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The Resolution of some Steps of the Reactions of Lactate Dehydrogenase with its Substrates

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1. The reaction of pig heart lactate dehydrogenase (EC 1.1.1.27) with NAD^+ and lactate to form pyruvate and NADH was followed by rapid spectrophotometric methods. The distinct spectrum of enzyme-bound NADH permits the measurement of the rate of dissociation of this compound. 2. The reduction of the first mole equivalent of NAD^+ per mole of enzyme sites can also be observed, and is much more rapid than the steady-state rate of NADH production. 3. At pH 8 the dissociation of the enzyme–NADH complex is rate-determining for the steady-state oxidation of lactate. At lower pH some other step after the interconversion of the ternary complex and before the dissociation of NADH is rate-determining. Other evidence for a compulsory-order mechanism is provided.

The results from extensive investigations on steady-state and equilibrium-exchange systems of LDH* with its two pairs of substrates have been reconciled by Schwert, Miller & Peanasky (1967) with a compulsory-order mechanism. Studies with enzymes at catalytic concentrations have given a framework into which any proposed mechanism has to fit. In spite of important pioneering experiments on alcohol dehydrogenase (Theorell & Chance, 1951; Geraci & Gibson, 1967) and LDH (Chance & Neilands, 1952), attempts at the direct observation of individual steps to learn something about the reactions of these enzymes with their substrates have lagged far behind the very detailed analysis of the indirect steady-state approach. Here we report some experiments on LDH using two approaches to the elucidation of enzyme mechanisms by observations of rapid transients. Enzyme concentrations are held sufficiently high so that spectral changes occurring during enzyme–substrate complex-formation and extinction changes due to the amount of product formed during a single turnover of the enzyme can be followed satisfactorily.

MATERIALS

Pig heart LDH H_4 was obtained from Boehringer Corp. (London) Ltd., London, W. 5 (LDH-1-iso 15353). The solid material was separated from the $(NH_4)_2SO_4$ suspension, dissolved in and dialysed against appropriate buffer. The specific activity was 300 units/mg. at pH 6 (0·2M-sodium phosphate, 0·08M-NaCl, 0·3mM-pyruvate, 0·15mM-NADH). Extinction at 280mμ of 1·38 for a 1mg./ml. solution was used to determine the enzyme concentration.

The number of NAD-binding sites on the enzyme was checked by the method of Pfleiderer, Jeckel & Wieland (1956) as modified by Holbrook (1966) with the NAD–sulphite complex. The enzyme had 1 binding site for 35000mol.wt.

NAD^+ and NADH were obtained from Boehringer Corp., and DL-lithium lactate was from Sigma Chemical Corp., St Louis, Mo., U.S.A. For experiments between pH 6 and pH 8, 0·2M-sodium phosphate buffers containing NaCl to adjust to constant ionic strength 0·3 were used. At pH 8·5, 0·2M-tris–HCl buffer was used. Oxamate was purchased from British Drug Houses Ltd., Poole, Dorset, as were the phosphate salts used.

METHODS AND RESULTS

Difference spectra between solutions of NADH and of LDH–NADH mixtures were recorded with a Cary spectrophotometer. Though the difference spectra varied in detail with pH, the one shown in Fig. 1 illustrates the essential features used for kinetic experiments.

The stopped-flow equipment used for recording rapid changes in percentage transmission was essentially that described by Barman & Gutfreund (1966). Interference filters whose pass bands are centred at 365mμ and 340mμ were used for following the degree of association of NADH with enzyme and for the control respectively. Measurements at 340mμ were also used for the calculation of the rate of formation of NADH from NAD^+ in the presence of enzyme and lactate.

The rate of displacement of NADH from its

* Abbreviation: LDH, lactate dehydrogenase.

enzyme complex was determined by mixing equal volumes of two solutions, made up in the appropriate buffer, in the stopped-flow apparatus. Solution A contained LDH (16mg./ml.) and NADH (0·45mM). Solution B contained NAD^+ (30mM).

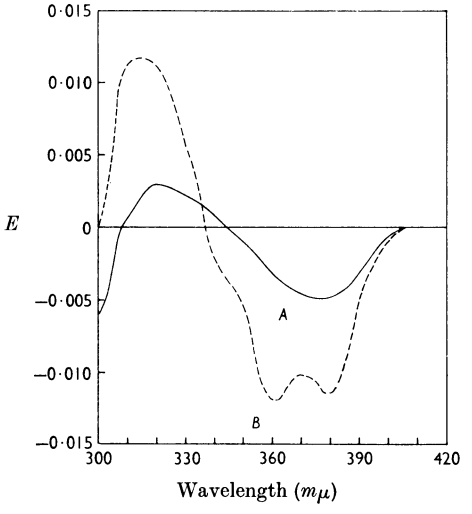

Fig. 1. Difference spectra recorded on solutions in 0·5cm. cells. Curve *A*, 0·03mM-LDH active sites and 0·05mM-NADH in phosphate buffer, pH6; curve *B*, as curve *A*, with the addition of 2mM-oxamate.

At each pH, experiments were carried out over a range of NAD^+ concentrations to determine that above this concentration no increase in the extent and rate of NADH displacement could be detected. The assumption is therefore made that we are measuring the rate of dissociation of NADH. It cannot be concluded that the presence of NAD^+ does not have a secondary effect on this rate.

In each buffer system, first-order rate constants were calculated from photographs from three stopped-flow experiments. In the phosphate buffers between pH6 and pH8·5, all results for the rate of NADH displacement from the enzyme were within the range $50 \pm 10 sec.^{-1}$. This is also the range of results obtained at any one pH.

The turnover of the enzyme at optimum lactate and NAD^+ concentrations in the phosphate buffers was found to be $8 sec.^{-1}$ at pH6, $21 sec.^{-1}$ at pH7, $31 sec.^{-1}$ at pH7·5, $36 sec.^{-1}$ at pH8 and $41 sec.^{-1}$ at pH8·5. In tris buffer our results on the pH-dependence of the turnover rate are in agreement with those of Schwert *et al.* (1967); at pH8·5 the turnover rate was $100 sec.^{-1}$. A noteworthy point is that the rate constant for NADH displacement from the enzyme in 0·2M-tris buffer, pH8·5, i.e. $100 \pm 10 sec.^{-1}$, was also higher than in phosphate buffer, and further it is again the same, within experimental error, as the turnover rate.

As shown in Fig. 1, the addition of oxamate to the LDH–NADH complex resulted in a change in the spectrum. The kinetics of the displacement of

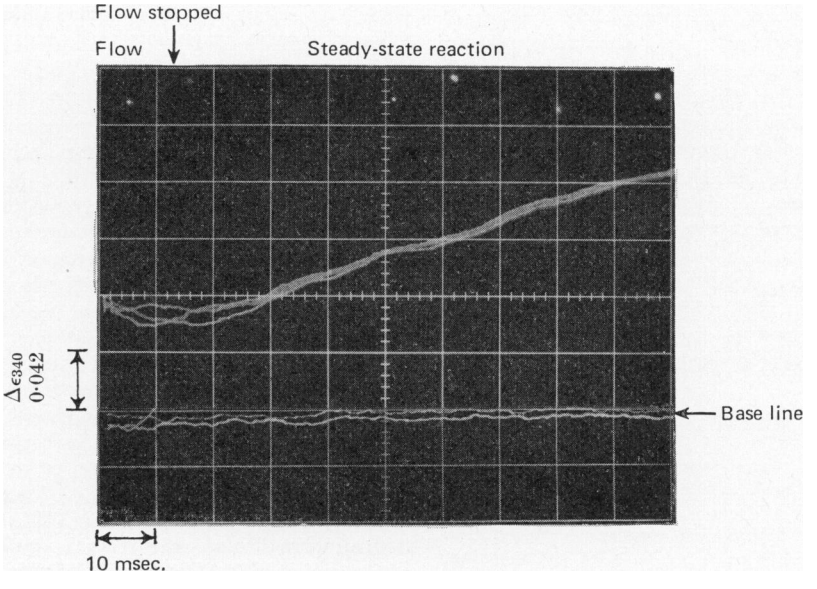

Fig. 2. Record of stopped-flow experiment indicating burst of NADH reaction during the 3msec. dead-time of the reaction of: 0·01mM-LDH active sites, 20mM-NAD^+, 30mM-lactate, phosphate buffer, pH8·0.

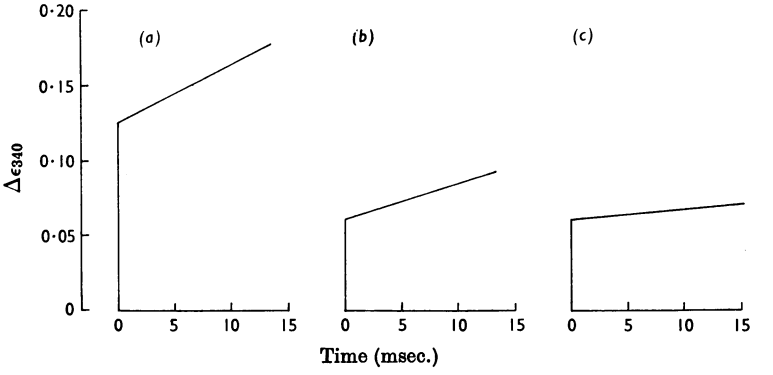

Fig. 3. Schematic record showing the extinction at 340mμ from observation in the stopped-flow apparatus. The initial extinction change, π, and the subsequent steady state were calculated from the difference in extinction between the substrate solution mixed with buffer and substrate solution mixed with enzyme. Concentrations in the final reaction mixtures were: (*a*) 0·02mM-LDH active sites, 20mM-NAD^+, 30mM-lactate, phosphate buffer, pH 8·0; (*b*) 0·01mM-LDH active sites, 20mM-NAD^+, 30mM-lactate, phosphate buffer, pH 8·0; (*c*) 0·01mM-LDH active sites, 20mM-NAD^+, 0·2M-lactate, phosphate buffer, pH 6·0.

NADH by NAD^+ were studied in the presence of oxamate at pH 7·0 in phosphate buffer. The rate of NADH displacement decreased as oxamate concentration increased. When the concentration of oxamate was 0·5mM, the rate of NADH displacement was 6sec.$^{-1}$; at 0·05mM it was 22sec.$^{-1}$. Such results indicate that the dissociation of NADH from the enzyme was at least slowed down, and possibly totally inhibited, by the presence of oxamate at the active site.

At pH 8, where the dissociation of NADH is rate-determining (see the Discussion section) for the overall reaction of oxidation of lactate, it was to be expected that the first mole of NADH per mole of enzymic active sites would be produced much more rapidly than the steady-state rate of NADH production. Fig. 2 shows an actual stopped-flow experiment with a number of identical experiments superimposed. The base line is taken at the extinction of the substrate solution mixed with an equal volume of buffer, whereas in the flow experiment the change in extinction is recorded of the reaction mixture consisting of an equal volume of substrate and enzyme solutions. The extinction of the enzyme solution was checked at 340mμ in the spectrophotometer and found to be negligible. The initial 15msec. (approx.) represents the extinction of the solution during flow as the oscilloscope is triggered 15msec. before flow stops. Fig. 3 shows that at pH 8 a burst of an equivalent amount of NADH, produced within the 3msec. between mixing and first observation of the extinction, was indeed observed at 0·01mM and 0·02mM active sites. As we cannot observe the first 3msec. after mixing, a suitable correction was made for the change in extinction due to the steady-state rate during this interval. Surprisingly, a similar experiment at pH 6, where the rate of dissociation of NADH is six times as fast as the turnover, still resulted in the initial rapid reduction of 1 mole of NAD^+ per mole of active sites (see Fig. 3*c*). The consequences of these findings are discussed below.

DISCUSSION

The resolution of individual steps of the overall reaction of an enzyme with its substrates and the evaluation of the rate constants associated with them is essentially preliminary information for two lines of investigation. First, it serves as a basis for studies of the chemical mechanism through detailed analysis of the primary events responsible for each step. Secondly, it gives information about the particular step that is rate-determining, and thus indicates where the control function of this particular enzyme lies. For the purpose of the present analyses, we consider a step as rate-determining if it is less than twice as fast as the overall reaction. This may appear arbitrary but the rationale behind this is that, if any other step were of the same rate, the overall rate would be half that of the rate-determining step. In a previous paper, Gutfreund, Cantwell, McMurray, Criddle & Hathaway (1968) gave some evidence that the dissociation of the enzyme–NAD^+ complex is rate-determining for the reduction of pyruvate to lactate.

In the present investigations, two rapid reactions occurring within the turnover of LDH were investigated and compared with the steady-state rate of lactate oxidation. The rate of dissociation of NADH from its complex with LDH is considerably faster than the turnover of the enzyme at pH 6.

Above pH 7 the rate of NADH dissociation becomes closely similar to the steady-state rate of lactate oxidation, and makes a major contribution to this rate. The dissociation constant K $3{\cdot}9 \times 10^{-7}$M previously determined for the ox heart enzyme (Anderson & Weber, 1965) was confirmed by us for the pig heart LDH–NADH complex. This value, together with our dissociation rate constant, gives a rate constant of 10^8M^{-1}sec.$^{-1}$ for the association of the enzyme with NADH, if this occurs in a single step. These values are not directly comparable with those of Czerlinski & Schreck (1964) because their experiments were carried out with rabbit skeletal-muscle LDH.

Our experiments on the dissociation of the ternary complex enzyme–NADH–oxamate are consistent with the conclusion that there is an obligatory order of dissociation; NADH only dissociates from the enzyme if no oxamate is bound. Oxamate appears to be a very good analogue for the substrate pyruvate.

A significant finding, even before a more precise evaluation of the rate of dissociation of LDH–NADH over an extended pH range, is the fact that the first mole of NADH per mole of enzyme sites is produced very much more rapidly than the subsequent steady-state rate of NADH production during lactate oxidation. A similar phenomenon has been observed for glutamate dehydrogenase by Iwatsubo & Pantaloni (1967). At the pH where the dissociation of NADH from the enzyme is the rate-determining step, the explanation for a burst of NADH equivalent to the enzyme site concentration is clear enough. All steps before the dissociation of NADH are fast, and after the first turnover NADH production is governed by the low rate of dissociation. At pH 6, however, the dissociation of NADH is about six times as fast as the rate-limiting step, but the burst of NADH still takes place and is still equivalent to the molarity of the enzymic sites. The compulsory sequence of the addition of NAD^+ or NADH to the enzyme before formation of the ternary complex has been discussed by Schwert *et al.* (1967) and has also been mentioned above. We are therefore in a position to use the sequence

$$\mathrm{LDH} \overset{k_1}{\rightleftharpoons} \mathrm{LDH}_{\mathrm{NAD}^+} \overset{k_2}{\rightleftharpoons} \mathrm{LDH}^{\mathrm{Lact}}_{\mathrm{NAD}^+} \overset{k_3}{\rightleftharpoons} \mathrm{LDH}^{\mathrm{Pyr}}_{\mathrm{NADH}} \overset{k_4}{\rightleftharpoons} \mathrm{LDH}_{\mathrm{NADH}} \overset{k_5}{\rightleftharpoons} \mathrm{LDH}$$

Scheme 1.

shown in Scheme 1 to discuss some aspects of the mechanism.

At pH 6, some step after the rapid interconversion of the ternary complex (k_3), but before the dissociation of NADH from the enzyme, must be rate-determining. It is not yet known whether the dissociation of pyruvate or a subsequent rearrangement of the enzyme–NADH complex is responsible for this rate-limiting step. The difference in the fluorescence and absorption spectrum between enzyme–NADH and the ternary enzyme–NADH–oxamate complex should permit distinction between the two steps in question by improved techniques. At pH 8 the step that is rate-limiting at pH 6 must be much faster than the turnover to result in k_5 being approximately equal to the turnover.

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