Transient-Kinetic Studies of Pig Muscle Lactate Dehydrogenase

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1. The very fast pre-steady-state formation of NADH catalysed by pig M_4 lactate dehydrogenase was equivalent to the enzyme-site concentration at pH values greater than 8.0 and to one-half the site concentration at pH 6.8. 2. The rate of dissociation of NADH from the enzyme at pH 8.0 (450s^{-1}) in the absence of other substrates is faster than the steady-state oxidation of lactate (80s^{-1}). The latter process is therefore controlled by a step before NADH dissociation but subsequent to the hydride transfer. 3. The oxidation of enzyme–NADH by excess of pyruvate was studied as a first-order process at pH 9.0. There was no effect of NAD on this reaction and it was concluded that the ternary complex undergoes a rate-limiting change before the hydride-transfer step. 4. Some conclusions about the reactions catalysed by the M_4 isoenzyme were drawn from a comparison of these results with those obtained with the H_4 isoenzyme and liver alcohol dehydrogenase.

Individual steps of the reactions of NAD-linked dehydrogenases can be studied by a number of rapid-reaction techniques (Gutfreund, 1971). In the present paper we describe observations from three types of stopped-flow experiments with the M_4 isoenzyme of pig lactate dehydrogenase: the formation of NADH, enzyme-bound and free, before the steady state is reached in the presence of excess of NAD^+ and lactate; the partial reaction of the oxidation of the enzyme–NADH complex by excess of pyruvate; the rates of formation and dissociation of the enzyme–NADH complex. From these observations we can draw some conclusions about the minimum number of distinct steps that must occur during the interconversion of NAD^+ and lactate to NADH and pyruvate and vice versa.

Comparison of the results previously obtained with the H_4 isoenzyme of lactate dehydrogenase (Criddle, McMurray & Gutfreund, 1968) and liver alcohol dehydrogenase (Shore & Gutfreund, 1970) with those reported here for M_4 lactate dehydrogenase show that qualitatively the mechanisms of these enzymes are very similar. There are quantitative differences in the various phenomena observed with members of this group of enzymes. These differences make one or other of the enzymes more suitable for the detailed study of each phenomenon. It is to be hoped that this wide approach to the study of NAD^+ -linked dehydrogenases will result in some understanding of the general mechanism of hydride transfer to and from the nicotinamide ring.

MATERIALS

Enzyme preparations. The M_4 isoenzyme of lactate dehydrogenase was prepared from pig skeletal muscle. A 5 kg portion of muscle was minced and extracted with twice its weight of 5mM-EDTA, pH 6.0. The meat was removed by filtration through linen and the extract diluted with an equal volume of water. The lactate dehydrogenase was concentrated by addition to the well-mixed extract of a slurry of CM-cellulose (Whatman) equilibrated with 5mM-EDTA, pH 6.0. Sufficient CM-cellulose was added to adsorb 90% of the lactate dehydrogenase. The CM-cellulose and adsorbed enzyme were removed by basket centrifugation. The lactate dehydrogenase was obtained in soluble form by two washes of minimal volume with 150mM- KH_2PO_4 –NaOH buffer, pH 8.5, containing 300mM-NaCl. The protein fraction that was precipitated between 45% and 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ was desalted by Sephadex G-25 gel filtration and applied to a column (16cm long $\times$ 1.4cm diam.) of CM-cellulose equilibrated with 15mM- KH_2PO_4 –NaOH buffer, pH 6.0. A linear gradient from 5 litres of 15mM- KH_2PO_4 –NaOH buffer, pH 6.0, to 5 litres of 15mM- KH_2PO_4 containing 300mM-NaCl, pH 6.0, was applied. Lactate dehydrogenase was eluted at 200–250mM-NaCl. The recovered enzyme was recrystallized three times with $(\text{NH}_4)_2\text{SO}_4$ and then subjected, with suitable controls, to cellulose acetate electrophoresis in 0.01M-barbitone–0.04M-sodium barbitone buffer, pH 8.6. Activity staining showed the preparation to be free of other isoenzymes of lactate dehydrogenase. The enzyme was stored at 4°C as a crystalline precipitate and was desalted just before use by either gel filtration on Sephadex G-25 or extensive dialysis. Protein concentration was

determined by the biuret method of Beisenherz *et al.* (1953) by using the factor 16.5 to convert extinction into mg of protein or by using $E_{1\text{cm}}^{0.1\%}$ 1.29 at 280 nm (Jécsai, 1962). The pure enzyme when assayed in 100 μM -NADH, 1.0 mM-pyruvate and 67 mM- KH_2PO_4 -NaOH buffer, pH 7.2, at 25°C converted 610 μmol of substrate/min per mg of protein. All enzyme concentrations expressed in this paper refer to active-site concentrations.

A millimolar extinction coefficient at 340 nm for M_4 lactate dehydrogenase-bound NADH was calculated from a difference spectrum of the bound and free nucleotide and found to be 5.9.

Reagents. Sodium pyruvate, NAD^+ and NADH were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. The NADH was dissolved in water and freeze-dried before use to remove traces of organic solvents. NADD was made by Dr J. D. Shore by using alcohol dehydrogenase to stereospecifically transfer deuterium from uniformly labelled ethanol to NAD^+ . Lithium DL-lactate was prepared from lactic acid and LiOH and recrystallized from ethanol-water. The buffer used was 67 mM- KH_2PO_4 -NaOH unless otherwise specified.

METHODS AND RESULTS

Steady-state rate measurements of the reduction of NAD^+ in the presence of lactate and enzyme and of the oxidation of NADH in the presence of pyruvate and enzyme were carried out by following the change in extinction at 340 nm in a Hilger-Gilford recording spectrophotometer at 25°C.

Rapid reactions were studied in a stopped-flow apparatus as described by Gutfreund (1965). Extinction changes in a 1 cm observation cell were measured with a quartz-iodine lamp and a grating monochromator as light-source. A Wratten 18A filter was used to minimize stray light. Rapid fluorescence changes were measured in a similar stopped-flow machine with the photomultiplier at 90° to an Hanovia 100W high-pressure mercury lamp. The 366 nm emission line was used to excite

NADH in a 2 mm-path-length cell and the fluorescence emission was monitored through either Kodak 98 and 2B filters or a 431 nm interference filter. Model experiments permitted an estimation of 1.3 ms as the dead time of the stopped-flow fluorimeter (S. J. Reynolds & D. Yates, unpublished work).

Stopped-flow spectrophotometer experiments similar to those reported by Heck, McMurray & Gutfreund (1968) for H_4 lactate dehydrogenase were done on the M_4 isoenzyme at saturating concentrations of NAD^+ and lactate. The results obtained with the two enzymes were similar. However, the burst of NADH production catalysed by the M_4 isoenzyme that occurred during the mixing time (approx. 3 ms) of the instrument was equivalent to the enzyme-site concentration only at pH values greater than 8.0. The relationship between the magnitude of this initial burst and pH is given in Fig. 1; the burst is one-half maximal at pH 6.8. The pH, then, controls the steady-state concentration of enzyme-NADH complexes. To test whether this control is reflected in the turnover rate of the enzyme, the effect of pH on the steady-state oxidation of lactate was determined. The turnover rates were calculated from the initial reaction velocities. The turnover rate of the enzyme under saturating lactate and NAD^+ concentrations is recorded, as controlled by pH, in Fig. 2. This rate profile is similar to that reported by Jécsai (1962). However, it is doubtful that the substrate concentrations employed by Jécsai were saturating at the lower pH values. It can be shown from Figs. 1 and 2 that over the pH range 6.3–8.0 the ratio of turnover rate to initial burst size is nearly constant.

Fig. 1. Effect of pH on the magnitude of the initial burst of NADH production. A solution of 40 mM- NAD^+ and 55 μM -lactate dehydrogenase was mixed with an equal volume of 0.50 M-lithium DL-lactate. The buffer was 67 mM- KH_2PO_4 and the temperature 22°C. The pH value was recorded after the reaction.

Fig. 2. Effect of pH on the turnover of pig M_4 lactate dehydrogenase. 20 mM- NAD^+ and 0.25 M-DL-lactate were equilibrated in 2.0 ml and the reaction was initiated with enzyme. $\circ$, Pyrophosphate buffer to ionic strength 0.150; Δ , 100 mM-glycine and NaCl to ionic strength 0.150. The temperature was 25°C.

Fig. 3. (a) Displacement of pig M₄ lactate dehydrogenase-bound NADH with NAD⁺. Enzyme-NADH (20 μ M) was mixed with an equal volume of 30 mM-NAD⁺ in the stopped-flow fluorimeter. The pH was 7.2 and the temperature 22°C. The time constant was 0.02 ms. (The traces of two identical experiments are superimposed.) (b) First-order Guggenheim plot of the reaction shown in (a).

The rate of dissociation of NADH from the enzyme was determined by displacing the bound NADH with NAD⁺. The diminished fluorescence of free NADH over enzyme-bound NADH allowed the reaction to be monitored in a stopped-flow fluorimeter. Fig. 3(a) shows the results of two identical experiments superimposed and Fig. 3(b) is a first-order plot (Guggenheim, 1926) of this record. The displacement at pH 7.2 was carried out over a sixfold range of NAD⁺ concentrations, and it was shown that as the NAD⁺ concentration was decreased the magnitude of the signal decreased but the rate of the reaction was unaffected. This procedure for measuring NADH dissociation from lactate dehydrogenase has been used previously (Heck *et al.* 1968) for the H₄ isoenzyme. Heck (1969) has shown that values obtained in this way

Fig. 4. Association reaction after the mixing of 8 μ M-enzyme with 8 μ M-NADH in the stopped-flow fluorimeter. The pH was 7.2 and the temperature 22°C. The time-constant was 0.02 ms.

agree with direct evaluation from relaxation measurements. At pH 6.0 the 'off' constant for NADH from M₄ lactate dehydrogenase was found to be 253 s⁻¹ and at pH 8.0 the 'off' constant was 458 s⁻¹.

The increased fluorescence of enzyme-bound NADH over free enzyme was also used to measure the rate of association of the M₄ isoenzyme with NADH. Fig. 4 is a record obtained when equal concentrations of enzyme and nucleotide were rapidly mixed together. Fluorescence titrations (R. A. Stinson & J. J. Holbrook, unpublished work) showed the dissociation constant for M₄ lactate dehydrogenase and NADH at pH 7.2 in 67 mM-KH₂PO₄-NaOH buffer to be 1.9×10^{-6} M. Thus under the conditions of this experiment the reaction would not go to completion and the record in Fig. 4 represents an approach to equilibrium. The second-order rate constant ($6.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) was calculated from the appropriate equation (Frost & Pearson, 1961). The 'on' and 'off' constants determined separately predict a dissociation constant of 6 μ M. This discrepancy with the value determined directly needs further explanation (see the Discussion section).

If enzyme-bound NADH is allowed to react with pyruvate under conditions of enzyme (26 μ M) in excess of NADH (20 μ M) the enzyme can turn over only once. At pH 9.0 the turnover of the enzyme is in the time-resolution of the instrument when enzyme-NADH is rapidly mixed with various concentrations of pyruvate. An example of the type of record obtained is shown in Fig. 5(a) and a first-order plot (Guggenheim, 1926) of this record is given in Fig. 5(b). The first-order rate constant for a single turnover of the enzyme was determined at a

Fig. 5. (a) Single-turnover experiment with enzyme-bound NADH. Pig M_4 lactate dehydrogenase at $28\mu\text{M}$ and NADH at $20\mu\text{M}$ were mixed with an equal volume of 2.0mM -pyruvate in the stopped-flow spectrophotometer. The buffer was 100mM -glycine- NaOH , pH 9.0. The time-constant was 1ms . The resulting reaction and end point are shown. The temperature was 22°C . (b) First-order Guggenheim plot of the reaction shown in (a).

number of pyruvate concentrations. In addition, the steady-state turnover of the enzyme as affected by pyruvate concentration was studied under the same conditions as used for the single-turnover experiments. The stopped-flow rate constants determined by first-order analyses and the steady-state turnover rates calculated from the initial reaction velocity at various pyruvate concentrations are plotted together in Fig. 6. Clearly the two sets of data are coincident and represent the same mechanistic step. The data in Fig. 6 give a K_m for pyruvate of $7.7 \times 10^{-3}\text{M}$ and a V_{max} of 246s^{-1} . The stopped-flow data alone give a K_m for pyruvate of $9.6 \times 10^{-3}\text{M}$ and a V_{max} of 264s^{-1} . When NADD was substituted for NADH in the stopped-flow experiment and the data were plotted as in Fig. 6(a) K_m for pyruvate of $9.0 \times 10^{-3}\text{M}$ and a V_{max} of 200s^{-1}

Fig. 6. Plot of steady-state turnovers and stopped-flow spectrophotometer single-turnover rate constants. Δ , Single-turnover data; $\circ$, steady-state data. The buffer for both experiments was 100mM -glycine- NaOH , pH 9.0, and the temperature was 23°C .

were obtained. Thus the ratio of the V_{max} with NADH to the V_{max} with NADD is 1.3.

DISCUSSION

The discussion of the experimental results reported above is best conducted around a model of the reaction path (Scheme 1). One can show that this model contains only steps that are both necessary and sufficient to accommodate all the kinetic information so far obtained.

Let us first consider that on mixing enzyme with saturating concentration of lactate and NAD^+ there is a rapid appearance of NADH before its slow steady-state rate of formation (Fig. 1). The amount of NADH produced within the dead time of the stopped-flow machine was dependent on pH and at a specified pH it was proportional to the concentration of enzyme active sites. At pH 8 and above the amount of NADH formed before the steady-state phase is essentially equivalent to the enzyme concentration. This means that the rate-limiting step for the steady-state turnover (80s^{-1} at pH 8) must occur after the hydride-transfer reaction (step 4 in Scheme 1). So far we have only been able to determine the rate of one subsequent step directly. The rate of dissociation of NADH from the enzyme (step 7) in the absence of other substrates except NAD^+ is 450s^{-1} . Further, the 'off' velocity for pyruvate (step 6 or 10) is inferred to be fast. By using the method of Gutfreund (1955) it is found that the 'on' velocity for pyruvate is larger than $10^6\text{M}^{-1}\cdot\text{s}^{-1}$. This, together with the K_m $7.7 \times 10^{-3}\text{M}$ (Fig. 6), gives an 'off' constant larger than $7.7 \times 10^3\text{s}^{-1}$. It is therefore clear that some

Scheme 1. E represents a subunit of lactate dehydrogenase and *E an altered form of E.

isomerization of the enzyme-NADH complex (step 5 or 11), after hydride transfer and either before or after pyruvate dissociation, must involve a slow step.

The decrease in the pre-steady-state burst of (enzyme-bound) NADH below pH 8 cannot be explained conclusively. In principle, the sum of the concentrations of all forms of enzyme-bound NADH in the steady state, in the presence of saturating concentrations of lactate and NAD, is controlled by two sets of conditions. First, the 'burst' size is proportional to the ratio of the rate of formation of the first enzyme-NADH complex to the rate of dissociation of NADH from its last enzyme complex. Secondly, it is dependent on the dynamic equilibrium:

during the transient phase. The steady-state concentration of enzyme-bound NADH is reached within the mixing time and its formation must be controlled by a rate constant larger than 1000s^{-1} . The rate-limiting step ($k = 80\text{s}^{-1}$ at pH 8) controls the dissociation of NADH from the enzyme over the range of pH investigated. It therefore appears most likely that the increase in the steady-state concentration of enzyme-bound NADH is due to an increase in the rate of the reverse reaction (step 4) below pH 8. This is consistent with our finding that for pyruvate reduction the interconversion of the ternary complex becomes more rapid with decreasing pH.

It is disappointing that the rapid rate of the pre-

steady-state phase of lactate oxidation by M₄ lactate dehydrogenase has made it impossible to analyse the kinetics of this process by the methods used so far. In the case of liver alcohol dehydrogenase Shore & Gutfreund (1970) have shown that a single first-order reaction ($k = 130\text{s}^{-1}$) is in control of the formation of enzyme-bound NADH on mixing enzyme with saturating amounts of NAD⁺ and ethanol. In the steady state both sites of the dimeric enzyme are occupied by NADH. In the case of the liver alcohol dehydrogenase the rate of appearance of enzyme-bound NADH exhibits a deuterium isotope effect of 6 and one can conclude that this transient reaction is the hydride-transfer step. These features, as well as large spectral changes and a wide range of substrates, make alcohol dehydrogenase a particularly good model for the development of methods for the study of NAD⁺-linked enzymes. For the present investigation the slower and relatively pH-independent transient with a large isotope effect serves as some guide as to the likely properties of the hydride-transfer step.

It has been reported by Criddle *et al.* (1968) that at pH 9 the interconversion of the ternary complex:

was rate-limiting for the reduction of pyruvate catalysed by H₄ lactate dehydrogenase. The experiments reported here were designed to investigate whether in the case of the M₄ isoenzyme the rate of oxidation of bound NADH was also the same as the steady-state rate of pyruvate reduction. Further, we wished to find out whether the rate of the interconversion of the ternary complex corre-

sponded to the rate of hydride transfer or whether it was controlled by an isomerization step. At pH 9 it was possible to make a direct comparison of the dependence on pyruvate concentration, of the rate of the single turnover partial reaction, with that of the steady-state velocity of NADH oxidation. The results summarized in Fig. 6 present clear evidence that the rates of the partial reaction between enzyme-bound NADH and pyruvate and the steady-state oxidation of NADH at pH 9.0 are limited by the same process. The very small isotope effect when NAD⁺ is used in place of NADH provides evidence that the hydride transfer is considerably faster than the observed interconversion of the ternary complex. The isomerization of the ternary complex (step 5) after addition of pyruvate to enzyme-NADH appears to be the reversal of the isomerization described above as the rate-limiting step in the dissociation of NADH during lactate oxidation.

Similar studies of the partial reaction of H₄ lactate dehydrogenase (H. Gutfreund, N. G. Bennett, C. H. McMurray & R. A. Stinson, unpublished work) and of liver alcohol dehydrogenase (Shore & Gutfreund, 1970) were also carried out under conditions of enzyme active-site concentration three times in excess of NADH concentration. Single exponentials were obtained with identical first-order rate constants independent of the number of sites occupied. The deuterium isotope effect for the liver alcohol dehydrogenase reaction was similar to that reported here for M₄ lactate dehydrogenase. With the H₄ isoenzyme no isotope effect could be detected for this reaction.

If one uses the absence of a large isotope effect in the reaction:

as evidence for a rate-limiting isomerization step one needs to have some information about the expected isotope effect for the hydride-transfer step. The large isotope effect for the hydride transfer from deuterioethanol to NAD⁺ should also be observed in the reverse direction if the transfer reaction itself is observed. N. G. Bennett (unpublished work) has found that there is no equilibrium isotope effect on the alcohol dehydrogenase reaction. It is reasonable to assume that the deuterium isotope effects on the hydride-transfer step of the alcohol dehydrogenase and the lactate dehydrogenase reactions will be very similar.

The comparison of the equilibrium dissociation

constant of the pig M₄ isoenzyme-NADH complex measured by R. A. Stinson & J. J. Holbrook (unpublished work) with the ratio of the measured rate constants for this step needs further explanation. Czerlinski & Schreck (1964) best explain their temperature-jump relaxation experiments on the rabbit M₄ isoenzyme-NADH system by a fast bimolecular step with a dissociation constant of 6 μM followed by a slower monomolecular interconversion with an equilibrium constant of 0.3. The result is an overall dissociation constant of 1.4 μM, which is in agreement with the thermodynamically determined value of 2.6 μM (R. A. Stinson & J. J. Holbrook, unpublished work) for the rabbit muscle enzyme. A two-step binding mechanism may explain the disagreement reported here between the thermodynamically and kinetically determined dissociation constants for NADH and pig muscle lactate dehydrogenase. No discrepancies between the results from the two methods have been reported for H₄ lactate dehydrogenase (Heck, 1969). One would infer a two-step process for the binding of NAD⁺ to dogfish M₄ lactate dehydrogenase from the combined inhibitor and X-ray data of McPherson (1970).

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