The Kinetics of the Interconversion of Intermediates of the Reaction of Pig Muscle Lactate Dehydrogenase with Oxidized Nicotinamide–Adenine Dinucleotide and Lactate

By NIGEL G. BENNETT and HERBERT GUTFREUND
*Molecular Enzymology Laboratory, Department of Biochemistry,
 University of Bristol, Bristol BS8 1TD, U.K.*

(Received 1 February 1973)

Oxamate competes with pyruvate for the substrate binding site on the E^{NADH} complex of pig skeletal muscle lactate dehydrogenase. When this enzyme was mixed with saturating concentrations of NAD^+ and lactate in a stopped-flow rapid-reaction spectrophotometer there was no transient accumulation of enzyme complexes with the reduced nucleotide. The steady-state rate of formation of free NADH was reached within the dead-time of the instrument (3 ms). When oxamate was added to inhibit the steady state and to uncouple the equilibration:

through the rapid formation of $E^{NADH}_{Oxamate}$, the rate of formation of E^{NADH} could be measured by observation of the first turnover. This pH-dependent transient is controlled by the rate of dissociation of pyruvate and the fraction of the enzyme in the form $E^{NADH}_{Pyruvate}$.

Stinson & Gutfreund (1971) carried out a detailed kinetic study of the reactions of the M_4 isoenzyme of pig lactate dehydrogenase. One of their findings was that mixing the enzyme and NAD^+ with lactate at pH values above 8 results in the formation of 1 mol of enzyme-bound NADH/mol of active sites within 2 ms. At lower pH values progressively smaller amounts of enzyme-bound NADH were formed in this 'instantaneous' phase. Since that time a split-beam stopped-flow reaction spectrophotometer (Gutfreund, 1972, p. 180) was developed in this laboratory by D. W. Yates, G. H. McMurray & H. Gutfreund (unpublished work). This instrument provides a record of the absolute difference in extinction between the solutions before and after mixing. Studies with this new method showed that a mixture of enzyme and NAD^+ without added lactate slowly produced NADH. This artifact is probably due to bound lactate and the amount of NADH produced at equilibrium increases with increasing pH. With the split-beam technique the base line (zero difference in extinction) is automatically provided and the enzyme solution is mixed with a solution containing NAD^+ and lactate. In further studies, reported in this paper, it was found that with the M_4 isoenzyme of lactate dehydrogenase a rapid transient formation of 1 mol of enzyme-bound NADH/mol of active sites could only be observed if the substrate solution also contained oxamate to inhibit the steady-state rate. The rate of interconversion of the ternary complex could thus be determined and further details of the mechanism of this enzyme could be added to the pro-

posals of Stinson & Gutfreund (1971). The use of inhibitors in the study of transient kinetics can be illustrated with experiments on lactate dehydrogenase.

Methods and Results

The M_4 isoenzyme of lactate dehydrogenase was prepared from pig skeletal muscle by the method of Stinson & Gutfreund (1971) and all other reagents were obtained and purified as described in this previous paper. The concentrations of this tetrameric enzyme are given as $\mu\text{equiv.}$ of active site concentrations. The split-beam reaction spectrophotometer was used to determine rapid changes in the extinction of enzyme–substrate mixtures on a millisecond time scale. The instrument was used and its dead-time determined as outlined by Gutfreund (1972, p. 180). Fig. 1 shows the records of reactions of M_4 lactate dehydrogenase for the first 30 ms after mixing the enzyme with lactate and NAD^+ in the presence and absence of oxamate. In the absence of oxamate the steady-state rate of NADH production was reached within the mixing time (approx. 3 ms). In the presence of oxamate there was a transient formation of enzyme-bound NADH through the accumulation of $E^{NADH}_{Oxamate}$ as the major steady-state intermediate and after that there is a much inhibited steady-state production of NADH. At 340 nm the molar extinction coefficients of NADH and its complexes with lactate dehydrogenase and lactate dehydrogenase–oxamate differed

Fig. 1. Records of the reactions of M_4 lactate dehydrogenase with lactate and NAD^+

(a) Records of $\Delta E_{340}^{1\text{ cm}}$ obtained by mixing solutions containing 25.0 μM (in sites), M_4 lactate dehydrogenase (upper trace), 50 μM (lower trace) with a solution containing 13.4 mM- NAD^+ and 66 mM-lithium-L-lactate in 100 mM-sodium-potassium phosphate (95 mM- Na_2HPO_4 , 5 mM- KH_2PO_4), pH 8, at 23°C in a split-beam stopped-flow spectrophotometer. (b) Shows a record under the same conditions for a solution of 25 μM enzyme mixed with a substrate solution as above, which also contains 8 mM-oxamate. The first-order plot of approach to the steady state was derived according to the method illustrated by Gutfreund (1972, p. 202).

by less than 5%. Oxamate exhibits non-competitive inhibition for the reaction:

and competitive inhibition for the reaction:

when studied by steady-state kinetics (see Fig. 2). Heck *et al.* (1968) showed that $NADH$ will not dissociate from the ternary complex $E_{\text{Oxamate}}^{NADH}$ in the case of H_4 lactate dehydrogenase and Holbrook & Stinson

(1970) showed that the affinity of $NADH$ binding to M_4 lactate dehydrogenase is at least 100-fold increased in the presence of oxamate. The transient accumulation of $E_{\text{Oxamate}}^{NADH}$ at high concentrations of NAD^+ , lactate and oxamate must be rate-limited by some step of the reaction:

The pH-dependence of this first-order process is shown in Table 1 together with the steady-state rates at substrate saturation in the absence of oxamate.

At pH 8 the effect of varying the oxamate concentration is mainly to increase the amplitude of the transient. At pH 6 there is more competition between oxamate and lactate and a corresponding decrease in the transient rate at increasing oxamate concentrations. The rate constants given were obtained by extrapolation to zero oxamate concentration. The fact that steady-state kinetic studies showed only non-competitive inhibition for lactate oxidation indicates the insensitivity of these measurements to the competitive inhibition of lactate binding, which can be estimated from the effect of oxamate on the transient rates.

Addition of pyruvate to the reaction mixture decreased the amplitude of the transient E^{NADH} production. The competition between pyruvate and oxamate for binding to E^{NADH} in this process can be compared with the competition obtained in steady-state measurements. The experiments shown in Fig. 3 represent steady-state rate measurements carried out at low and high enzyme concentrations. The three experiments at 15 $\mu\text{equiv.}$ of active sites and 20 mM-lactate were also used to compare the amplitude of the transient E^{NADH} production with the steady rate. It was found that these quantities were directly proportional.

Deuterium-labelled lactate was prepared by alkaline hydrolysis of diethyl 2-bromo-2-methylmalonate in water and subsequent acid-catalysed decarboxylation in 2H_2O . The product was characterized by proton magnetic resonance spectroscopy. The rate of the transient was only about 10% lower when deuterio-lactate was used as substrate. This lower rate could well be due to impurities in the material prepared by us.

Discussion

Scheme 1 will be used to discuss the results reported in this paper as well as those obtained by Stinson & Gutfreund (1971). The results will be used in turn to show that the steps in this scheme are necessary and sufficient to account for the steady-state and transient kinetic behaviour of the reactions of M_4 lactate dehydrogenase with its substrates.

The transient production of enzyme-bound $NADH$ at high substrate concentration, which occurs in the

Fig. 2. Inhibition by oxamate of reactions of M_4 lactate dehydrogenase in the steady state

(a) The inhibition of NAD^+ (2.5mM) reduction by oxamate in 0.1M-sodium-potassium phosphate (95mM- Na_2HPO_4 , 5mM- KH_2PO_4) buffer, pH8. The points (with a range of three measurements) are experimental observations and the lines are those calculated for K_m lactate = 5 mM, K_i oxamate = 0.25mM, $k_{\text{cat.}}$ = 80s⁻¹. The oxamate concentrations (mM) were: curve (A), 0.5; curve (B), 0.3; curve (C), 0.1; curve (D), 0. (b) The inhibition of NADH (0.2mM) oxidation by oxamate in 0.1M-sodium-potassium phosphate buffer, pH7.5. The points are experimental observations and the lines are those calculated for K_m pyruvate = 0.6mM, K_i oxamate = 0.15mM and $k_{\text{cat.}}$ = 450s⁻¹. The oxamate concentrations (mM) were: curve (A), 1.0; curve (B), 0.5; curve (C), 0.3; curve (D) 0.1; curve (E), 0.

Table 1. pH-dependence of some rate-constants during lactate oxidation by M_4 lactate dehydrogenase

The rate of transient approach ($k_{\text{trans.}}$) to the steady-state was measured in the presence of oxamate but extrapolated back to zero oxamate concentration; k_{off} is the rate of dissociation of NADH from its complex with enzyme (data from Stinson & Gutfreund, 1971).

pH	$k_{\text{cat.}}$ (s ⁻¹)	$k_{\text{trans.}}$ (s ⁻¹)	k_{off} (s ⁻¹)
6	9	165	250
7	30	220	330
8	83	300	470

presence of oxamate, must be a measure of the rate of formation of E^{NADH} or E^{NADH} from the first ternary complex, since these are the only forms of the enzyme with a high affinity for oxamate. At pH8 the rate of formation of the E^{NADH} complexes is 300s⁻¹, the rate of dissociation of NADH (step 11) is 450s⁻¹ (Stinson & Gutfreund, 1971) and the steady-state rate of NADH formation is 83s⁻¹. In the absence of oxamate the concentrations of all the intermediates on the right-hand half of Scheme 1 (which absorb light at 340nm) are very low, yet the formation of enzyme-bound NADH (in the presence of oxamate) is nearly 4 times faster than the steady-state rate of NADH formation. It must be concluded that oxamate can

Fig. 3. Study of the inhibition of lactate oxidation by pyruvate

The formation of NADH from 2 mM-NAD⁺ was followed during lactate oxidation in 100 mM-sodium-potassium phosphate (95 mM-Na₂HPO₄, 5 mM-KH₂PO₄) buffer at pH 8. ●, Measurements made in the presence of 0.1–1.0 μg of enzyme/ml (in a recording spectrophotometer); ○, measurements made in the presence of 0.6 mg enzyme/ml in a stopped-flow spectrophotometer. All velocity measurements were normalized to unit enzyme concentration. The points represent experimental measurements and the lines are calculated for K_m (lactate) = 5 mM, K_i (pyruvate) = 0.35 mM and $k_{cat.} = 80 \text{ s}^{-1}$. The pyruvate concentrations (mM) were as follows: curve (A), 0.6; curve (B), 0.3; curve (C), 0.

prevent the rapid equilibration of the oxidation/reduction of enzyme-bound nucleotides, with the equilibrium of the ternary complexes being essentially completely on the side of oxidized nucleotide. The transient of the formation of enzyme-bound NADH in the presence of oxamate can be represented by a single exponential. This confirms the equilibrium position of the ternary complexes and the conclusion of Stinson & Gutfreund (1971) that the four sites of this tetrameric enzyme are equivalent. N. G. Bennett & H. Gutfreund (unpublished work) found that in the case of H₄ lactate dehydrogenase the four sites are also kinetically equivalent, but the environment in the enzyme complexes is such as to give pH-dependent but observable equilibria between the ternary complexes with oxidized and reduced nucleotides. Stinson & Holbrook (1973) have shown that the four NADH binding sites on H₄ and M₄ lactate dehydrogenase are identical and independent.

Stinson & Gutfreund (1971) reported that the rate of oxidation of the enzyme-NADH complex caused by the addition of a large excess of pyruvate was not significantly affected by the substitution of specifically deuterated NAD²H for NADH. It was concluded that the rate-limiting step for the interconversion of the ternary complex was an isomerization after pyruvate binds to E^{NADH}, and the chemical reaction:

was very fast. We have now shown that in the reverse reaction the absence of a deuterium isotope-effect also

suggests a rate-limiting step distinct from the fast chemical reaction of the interconversion of the ternary complex. It is argued below that the rate of the transient is probably controlled by the dissociation of pyruvate.

The addition of increasing concentrations of pyruvate, to the reaction mixtures used for the study of the transient formation of E^{*NADH}_{Pyruvate}, provides information about the affinity of E^{*NADH} for pyruvate as compared with oxamate. Since, however, there will be some E^{NADH} present the $K_i = 0.35 \text{ mM}$ (Fig. 3) only gives an upper limit for the dissociation constant:

The K_m for pyruvate in the reaction:

(single-turnover experiments of Stinson & Gutfreund, 1971) is approximately 1 mM at pH 8. According to the mechanism proposed by Stinson & Gutfreund (1971) and elaborated here, this K_m should correspond to the dissociation constant:

The amplitude of the transient production of E^{NADH}_{Oxamate} can be used to estimate the proportion of the enzyme sites that are converted into this form during the approach to the inhibited steady state. The total optical density change from the base-line to the intercept of the steady-state rate extrapolated back to the zero-time axis corresponds to 0.9 equiv. of

Scheme 1. A representation of the steps in the reaction of M_4 lactate dehydrogenase with its substrates

The number of steps included in this scheme are necessary and sufficient to accommodate the observations from the transient kinetic experiments described.

NADH per enzyme site in solution at high oxamate concentrations. This indicates full occupancy of enzyme sites with NADH in the steady-state (Gutfreund, 1965, p. 61, 1971, 1972, p. 200). The whole of the transient is described by a single exponential. This indicates that the four sites of the tetrameric enzymes are kinetically independent and identical.

Inhibitors that compete for the product-binding site but compete only slightly or not all for the substrate binding site provide a useful tool for the isolation and kinetic investigation of individual steps. This phenomenon can occur for two reasons in NAD^+ -linked dehydrogenases. First, if the rate of formation of enzyme-bound NADH is slower than the dissociation of NADH from the complex, no transient formation of enzyme-bound NADH can be observed. If, however, the dissociation of NADH can be slowed down by the addition of an inhibitor the transient rate can be measured. The second condition under which an inhibitor can isolate a transient is the one applicable to the M_4 lactate dehydrogenase system described here. The equilibration:

can be uncoupled by the addition of oxamate to form $E^*NADH_{Oxamate}$. The pH-dependent transient rates

listed in Table 1 can be due either to the isomerization to form the reactive ternary complex:

or due to the rate of dissociation of pyruvate from $E^*NADH_{Pyruvate}$ which occurs at low equilibrium concentration. In such a case the observed first-order rate-constant for the dissociation of pyruvate would be the true rate constant times the fraction of the total enzyme in the form before pyruvate dissociation. Both these alternatives would account for the absence of a deuterium isotope-effect.

References

- Gutfreund, H. (1965) *An Introduction to the Study of Enzymes*, Blackwell Scientific Publications, Oxford
- Gutfreund, H. (1971) *Annu. Rev. Biochem.* **40**, 315-344
- Gutfreund, H. (1972) *Enzymes: Physical Principles*, Wiley Interscience, London and New York
- Heck, H. d'A., McMurray, C. H., & Gutfreund, H. (1968) *Biochem. J.* **108**, 793-796
- Stinson, R. A. & Gutfreund, H. (1971) *Biochem. J.* **121**, 235-240
- Stinson, R. A. & Holbrook, J. J. (1973) *Biochem. J.* **131**, 719-728