at 340 nm as a function of pyruvate concentration and pH.* Buffers used were all $0.1 M$ -phosphate ranging from pH 6.0 to 8.5 in $0.5 pH$ steps. No attempt was made to correct for changes of ionic strength with various pH values. Enzyme ($14 \mu M$) was premixed with $10 \mu M$ -NADH, and mixed with various concentrations of pyruvate in the stopped-flow spectrophotometer. A similar experiment has been described by Whitaker *et al.* (1974) at pH 9.0. In all cases a single first-order trace was obtained, yielding a linear plot of logarithm of extinction against time. A typical example is given in Fig. 7. The dependence of the observed rate constant on pyruvate concentration is given for each pH in double-reciprocal plots (Fig. 8). The linearity of these plots shows that we are observing a rate-limiting step for hydride transfer that occurs after pyruvate binding, and not the rate of binding itself. The intercept in each case

gives the rate constant for the step limiting the rate of NADH oxidation under conditions of pyruvate saturation, and the ratio of the slope to the intercept will give the K_m value for that step. Since the value obtained at pH 8.0 was close to the known value for the catalytic-centre activity, $k_{cat.}$ (equivalent to 360 units/mg), it was decided to test whether the rate-limiting step for single-turnover experiments might also be rate-limiting for steady-state conditions. If this were not the case, then the step observed in the single-turnover experiments would appear as a pre-steady-state transient in steady-state experiments. When the experiment was repeated in the same solutions at pH 6.0 and 8.0, but with 200 μ M-NADH in the substrate syringe, no sign of a pre-steady-state transient could be seen, and the derived $k_{cat.}$ was in good agreement with the corresponding rate constant for the single-turnover experiment. Therefore the derived $k_{cat.}$ and K_m values from Fig. 8, which are shown in Table 2, can be considered to be applicable to the steady-state reaction.

(b) *Observation of H^+ uptake at pH 8.0 in Phenol Red.* All solutions were made up in 0.5M-phosphate buffer containing 0.1M-NaCl to maintain enzyme stability. Each solution also contained 20 μ M-Phenol Red. Enzyme, NADH and pyruvate concentrations were as used for the experiments just described. The pH of each solution was finally adjusted with 0.1M-NaOH or -HCl immediately before loading into the appropriate syringe.

A typical trace at E_{560} is shown in Fig. 9. The extinction increases exponentially. The amplitude is a function of the NADH concentration initially present and the buffering capacity of the solution. Plots of $\log(E_{t=\infty} - E_t)$ (final absorbance—

absorbance at time t) against t are linear, as shown in the example in Fig. 9, and the double-reciprocal dependence of the observed rate constant on pyruvate concentration is given in Fig. 10, together with the observed rate constants under the same conditions for NADH oxidation observed at 340 nm

Fig. 6. Dependence of the fast rate constant of nucleotide fluorescence quenching on pyruvate concentration

Conditions are as given in Fig. 5 but with various pyruvate concentrations. The slope gives a second-order rate constant for pyruvate binding to the enzyme-NADH complex of approx. $3 \times 10^6 M^{-1} \cdot s^{-1}$, and the intercept gives a value for the sum of the dissociation rate constant and the turnover rate constant of approx. $285 s^{-1}$. Since the turnover rate at pH 8.0 is $210 s^{-1}$ (Table 2) the dissociation rate constant must be approx. $75 s^{-1}$, giving a dissociation constant of about 25 μ M for pyruvate, and a K_m value of 95 μ M.

Fig. 7. Reaction of lactate dehydrogenase with NADH and pyruvate under single-turnover conditions observed by disappearance of extinction at 340 nm

The reaction mixture contained 7 μ M pig heart lactate dehydrogenase and 5 μ M-NADH from one syringe, and 30 μ M-sodium pyruvate from the other, in 0.1M-phosphate buffer at pH 7.5, 20°C. (a) Duplicate oscilloscope traces are superimposed. (b) Graph of logarithm (extinction) against time.

but in stronger buffer, as described in the previous section. The possibility of a very rapid transient which might occur in the dead-time of the machine was checked as follows. A trace was recorded under single-turnover conditions as just described, and immediately the enzyme solution was replaced with a solution containing enzyme at one-hundredth the concentration, but in the same buffer containing the same amount of NADH. A steady-state experiment was then carried out, and the amplitude of the change in extinction was compared

Fig. 8. Double-reciprocal dependence on pyruvate concentration of rate constants for single-turnover NADH oxidation by lactate dehydrogenase

Conditions are as described for Fig. 7, but at various pyruvate concentrations. Experiments were carried out at pH 6.0 (○), pH 6.5 (△), pH 7.0 (□), pH 7.5 (●), pH 8.0 (▲) and pH 8.5 (■).

with that of the single-turnover experiment. The amplitudes were the same within experimental error, that is about $\pm 10\%$. This experiment shows, not only that there is no 'instantaneous' transient of significant size, but also that decreasing the enzyme concentration 100-fold has little effect on the buffering capacity of the solution. In either case the effect would be to give a much larger amplitude for the steady-state experiment.

The possibility of indicator binding to the enzyme-NADH complex was also checked. Enzyme and NADH in concentrations similar to those used for single-turnover experiments, and in the same dilute buffer, were placed in one side of a centrally bisected 10mm spectrophotometer cuvette, and an equal volume of buffer containing Phenol Red at double the concentration used for the single-turnover experiments was placed in the other side. The spectrum of the indicator was recorded, and then the two sides were mixed and the spectrum was re-recorded. Apart from a slight difference in the intensity of the spectrum (less than 1%) probably due to a slight difference in pH between the two solutions, there was otherwise no change. Indicator binding to

Table 2. pH-dependence of single-turnover rate and K_m value for pyruvate reduction

pH	K_m (μM)	V_{max} (s^{-1})
6.0	89	625
6.5	55	410
7.0	46	400
7.5	38	200
8.0	114	210
8.5	284	175

Fig. 9. H^+ uptake by lactate dehydrogenase during single-turnover pyruvate reduction monitored by Phenol Red extinction at 560 nm, pH 8.0

The solution contained $6.5 \mu\text{M}$ pig heart lactate dehydrogenase and $5 \mu\text{M}$ -NADH added from one syringe, and $80 \mu\text{M}$ -sodium pyruvate from the other, in 0.5 mM -phosphate buffer- 0.1 M -NaCl adjusted to pH 8.0 immediately before the experiment. The solution also contained $20 \mu\text{M}$ -Phenol Red. The experiment was carried out at 20°C . (a) Duplicate oscilloscope traces superimposed. (b) Semi-logarithmic graph of the approach of extinction at 560 nm to the final value against time.

the enzyme would be expected to show a shift in the extinction maximum and possibly a peak sharpening owing to a less-polar environment, or alternatively a shift in pK_a of the indicator may occur which would greatly alter the maximum extinction. It is therefore concluded that Phenol Red does not bind significantly to lactate dehydrogenase under the conditions described above.

From Fig. 10 it must be concluded that at high pH the H^+ required by the stoichiometry of the reaction is taken up after pyruvate binding and at a rate governed by a step that is also rate-limiting for NADH oxidation. Consequently pyruvate must be able to bind to the unprotonated enzyme.

(c) *Observation of NADH oxidation and H^+ uptake in 2H_2O .* Single-turnover pyruvate reduction was carried out in a solvent that contained approx. 86% 2H_2O . The buffer used was 0.5 mM-phosphate in 2H_2O , also containing 0.1 M-NaCl. NADH and pyruvate were also made up in 2H_2O , but the enzyme stock solution was in aqueous buffer as the large volumes of solvent involved in the preparation of the enzyme for this work rendered preparation in 2H_2O impractical. Indicator was also used from an aqueous stock, and aqueous 0.1 M-NaOH and -HCl were used for final pH adjustment.

Fig. 10. Double-reciprocal dependence on pyruvate concentration of the rate constants for H^+ uptake and NADH oxidation by lactate dehydrogenase under single-turnover conditions at pH 8.0, 20°C

○, Rate constants for NADH oxidation obtained as described in Fig. 7. △, Rate constants for H^+ uptake obtained as described in Fig. 9. The values obtained in these experiments give a K_m for pyruvate of 102 μM and a k_{cat} of 190 s^{-1} , in reasonable agreement with values of 114 μM and 210 s^{-1} obtained in Fig. 8 for pH 8.0.

Fig. 11. Reaction of lactate dehydrogenase with NADH and pyruvate in 2H_2O , pD 8.4, observed by extinction at 340 nm and by change in extinction of added Phenol Red at 560 nm

The reaction mixture contained 2.1 μM pig heart lactate dehydrogenase from one syringe, and 1.75 μM -NADH and 500 μM -pyruvate from the other, in a buffer comprising 0.5 mM-phosphate-0.1 M-NaCl in 86% 2H_2O at a pD of 8.4 and containing 20 μM -Phenol Red. (a) Oscilloscope trace at 560 nm. (a') Semi-logarithmic graph of approach of extinction at 560 nm to final value against time. (b) Oscilloscope trace at 340 nm. (b') Graph of the logarithm of the extinction at 340 nm against time. The rate constants obtained are 56 s^{-1} for (a) and 24 s^{-1} for (b).

Fig. 12. Single-turnover reaction of lactate dehydrogenase with pyruvate and NADH at pH 8.0, 6°C, observed by extinction at 340nm and by change in extinction at 560nm of added Phenol Red

The final solution contained 24 μM pig heart lactate dehydrogenase from one syringe, and 20 μM -NADH and 500 μM -sodium pyruvate from the other, in 0.5mm-phosphate buffer-0.1M-NaCl, pH 8.0 at 6°C. Phenol Red was also added at a concentration of 20 μM . (a) Oscilloscope trace at E_{560} . (b) Oscilloscope trace at E_{340} . (c) Semi-logarithmic graphs showing the disappearance of absorbance at 340nm with time ($\circ$) and the approach of absorbance at 560nm to the final value (Δ). (d) Semi-logarithmic graph of the approach of extinction change at 560nm (E) to the back-extrapolated ($E_{\text{extrap.}}$) slow phase with time. The rate constant for the slow phase at 560nm and for the trace at 340nm is 73 s^{-1} and that for the fast phase at 560nm is 420 s^{-1} .

The combined contribution of these last-mentioned solutions is only 3% of the total volume in any case. The final reaction mixture contained 2.1 μM enzyme, 1.75 μM -NADH and 500 μM -pyruvate, concentrations well in excess of the K_m value for pyruvate, and slightly above the dissociation constant for NADH. The following protocol was adopted.

Enzyme solution in one syringe and pyruvate plus NADH in the other had been adjusted to give a pH-meter reading of 8.0, corresponding to a pD of approx. 8.4 (Gutfreund, 1972). This value was used because in $^2\text{H}_2\text{O}$ the $\text{p}K_a$ of the indicator was also shifted up by an amount comparable with the response of the glass electrode. It is possible that the $\text{p}K_a$ values of many ionizing side chains in the enzyme were similarly shifted and that the ionic state of the solution was similar to that in water at pH 8.0. Only one syringe full of each solution was used for each set of experiments, and the following results were obtained: first a good trace of NADH oxidation monitored at E_{340} was recorded, then two good traces at E_{560} (showing H^+ uptake) were recorded, and finally another trace was recorded at E_{340} .

Typical traces at both wavelengths are shown in Fig. 11, with corresponding semi-logarithmic plots. The rates obtained for each pair of results at the same wavelength agreed to within 10%, but the rate constant of NADH oxidation was $24 \pm 2\text{ s}^{-1}$ and that observed for H^+ uptake was $56 \pm 4\text{ s}^{-1}$.

These results clearly show that in $^2\text{H}_2\text{O}$ a step that occurs subsequent to H^+ uptake has been slowed down to a rate slower than that of the step governing H^+ uptake under these conditions. This step has become rate-limiting for NADH oxidation. These results are in contrast with those for similar experiments in water, when the step that is rate-limiting for H^+ uptake also governs the rate of NADH oxidation.

When the experiment was repeated at a pH-meter reading of 6.0, that is a pD of 6.4, in a solvent containing 83% $^2\text{H}_2\text{O}$ and with Chlorophenol Red as an indicator, the observed rates of NADH oxidation at 340nm and of H^+ uptake at 580nm were identical within a 10% error margin.

(d) *Observation of NADH oxidation and H^+ uptake at pH 8.0, low temperature.* Experiments were carried out in a cold-room at 6°C, with observation of NADH oxidation at 340nm and H^+ uptake at 560nm with Phenol Red as an indicator. For these experiments the final concentration of enzyme was 24 μM , and that of NADH was 20 μM . Otherwise solutions were prepared as described above.

Typical results are shown in Fig. 12, with accompanying semi-logarithmic plots. In these experiments it can be seen that the NADH oxidation is first-order as observed at 20°C, although considerably slower, but the H^+ uptake is now biphasic, with the slower rate corresponding to the rate of

Fig. 13. Double-reciprocal dependence on pyruvate concentration of slow and fast rate constants for the observed two phases of H^+ uptake by lactate dehydrogenase under single-turnover conditions, pH 8.0, 6°C

Experimental conditions are described in Fig. 12 except that the pyruvate concentration was varied. In all cases the slow rate constant was the same as that for the disappearance of extinction at 340 nm.

NADH oxidation. A value for the fast rate constant can be obtained from a plot of the logarithm of the difference between the observed extinction and that predicted by back extrapolation of the slow phase, against time. Both rates show a double-reciprocal dependence on pyruvate concentration (Fig. 13). This shows that the fast rate observed represents a step that occurs after pyruvate binding and does not reflect the binding rate itself.

Discussion

The results obtained in this work can be discussed in terms of three reactions: lactate oxidation, pyruvate reduction at low pH and pyruvate reduction at high pH.

The oxidation of lactate will be as follows:

Because of the relatively high concentrations of lactate and NAD^+ used, the binding of these ligands to the enzyme will be very rapid and complete within the dead-time of the apparatus. The hydride-transfer step, step (1), shows up as the instantaneous

step of protein fluorescence quenching and increase in extinction at 340 nm which occurs within the dead-time of the apparatus (Whitaker *et al.*, 1974). The relative amplitude of this step compared with the total fluorescence change indicates that the equilibrium of this step is in favour of the enzyme- NAD^+ -lactate complex, although the amount varies with pH (see results quoted in Table 1). It has previously been assumed that the step immediately after hydride transfer is pyruvate dissociation. This step gives rise to the observed transient in the protein fluorescence experiments, which appears to have the same rate as the transient observed in nucleotide fluorescence experiments. However, the pyruvate inhibition experiments show that at pH 8.0 the pyruvate dissociation step, observed by nucleotide fluorescence, can be almost completely inhibited, but the observed protein fluorescence signal remains virtually unchanged. This indicates an isomerization step before pyruvate dissociation. The partial inhibition of this step by pyruvate at lower pH is believed to show that the overall equilibrium of steps (1) and (2) varies from strongly favouring the enzyme-NADH-pyruvate complex at pH 8.0 to weakly favouring the enzyme-NAD $^+$ -lactate complex at pH 6.0.

When lactate oxidation catalysed by lactate dehydrogenase at pH 8.0 is inhibited by added pyruvate, no pre-steady-state release of a H^+ is seen. This implies that the species that accumulates in the steady state under these conditions retains the H^+ produced by the reaction, that is, if the main species of enzyme complex in the steady-state reaction contains pyruvate, it is also protonated. If the reaction is carried out in the reverse direction, however, pyruvate will bind to the unprotonated enzyme-NADH complex. It can also be seen that the dissociation rate constant and the dissociation constant for pyruvate as a substrate in pyruvate reduction experiments (Fig. 6) differ from values obtained when pyruvate acts as an inhibitor of lactate oxidation (Table 1). It is therefore concluded that a further isomerization step occurs after pyruvate dissociation, resulting in a change of pK_a of a group

in the enzyme that consequently releases a H^+ . This step could also alter the binding parameters for pyruvate. The step is designated step (4) in the above scheme.

Pyruvate reduction at high pH will be as follows:

Pyruvate binding, step (8), was investigated by using nucleotide fluorescence quenching as a probe. The results of experiments at pH 8.0 are shown in Fig. 6, and values of $3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the association rate constant and $75 \pm 25 \text{ s}^{-1}$ for the dissociation rate constant were obtained. Step (7) is the rate-limiting step for pyruvate reduction at high pH under the usual conditions in which this reaction is carried out, and hence this rate constant determines the observed rate constant for NADH oxidation or H^+ uptake. The step has no effect on the stoichiometry of the bound reactants, but seems to cause a perturbation of the pK_a of some group in the ternary complex, possibly the side chain of histidine-195, from below 8.0 to above 8.0. This step is identified as some sort of conformational change or rearrangement.

In water at room temperature the rate constant of step (7) corresponds fairly well to k_{cat} , the limiting catalytic rate constant for steady-state turnover. Therefore all subsequent steps in the above scheme must be considerably faster than step (7), which implies a minimum value of approx. 1000 s^{-1} for the rate constant of each of these steps at pH 8.0. If the reaction is carried out in $^2\text{H}_2\text{O}$, however, the observed rate of NADH oxidation is significantly slower than that of H^+ uptake, indicating the presence of another step between step (6) and step (1) which has been greatly slowed down in $^2\text{H}_2\text{O}$. This step coincides with the step in lactate oxidation responsible for the production of enzyme-bound NADH from lactate in the presence of saturating pyruvate, and so is identified as step (2). This step, like step (7), involves no change in the stoichiometry of the reactants and again must be labelled as some kind of rearrangement.

The pyruvate reduction experiment at low temperature can also be explained by this scheme if it is assumed that the perturbation of a pK_a caused by step (7) is not as great at low temperature. This would lead to a fractional H^+ uptake, and the remaining fraction of H^+ uptake would be governed by step (2), which must also be slowed down considerably at low temperature.

Pyruvate reduction at low pH proceeds according to:

This scheme is the same as that suggested by Holbrook & Stinson (1973) and Holbrook & Gutfreund (1973), except that the rearrangement step has been split into two parts. There is no solid evidence that this step should have two parts to it, but general analogy with the reaction at high pH

suggests that it should be so. Whether step (9) or step (2) or both are rate-limiting for this reaction is indeterminable, as neither of the steps can be directly observed at present. The H^+ -uptake step at low pH occurs after hydride transfer and possibly even after lactate dissociation according to this model, and this is consistent with the identity of the rates of NADH oxidation and H^+ uptake in $^2\text{H}_2\text{O}$ at low pH.

When all the preceding partial reactions are combined, the mechanism of Scheme 1 is reached. A noteworthy feature of the mechanism is the loop described by reactions (3), (4), (10) and (9). This loop implies that the enzyme only goes to the starred form under normal conditions for pyruvate reduction, after pyruvate has bound. However, it appears only to revert to the unstarred state after dissociation of pyruvate in lactate oxidation experiments. This type of behaviour was first suggested by Gutfreund (1971) for this enzyme, and has been demonstrated by Bennett & Gutfreund (1973) for pig muscle lactate dehydrogenase also.

It will be noted that the part of Scheme 1 describing pyruvate reduction at high pH is at variance with the suggestion by Holbrook & Stinson (1973) and Holbrook & Gutfreund (1973) that pyruvate will only bind to the protonated form of the enzyme-NADH complex. A similar argument has been put forward by Bloxham *et al.* (1975) based on the pH-dependence of binding of epoxide inhibitors to pig heart lactate dehydrogenase. Although the evidence that this is the case for oxamate binding is good, the only real evidence for this type of behaviour in the case of pyruvate binding comes from the pH-dependence of the K_m value for pyruvate binding. Schwert *et al.* (1967), using the ox heart enzyme, showed that the K_m for pyruvate for steady-state experiments rises above about pH 7 at a rate that suggests the ionization of a binding group, the low pH form of which is necessary for pyruvate binding. Subsequent examination of these results shows that the pK_a of such a group would be in the range 7.4–7.7, somewhat higher than the pK_a of the essential histidine residue measured as 6.7 ± 0.2 in the enzyme-NADH complex (Holbrook & Ingram, 1973). Results of single-turnover studies in Table 2

show an upward trend in K_m for pyruvate in the range pH 7.4–7.7 also, with a small upward shift at lower pH as well.

K_m for pyruvate is given by the expression:

$$K_m = \frac{k_{\text{off}} + k_r}{k_{\text{on}}}$$

(The meaning of the various rate-constant symbols is given elsewhere in this paper.) Results in Fig. 6 show that, at pH 8.0, k_r is about twice as fast as k_{off} . Therefore the turnover rate constant, k_r , has an important effect on K_m at pH 8.0, and since k_r increases with decreasing pH this effect is likely to be significant at lower pH also. To determine the effect of pH on the binding of pyruvate, it is therefore necessary to determine the dissociation constant for pyruvate at each pH. Since at pH lower than 8.0 the rate of pyruvate binding is too fast to be measured, there is no way of telling whether the effect of changing pH on K_m is due entirely to a change of k_r or whether k_{off} or k_{on} are also pH-dependent. Consequently there is no real evidence to support the binding of pyruvate to the protonated enzyme only.

In Scheme 1 the steps of obvious interest are those labelled (2) and (7) (or 9). One of these steps is rate-limiting for pyruvate reduction under any set of conditions so far studied, and the principle of biological parsimony suggests that they must be essential to the reaction.

X-ray-crystallographic experiments with dogfish muscle lactate dehydrogenase (Adams *et al.*, 1973) show that between the apoenzyme and a ternary complex (either enzyme-NAD⁺-pyruvate, enzyme-NAD⁺-oxalate or enzyme-NADH-oxamate) there is a conformational change in which a loop of residues from residue 98 to residue 114 moves through a relatively large distance to close over the active centre in the ternary complex. It would be of interest to know if this type of conformational change is responsible for step (7) or step (2) in the catalytic sequence. Step (7) is a particularly attractive candidate for this type of change because it provides a means of explaining the pK_a perturbation of the histidine-195 side chain which could then take up a H⁺. Correlation of kinetically observed 'isomerizations' with known crystallographic structures, however, remains purely speculative until a means can be found of directly observing conformational changes during reaction.

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