Biochem. J. (1978) **175**, 987–998
Printed in Great Britain

Malate Dehydrogenase of the Cytosol

A KINETIC INVESTIGATION OF THE REACTION MECHANISM AND A COMPARISON WITH LACTATE DEHYDROGENASE

By ALBERTO LODOLA,* JOSEPH D. SHORE,† DAVID M. PARKER* and J. JOHN HOLBROOK*
**Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K., and †Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, U.S.A.*

(*Received* 24 *February* 1978)

1. The mechanisms of the reduction of oxaloacetate and of 3-fluoro-oxaloacetate by NADH catalysed by cytoplasmic pig heart malate dehydrogenase (MDH) were investigated. 2. One mol of dimeric enzyme produces 1.7 ± 0.4 mol of enzyme-bound NADH when mixed with saturating NAD^+ and L-malate at a rate much higher than the subsequent turnover at pH 7.5. 3. Transient measurements of protein and nucleotide fluorescence show that the steady-state complex in the forward direction is MDH–NADH and in the reverse direction MDH–NADH–oxaloacetate. 4. The rate of dissociation of MDH–NADH was measured and is the same as $V_{max.}$ in the forward direction at pH 7.5. Both NADH-binding sites are kinetically equivalent. The rate of dissociation varies with pH, as does the equilibrium binding constant for NADH. 5. 3-Fluoro-oxaloacetate is composed of three forms (F1, F2 and S) of which F1 and F2 are immediately substrates for the enzyme. The third form, S, is not a substrate, but when the F forms are used up form S slowly and non-enzymically equilibrates to yield the active substrate forms. S is 2,2-dihydroxy-3-fluorosuccinate. 6. The steady-state compound during the reduction of form F1 is an enzyme form that does not contain NADH, probably MDH–NAD^+–fluoromalate. The steady-state compound for form F2 is an enzyme form containing NADH, probably MDH–NADH–fluoro-oxaloacetate. 7. The rate-limiting reaction in the reduction of form F2 shows a deuterium isotope rate ratio of 4 when NADH is replaced by its deuterium analogue, and the rate-limiting reaction is concluded to be hydride transfer. 8. A novel titration was used to show that dimeric cytoplasmic malate dehydrogenase contains two sites that can rapidly reduce the F1 form of 3-fluoro-oxaloacetate. The enzyme shows 'all-of-the-sites' behaviour. 9. Partial mechanisms are proposed to explain the enzyme-catalysed transformations of the natural and the fluoro substrates. These mechanisms are similar to the mechanism of pig heart lactate dehydrogenase and this, and the structural results of others, can be explained if the two enzymes are a product of divergent evolution.

The detailed investigation of the reaction mechanism of the malate dehydrogenase of the cytosol which is reported in this and earlier papers (Lodola *et al.*, 1978*a*,*b*; Parker *et al.*, 1978) has been undertaken because we wished to examine the extent to that two enzymes, pig heart lactate dehydrogenase and malate dehydrogenase, which have superimposable three-dimensional structures (Hill *et al.*, 1972; Rao & Rossmann, 1973), would also have similar catalytic mechanisms. The investigation was also made because we wished to discover whether any of the features of the 'oil–water–histidine' mechanism that has been proposed for lactate dehydrogenase (Parker & Holbrook, 1977) would apply to other enzymes of the same class.

Abbreviation used: MDH–NADH, malate dehydrogenase–NADH complex.

There have been investigations of the mechanism of malate dehydrogenase from pig (particularly Frieden & Fernandez-Souza, 1975) and from ox (Raval & Wolfe, 1962; Cassman & Englard, 1966; Silverstein & Sulebele, 1969), but none of these was in sufficient molecular detail to allow direct comparison with the lactate dehydrogenase mechanism (Holbrook & Gutfreund, 1973). From their analysis of complete progress curves Frieden & Fernandez-Souza (1975) concluded that the cytosol malate dehydrogenase bound first the cofactor and then the substrate in an ordered reaction mechanism leading to a ternary complex. These workers also concluded that the rate-limiting steps in both the oxidation of malate and the reduction of oxaloacetate were the rates at which the cofactors were released from their binary complexes with the enzyme. Many of the

results from the transient-kinetic investigation are in agreement with those of Frieden & Fernandez-Souza (1975), but we show below that the rate-limiting complex for oxaloacetate reduction is an enzyme form containing NADH (not NAD^+). To obtain a direct demonstration of the catalytic process in malate dehydrogenase we have had to use a 'slower' substrate than oxaloacetate, 3-fluoro-oxaloacetate.

Materials and Methods

The sources of chemicals and enzymes are described in the previous papers of this series (Lodola *et al.*, 1978*a*,*b*; Parker *et al.*, 1978). 3-Fluoro-oxaloacetic acid was synthesized by Lancaster Synthesis, Lancaster, U.K., by the method of Kun *et al.* (1958). The deuterium analogue of NADH was prepared from the barium salt by precipitating the Ba^{2+} by the addition of Na_2SO_4. Tris buffers were prepared by adjusting a solution of Tris base of the named molarity to the named pH with acetic acid or 11M-HCl.

Heat-inactivation experiments

A solution (0.2ml of 1–2mg/ml) of enzyme was incubated at 65°C in 50mM-H_3PO_4 adjusted with 10M-NaOH to pH 6. At intervals the vial containing the enzyme solution was cooled in an ice bath and a sample of the enzyme assayed in the malate-to-oxaloacetate direction. The various protecting and labilizing agents shown in Fig. 1 were included in the enzyme solution.

Steady-state kinetic experiments

The optimal rate for the oxidation of malate was estimated by determining the rate at which the enzyme catalysed the reduction of 2.5mM-, 5mM- and 10mM-NAD^+ at five concentrations of L-malate (0.63, 1.25, 2.5, 5 and 10mM). The initial rates were converted into mol of NADH produced/mol of enzyme (mol.wt. 35000)/s. By inspection of the grid of values, $V_{max.}$ was estimated to be $30s^{-1}$ at about 7mM-NAD^+ and 1mM-L-malate. The buffer was 0.1M-Tris/acetate, pH 8. $V_{max.}$ in the reverse direction ($V_{max.,r}$) was estimated from the initial rates of oxidation of NADH (0.05, 0.1, 0.2 and 0.4mM) by each of a range of oxaloacetate concentrations (0.25, 0.5, 1 and 2mM). $V_{max.,r}$ was $430s^{-1}$ at 0.5mM-oxaloacetate and 0.2mM-NADH. Both measurements were at 21°C, the temperature of the transient-kinetic measurements.

Transient-kinetic studies

The stopped-flow device was described by Shore *et al.* (1975) and enabled measurements of two optical signals to be made at the same time [any two of protein fluorescence (excited at 297nm, emission via Wratten 18A filter), nucleotide fluorescence (excited at 366 or 340nm, emission via Kodak Wratten filter 98) or absorbance]. The path length for fluorescence was 1mm, and for absorbance was 1cm. The dead time in the dual-wavelength observation cell was about 2.4ms. For some measurements a Durrum–Gibson stopped-flow device was used with a path length of 1.6cm and a dead time of 2.5ms. In both cases the transient changes in optical signals were captured by a Neurograph transient recorder (Transidyne General Corp., Ann Arbor, MI, U.S.A.) with a precision of 2k of 10-bit bytes. The stored signals were then either displayed on an x–y recorder or on an oscilloscope. A simple analogue circuit was used simultaneously to display the log(optical signal) to facilitate the direct read-off of first-order rate constants from plots (this circuit was originally suggested to us by Dr. D. Ballou, Department of Biochemistry, University of Michigan, Ann Arbor, MI, U.S.A.). Unless otherwise stated the reactant concentrations mentioned are those in the syringes before the 1:1 dilution caused by the mixing device.

Results and Discussion

Thermal inactivation experiments

Coenzymes bind to malate dehydrogenase. Thus the proof of the compulsory ordered reaction mechanism depends on the demonstration that the substrates do not bind to the enzyme in the absence of added coenzyme, and Frieden & Fernandez-Souza (1975) failed to detect significant oxaloacetate binding. Fig. 1(*a*) shows that, as expected, added NADH protects the enzyme from thermal deactivation. Fig. 1(*b*) shows that added oxaloacetate labilizes the enzyme for thermal deactivation. This latter result implies that the added oxaloacetate can combine with the enzyme and that half-maximum increase in labilization will take place at about 2mM-oxaloacetate. Such weak binding would not have been detected by Frieden & Fernandez-Souza (1975). Even if the enzyme–oxaloacetate complex with K_d=2mM were to be kinetically competent, the dissociation constant is so much larger than the 40μM estimated by Frieden & Fernandez-Souza (1975) for the binding of oxaloacetate to MDH–NADH that the current view (Raval & Wolfe, 1962) that the order of binding is 'compulsory' is not challenged. Since oxaloacetate is a complex mixture of keto, enol and hydrate, it is by no means certain that the complex is kinetically competent. There is no agreement in the literature whether the enzyme from the cytosol is inhibited by oxaloacetate (Kitto & Kaplan, 1966; Kitto & Lewis, 1967; Francis & Coughlan, 1976) or whether this inhibition is limited

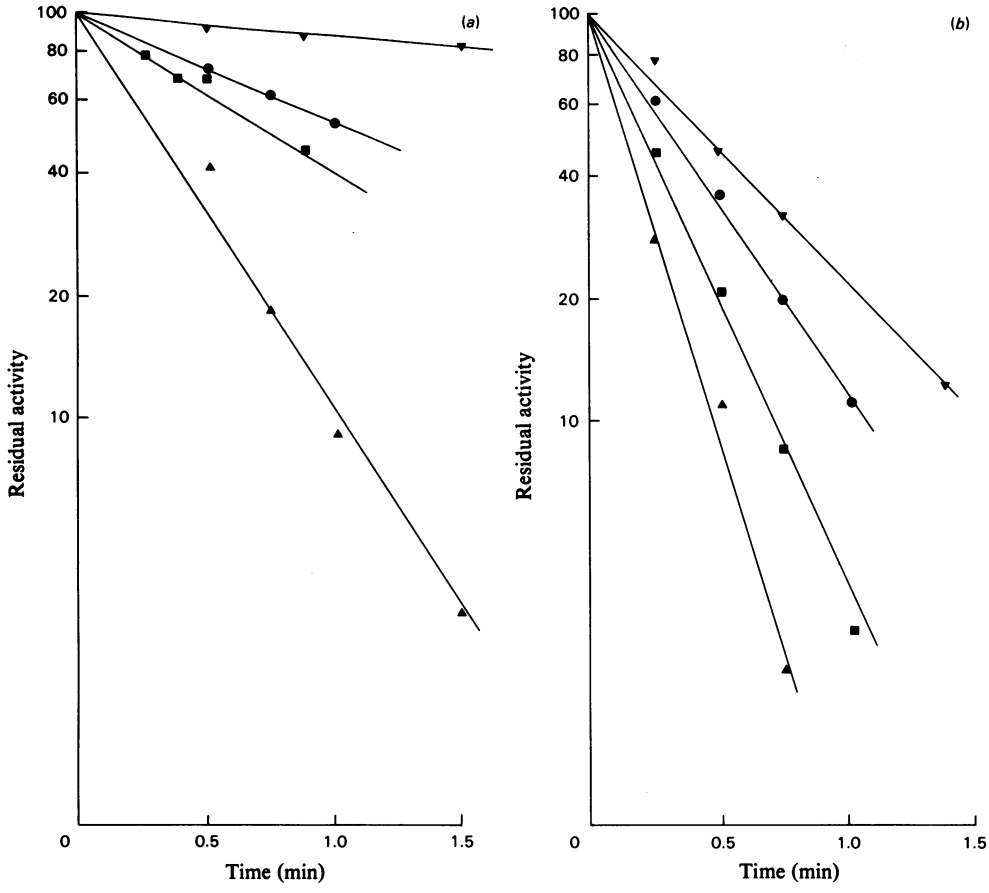


Fig. 1. *Thermal inactivation of malate dehydrogenase*

Solutions of enzyme (0.2ml of 1–2mg/ml in 50mM-H_3PO_4 adjusted to pH 6 with 10M-NaOH) were incubated with (*a*) (▼) 0.2mM-, (●) 0.1mM-, (■) 0.05mM- and (▲) 0mM-NADH or (*b*) (▼) 0mM-, (●) 0.7mM-, (■) 1mM- or (▲) 5mM-oxaloacetate at 65°C. At the times shown the reaction mixture was cooled to 0°C and a sample of enzyme was assayed. The presence of NADH protected and that of oxaloacetate labilized the enzyme to heat inactivation. Residual activity is expressed as percentage of an unheated control.

to the enzyme from the mitochondrion (Englard *et al.*, 1960).

Kinetics of NADH association with malate dehydrogenase

The experiments in this section attempt to discover whether the equilibrium between the coenzyme and enzyme may be described by:

$$\text{MDH} + \text{NADH} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{MDH–NADH}$$

The rate of approach to equilibrium of this process under conditions where [NADH] is held constant is first-order and is described by $k_{app.} = k_{+1}[\text{NADH}] + k_{-1}$. In rapid-mixing experiments where the enzyme is mixed with increasing concentrations of NADH, k_{+1} can be determined as the slope of a plot of $k_{app.}$ against [NADH], as was done for lactate dehydrogenase (Holbrook *et al.*, 1977). In this case k_{+1} turns out to be fast and in practice the experiments are limited to a narrow range of NADH concentrations, the lower limit set by the need to maintain first-order conditions and the upper limit set by the dead-time of the apparatus (1ms). Fig. 2 shows a typical result of the increased NADH fluorescence that takes place when 2μM-MDH is mixed with 8μM-NADH. Within the noise level of the experiment the reaction is first-order up to at least $t_{0.9}$ (the times for 90% completion of a first-order reaction). From nine traces at pH 6 the second-order rate constant was calculated to be $5.7 \times 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$, and, from

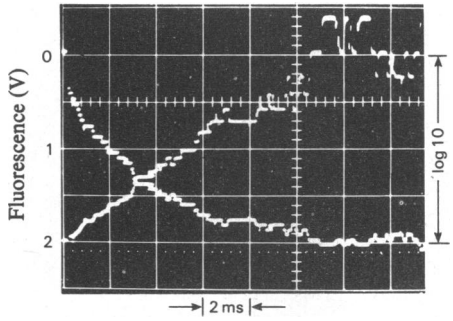


Fig. 2. *Rate of association of NADH with malate dehydrogenase*
Enzyme (2μM-sites) was rapidly mixed with NADH (8μM) in a stopped-flow fluorimeter monitoring fluorescence excited at 366nm and emitted at 430nm (Wrattan 98 filter) in a path length of 2mm, in 50mM-H_3PO_4 adjusted to pH 6 with 10M-NaOH. The curve is the increase (inverted) in fluorescence when NADH is bound to the enzyme. The line is a plot of $\log[(\Delta F_{max.}-\Delta F)/\Delta F_{max.}]$ on a scale such that $\log 10 = 4$ boxes. The log plot is linear up to at least $t_{0.9}$ and corresponds to a first-order rate constant of $210\,s^{-1}$. $k_{+1} = 5\times10^7\,M^{-1}\cdot s^{-1}$.

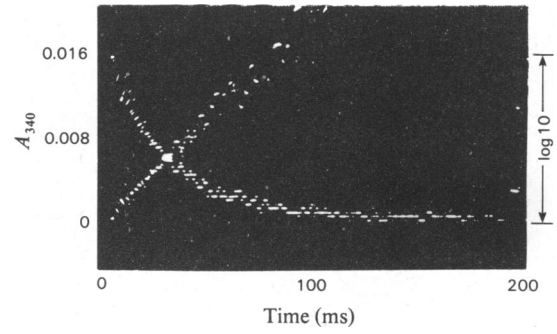


Fig. 3. *Rate of dissociation of NADH from MDH–NADH*
A solution of 6μM-malate dehydrogenase sites and 10μM-NADH was rapidly mixed with an equal volume of alcohol dehydrogenase (70μM-sites) and 20mM-acetaldehyde in 50mM-H_3PO_4 adjusted to pH 7.5 with 10M-NaOH. The curve shows the ΔA_{340} (1cm path length) corresponding to the dissociation of NADH from the enzyme (the excess free NADH is oxidized within the dead-time of the apparatus). The reaction is first-order and the line is a plot of $\log\Delta A$ on a scale of $\log 10 = 4$ boxes. $k_{-1} = 28\,s^{-1}$.

24 results at pH 7, $6.5\times10^7\,M^{-1}\cdot s^{-1}$. The bimolecular rate was not limited by any process with a first-order rate constant of less than $400\,s^{-1}$. Since the concentrations of NADH used in these experiments saturate more than 90% of the two NADH-binding sites of the dimeric enzyme (Lodola *et al.*, 1978*a*), we conclude that the two sites are also kinetically equivalent. The rate constants for the formation of MDH–NADH that we determined are similar to that reported by Frieden & Fernandez-Souza (1975). This suggests that the high-fluorescence nucleotide compound MDH–NADH is kinetically competent.

Dissociation of NADH from MDH–NADH

We measured the rate at which NADH can dissociate from MDH–NADH by rapidly mixing a solution of complex with a trapping solution (70μM-alcohol dehydrogenase sites and 20mM-acetaldehyde) that reacts rapidly with free NADH so that the equilibrium:

$$\text{MDH–NADH} \underset{}{\overset{k_{-1}}{\rightleftharpoons}} \text{MDH} + \text{NADH}$$

goes completely to the right. The decrease in the NADH concentration was monitored either by the decrease in NADH fluorescence as NADH is consumed or by the decrease in A_{340}. For both monitoring signals we observed a simple exponential decrease in $[NADH]_{total}$ after mixing, and an example is shown in Fig. 3. Fig. 4 summarizes the rate constants for the decomposition of MDH–NADH measured between pH 6 and 7.8. The value at pH 7.5 is close to the value calculated by Frieden & Fernandez-Souza (1975) and is also close to the value of $V_{max.}$ at this pH, which we calculated by assuming that both subunits in the dimer are active ($31\,s^{-1}$) (if only one of the two subunits were active k_{-1} would have to be $62\,s^{-1}$ to account for a $V_{max.}$ of $31\,s^{-1}$ at pH 7.5).

The increase in the rate of dissociation of NADH with increasing pH is expected from the increase in $K_{E,\,NADH}$ with pH (Lodola *et al.*, 1978*b*). If only k_{-1} (i.e. not k_{+1}) were pH-dependent (as observed by Frieden & Fernandez-Souza, 1975) then k_{-1} at high pH should be 10 times k_{-1} at low pH, i.e. $10\times6\,s^{-1} = 60\,s^{-1}$, and should be $[(60-6)/2]+6 = 33\,s^{-1}$ at pH 7.4. Although the value of $k_{-1} = 25\,s^{-1}$ at pH 7.4 in Fig. 4 is approximately as expected, it should be remembered that our results do not extend to sufficiently high pH or to sufficiently high rates for there to be confidence that the value of k_{-1} at pH 7.8 is approaching an alkaline asymptote of about $60\,s^{-1}$.

The ratio $k_{-1}/k_{+1} = 10^{-7}\,M$ at pH 6 is appreciably lower than the value of $K_{E,\,NADH}$ measured by equilibrium titration (0.3μM) ($K_{E,\,NADH}$ is the equilibrium constant for reaction 1). Part of the difference is due to the temperature of the kinetic experiments being ambient (18–22°C) whereas the equilibrium titrations were at 25°C. Although we have not made the systematic investigation of the effects of ionic strength and temperature that we did for lactate dehydrogenase (Stinson & Holbrook,

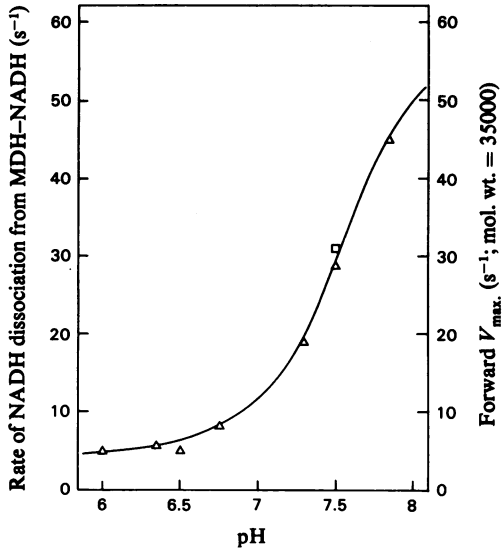


Fig. 4. *Variation of k_{-1} for MDH–NADH dissociation with pH*

The rate constants for NADH dissociation (△) were determined as described in the legend to Fig. 3, but in a series of buffers of I=0.1 made from H_3PO_4 and 10M-NaOH. Also shown is the initial rate of formation of NADH when 1μM-malate dehydrogenase sites catalyse the reduction of 3.7mM-NAD^+ by 5mM-L-malate at pH 7.5 (□).

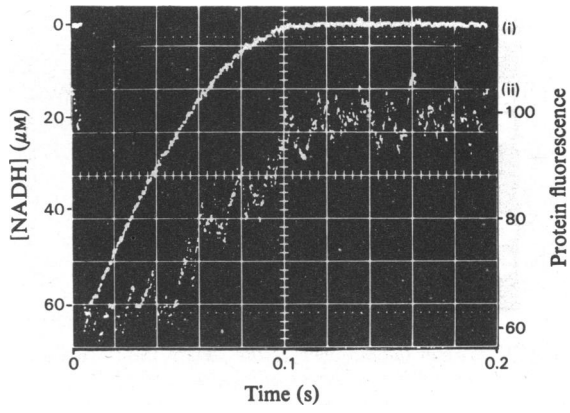


Fig. 5. *Protein fluorescence of the steady state compound during the reduction of oxaloacetate by NADH*

A solution of malate dehydrogenase (4μM-sites) was rapidly mixed with an equal volume of 135μM-NADH and 500μM-oxaloacetate in 50mM-Tris/acetate, pH 8. The A_{340} (curve i, inverted) decrease and the protein fluorescence (curve ii) were simultaneously measured in a dual-wavelength stopped-flow spectrophotometer. During the first 40ms while the initial steady state is maintained (500s^{-1}), the enzyme has the decreased protein fluorescence characteristic of compounds of malate dehydrogenase containing NADH. As the NADH is used up and the steady state decays the protein fluorescence returns to a value characteristic of the free enzyme (set at 100 units).

1973), we have determined $K_{E,\,NADH}$ for malate dehydrogenase as 80nM at pH 6 at 6°C and at low ionic strength. A difference between kinetic and equilibrium values for a binding constant would, if measured under identical conditions, indicate an extra step in the binding reaction.

The amplitude of the decrease in A_{340} as NADH dissociates from malate dehydrogenase may be used to calculate the concentration of NADH that is bound to the enzyme. For 3μM-enzyme (mol.wt. 35000) after mixing we observed $\Delta A_{340} = 0.018 \pm 0.003$. The value is very close to that expected if there are two sites on each malate dehydrogenase dimer that lose NADH at indistinguishable rates.

Protein fluorescence of malate dehydrogenase in its steady-state complexes

The intrinsic protein fluorescence is an indicator of whether the enzyme exists in a complex with NADH. The free enzyme and its complex with NAD^+ have unquenched fluorescence. Both the MDH–NADH complex and the ternary complex with hydroxymalonate have protein fluorescence quenched by about 40% (Lodola *et al.*, 1978*b*; Holbrook & Wolfe, 1972). The steady-state conversion of oxaloacetate into malate was established by rapidly mixing equal volumes of malate dehydrogenase (4μM; mol.wt. 35000) and NADH (0.13mM) with 1mM-oxaloacetate in 0.1M-Tris/acetate buffer, pH 8. Fig. 5 shows that over the first 30ms the concentration of NADH, as measured by A_{340}, decreased at the zero-order rate characteristic of the steady state. The protein fluorescence was monitored at the same time as the absorbance, and Fig. 5 shows that during the initial 30ms the intrinsic fluorescence is quenched, at a value (0.55) characteristic of an enzyme complex containing NADH. Only as the NADH is used up, and the steady state decays, does the fluorescence increase to the value characteristic of either free enzyme or MDH–NAD^+. What then is the rate-limiting step that causes the enzyme to pile up as a complex containing NADH in the steady state? It cannot be the rate at which NADH binds, as this would mean that the enzyme would either be free or in a complex with NAD^+ in the steady state, and these complexes have high fluorescence (Holbrook & Wolfe, 1972). It cannot be the rate at which C–H bonds are broken when NADH is converted into NAD^+, since $V_{max.}$ with NADH (500s^{-1}) and with NADD (460s^{-1}) were indistinguishable when

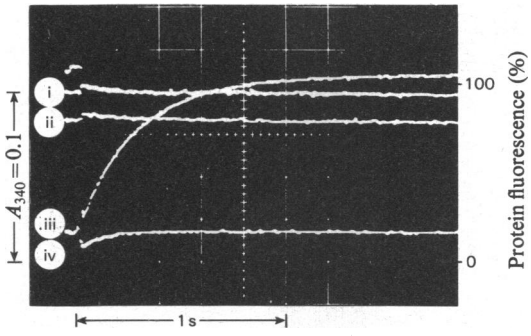


Fig. 6. *Protein fluorescence of the steady-state compound during the oxidation of malate by NAD+*

A solution of 2 μM-malate dehydrogenase sites was rapidly mixed with an equal volume of 4.3 mM-NAD^+ with (ii, iii) or without (i, iv) 4 mM-L-malate in 50 mM-Tris/HCl, pH 8, in a dual-wavelength stopped-flow fluorimeter cell that monitored protein fluorescence (curves i and ii, excited at 297 nm, emission via Wrattan 18A filter) and A_{340} (curves iii and iv) simultaneously. The enzyme, both in the initial steady state and at all points until equilibrium is established, exists as a compound with decreased protein fluorescence, MDH–NADH.

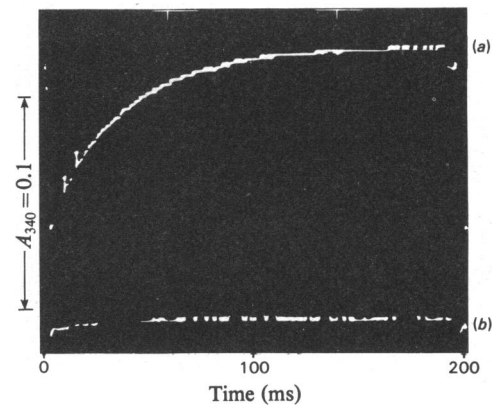


Fig. 7. *'Instant' production of 1 mol of bound NADH/mol during the malate dehydrogenase forward reaction*

Two experiments are shown. In curve (*a*) A_{340} was measured after rapidly mixing equal volumes of 20 μM-malate dehydrogenase (mol.wt. 35000) with 7.3 mM-NAD^+ and 10 mM-L-malate in 50 mM-H_3PO_4 adjusted to pH 7.5 with 10 M-NaOH. The initial rate is 320 μM-NADH/s by 10 μM-malate dehydrogenase sites. Curve (*b*) is a record of the same experiment without malate. Notice that 8 μM-NADH is produced in the complete experiment at a rate much higher than the mixing time and much higher than the subsequent steady state.

compared. It must therefore be a step in the reaction associated with the binding of oxaloacetate to MDH–NADH. The step might be a rearrangement of MDH–NADH required before oxaloacetate can bind, or it might be, as with lactate dehydrogenase, a rearrangement of an initially formed MDH–NADH–oxaloacetate ternary complex. Our conclusion on the nature of the steady-state complex differs from that of Frieden & Fernandez-Souza (1975). These workers calculated that the rate of dissociation of NAD^+ was the slowest step in the reverse reaction catalysed by the enzyme; however, the rate of NAD^+ dissociation was not measured directly: it depended on an assumed mechanism and was only obtained as the complex function of a number of steady-state constants, all of which would have contained some error. The more direct value for the rate constant for NAD^+ dissociation from the enzyme obtained by multiplying the equilibrium binding constant by the bimolecular association rate constant (Parker *et al.*, 1978) suggests that the rate of NAD^+ dissociation is much higher than $V_{max.}$ and agrees with the observation that the enzyme in the reverse steady-state complex has quenched protein fluorescence, i.e. contains NADH.

It has already been suggested that the rate of dissociation of an MDH–NADH binary complex is the slowest step in the forward reaction (Frieden & Fernandez-Souza, 1975). We have confirmed this by rapidly mixing malate dehydrogenase with NAD^+ and malate (10 mM) and observing both ΔA_{340} and the protein fluorescence of the enzyme (Fig. 6). During the initial, zero-order, production of NADH the enzyme has quenched protein fluorescence. We interpret this to mean that the enzyme is in a complex containing NADH, and this conclusion is in agreement with that of Frieden & Fernandez-Souza (1975). The protein fluorescence does not decrease further as the concentration of malate is increased, but it was only half-maximally quenched when the [malate] was only 1 mM. When the experiment was repeated at much higher enzyme concentrations (10 μM-sites after mixing), it was noted that the initial value of A_{340} was higher than would be expected from simply mixing together the reactants, and thus some NADH is formed within the mixing time of the apparatus. Compared with a blank from which malate was omitted, Fig. 7 shows that the complete reaction mixture 'instantly' produces 1.7 ± 0.4 mol of NADH/mol of malate dehydrogenase dimer. We cannot improve the precision of this estimate, but it is clearly greater than the value of 1 mol of NADH/mol of dimer expected if this were a 'half-of-the-sites' enzyme. We have also characterized the nucleotide fluorescence of the compound of malate dehydrogenase and NADH that is rapidly formed in the initial turnover and that is present in the forward

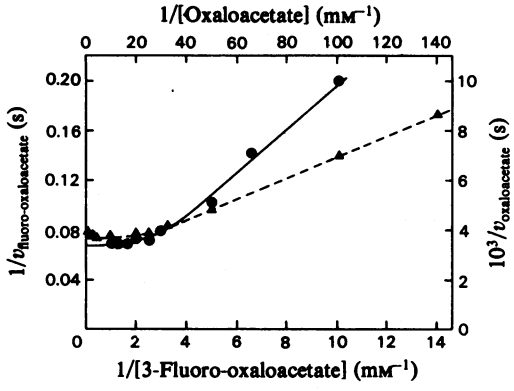


Fig. 8. *Comparison of the steady-state kinetics of oxaloacetate and 3-fluoro-oxaloacetate*

Double-reciprocal plots of the initial rate (v, calculated as s^{-1}, by using mol.wt. 35000 for the enzyme) for the steady-state oxidation of 0.15mM-NADH by either oxaloacetate (▲) or 3-fluoro-oxaloacetate (●) in 50mM-H_3PO_4 adjusted to pH 7 by 10M-NaOH.

steady state. By measuring ΔA_{340} during the steady state and the change of fluorescence in the same stopped-flow cell we can determine the fluorescence of a given concentration of free NADH. The NADH on the enzyme immediately after mixing was 3.2 times as fluorescent as the same concentration of free NADH [excitation at 366nm, emission via a Wratten no. 98 filter (about 430nm)]. Similarly when the fluorescence of the NADH was sensitized by radiation absorbed by the protein (excitation at 297nm, emission via Wratten no. 98) the fluorescence of the NADH formed in the first turnover was 12 times that of free NADH. Both of these enhancements are those characteristic of the MDH–NADH binary complex formed when the enzyme is titrated with NADH (Holbrook & Wolfe, 1972). It is unlikely, when one recalls the properties of the ternary keto acid–NADH complexes of lactate dehydrogenase and glutamate dehydrogenase (Holbrook & Stinson, 1973; di Franco & Iwatsubo, 1972), that NADH in the MDH–NADH–oxaloacetate complex would have the same environment and fluorescence as NADH in the binary complex. In Scheme 1 we show the complex as MDH–NADH. The coincidence of k_{off} (the rate constant for coenzyme dissociation) for NADH and $V_{max.f}$ ($V_{max.}$ in the forward reaction) is further support for this conclusion.

Steady-state kinetics with 3-fluoro-oxaloacetate

$V_{max.}$ with oxaloacetate (500s^{-1}) was too great to obtain the fluorescence characteristics of the steady-state compound, so we chose a 'slower' substrate, 3-fluoro-oxaloacetate, for a direct demonstration of an active NADH–keto substrate–enzyme ternary complex.

Kun (1963) reported that 3-fluoro-oxaloacetate was a substrate for malate dehydrogenase and we have confirmed this. The fluoro substrate was a competitive inhibitor for L-malate oxidation [assayed in 0.1M-glycine adjusted to pH 10 with 10M-NaOH and 10mM-NAD^+, with $K_i = 80\mu M$; cf. Kun *et al.* (1958), 7.4μM] and for oxaloacetate reduction [assayed with 0.15mM-NADH, and 50mM-H_3PO_4 adjusted to pH 7 with 10M-NaOH, with $K_i = 6\mu M$; cf. Kun *et al.* (1958), 28μM]. Competition with oxaloacetate is expected. The competition with malate is suggested to arise because most of the fluoro substrate is present as the hydrate, which is a structural analogue of malate. A comparison of oxaloacetate and its fluoro analogue as substrates is shown in the double-reciprocal plots of Fig. 8. Both curves are non-linear. For the fluoro-oxaloacetate the rate at high substrate concentration reaches a limit value (rather than decreasing, had there been normal substrate inhibition), which is equivalent to 14.5mol of NADH/s per mol of malate dehydrogenase (mol.wt. 35000) at pH 7.

Demonstration of active ternary complexes with 3-fluoro-oxaloacetate

Fig. 9 shows results from preliminary stopped-flow experiments in which various concentrations of fluoro-oxaloacetate were used to oxidize 0.15mM-NADH in 50mM-phosphate buffer, pH 7. Similar results were obtained at pH 6. At the two highest concentrations of substrate all the NADH was oxidized in a fast zero-order phase (FP), which only decayed as the NADH was used up. As the concentration of substrate decreased part of the NADH was oxidized in a fast phase, whereas the remainder was oxidized in a slow phase (SP). In the presence of excess NADH and given enough time, all the fluoro substrate was reduced. Two explanations were considered to account for the biphasic reaction profiles: (i) the fluoro substrate is a mixture of rapidly and slowly enzymically reducible forms; and (ii) the fluoro substrate is a mixture of two forms, one that is immediately and rapidly enzymically reduced and a second form that is not a substrate but that isomerizes in a slow non-enzymic process to give the active substrate. The two explanations could be distinguished, since if explanation (i) were correct then increasing the enzyme concentration should increase the rate of both phases, whereas if explanation (ii) were correct then increasing the enzyme concentration should only increase the rate of the fast enzyme-catalysed process. Fig. 10 clearly demonstrates that a 10-fold increase in enzyme concentration has no effect on the slow phase and is exactly as predicted by hypothesis (ii).

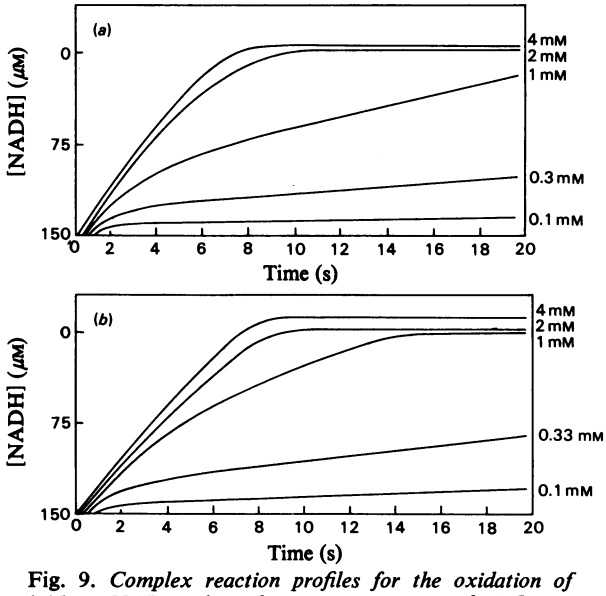
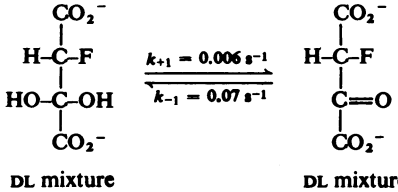


Fig. 9. *Complex reaction profiles for the oxidation of 0.15 mM-NADH by the concentrations of 3-fluoro-oxaloacetate shown and 2 μM-malate dehydrogenase (mol.wt.=35000)*

Each curve is a tracing of $-\Delta A_{340}$ (slightly displaced along the time axis for clarity) after enzyme had been rapidly mixed with NADH and 3-fluoro-oxaloacetate to give the concentrations shown on the graphs: (*a*) at pH 6; (*b*) at pH 7 (both 50 mM-H_3PO_4 adjusted with 10 M-NaOH). At pH 7 (values at pH 6 in parentheses) 4% of the total fluoro-oxaloacetate is reduced at a zero-order rate of 12 s^{-1} (13 s^{-1}), which is constant down to 100 μM-substrate, indicating $K<100$ μM; 4% of the fluoro substrate is then reduced in a phase of initial rate 10 s^{-1} (12 s^{-1}) that is appreciably less at [S] <0.5 μM; 92% of the 3-fluoro-oxaloacetate is inactive as a substrate and is only converted into active substrate at a low zero-order rate, which is 0.006 s^{-1} (0.0045 s^{-1}), based on the starting concentration of the fluoro substrate.

The equilibrium mixture of analytically pure 3-fluoro-oxaloacetate contains at pH 7 8% active substrates and 92% inactive isomer. Since the active form of oxaloacetate is the keto form (Loewus *et al.*, 1955) and since 3-fluoro-oxaloacetate contains very little enol form (Kun *et al.*, 1958), the 92%-inactive isomer is the hydrate (possibly stabilized by hydrogen bonds as shown by Kun *et al.*, 1958) and the equilibria are:



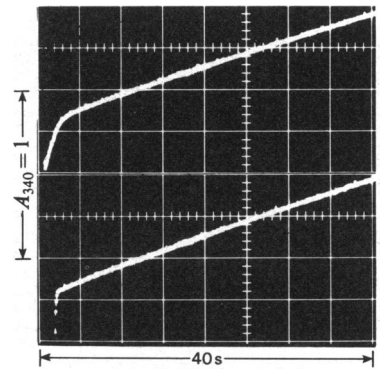


Fig. 10. *Characterization of the slow phase during the reduction of 92% of 3-fluoro-oxaloacetate*

Two experiments are shown in which 1 mM-3-fluoro-oxaloacetate and 0.3 mM-NADH in 50 mM-H_3PO_4, pH 6, were mixed with malate dehydrogenase (4 μM-sites in the upper curve and 40 μM-sites in the lower curve). Note that the rate of the slow reaction does not depend on [enzyme]. The rate of the initial fast phase increases 10-fold. ΔA_{340} was monitored. The amplitude of the fast reaction is 8.5% of the total fluoro-oxaloacetate. The rate of the slow phase is 0.0058 s^{-1}, based on the fluoro substrate concentration.

The solution of the fluoro-oxaloacetate alone is optically inactive, as would be expected, since it is the product of a chemical synthesis.

A close examination of the rapid initial fast phase (that is at shorter times and with increased absorbance sensitivity) showed that this phase was itself composed of two zero-order phases of approximately equal amplitude (FP1 and FP2) and separated by a most unexpected discontinuity. The two phases and the discontinuity have been observed on two differently designed stopped-flow machines and are thus not likely to be machine or mixing artifacts [Fig. 11(*a*), trace i]. By simultaneous measurement of both A_{340} and the intrinsic fluorescence of the enzyme we were able to use the same arguments as we have previously used with oxaloacetate to determine the state of the enzyme in the steady states FP1 and FP2. Fig. 11(*a*) shows that during FP1 the enzyme has unquenched protein fluorescence and thus cannot contain bound NADH. Since we know that both the rate of dissociation of NAD^+ from MDH–NAD^+ (2400 s^{-1}; Parker *et al.*, 1978) and the rate of combination of the free enzyme with NADH (7500 s^{-1} at 160 μM-NADH) are both faster than $V_{max.}$ for FP1 (13 s^{-1}), we conclude that the rate-limiting step is the rate of dissociation of MDH–NAD^+–3-fluoromalate. The steady-state compound is unlikely to be MDH–NAD^+–2,2′-(dihydroxy)-3-fluorosuccinate (the hydrate) because the steady-state decays as the

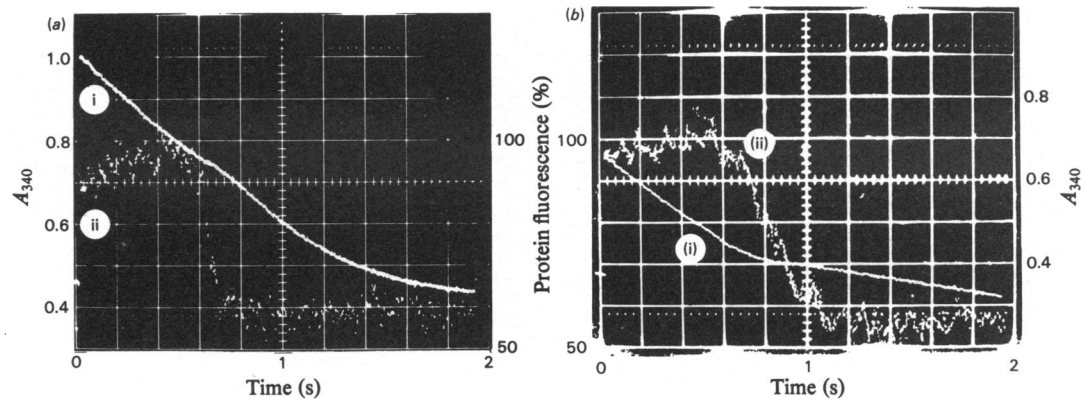


Fig. 11. *Deuterium isotope rate effects with 3-fluoro-oxaloacetates*

Two stopped-flow experiments are shown and in each A_{340} (i) and protein fluorescence (ii) were monitored. A solution of 8μM-malate dehydrogenase sites in 50mM-H_3PO_4 adjusted to pH 7 with 10M-NaOH was mixed with an equal volume of solution containing 2mM-3-fluoro-oxaloacetate and in (*a*) 0.32mM-NADH and in (*b*) 0.2mM-NAD^2H. During the initial zero-order reaction (before the discontinuity in the A_{340} trace) the protein has high protein fluorescence. During the discontinuity there is a rapid decrease in the enzyme protein fluorescence as it enters a new steady state (FP2). The rate of the first steady state is the same with both kinds of NADH. The rate of the second steady state is 4 times slower with NAD^2H than with NADH. In this steady state C–H bond scission is partially rate-limiting.

kinetically tight (i.e. with low K_m rather than low K_s) substrate is exhausted.

After one isomer of the fluoro substrate has been used up in FP1 there is a discontinuity that reflects the enzyme changing to a second steady-state complex during FP2. In this complex (Fig. 11) the intrinsic protein fluorescence is quenched and thus the complex contains NADH. When NADH was replaced by NAD^2H (Fig. 11*b*) we observed no change in the rate of FP1 ($12s^{-1}$) but noted that the rate of FP2 was decreased from $9.8s^{-1}$ (NADH) to $2.4s^{-1}$ (NAD^2H). Thus the decomposition of the steady-state complex containing NADH in FP2 is limited by the rate of C–H bond scission and we conclude that the MDH–NADH–3-fluoro-oxaloacetate → MDH–NAD^+–fluoromalate conversion is rate-limiting in FP2. We have no direct proof of the structures of the two forms of oxaloacetate that are active substrates for MDH and that are each present as 4% in fluoro-oxaloacetate. Our working hypothesis is that they are D- and L-isomers at C-3. This work clearly points to the danger of assuming that different substrates are transformed by the same steady-state complex of an enzyme.

The discontinuity in traces such as Fig. 11 could not arise of the two active forms of 3-fluoro-oxaloacetate were transformed by the same steady-state complex if the enzyme. The discontinuity is expected if the substrate transformed during FP1 has a very much lower K_m than the substrate transformed in FP2. The rapid exhaustion of the (kinetically) tight substrate and the slow dissociation of enzyme–NAD^+–3-fluoromalate leads to the lag phase before the enzyme can bind NADH and enter the second steady state.

Kinetic determination of the number of active subunits in the malate dehydrogenase dimer

At high concentrations of 3-fluoro-oxaloacetate (16mM) there is sufficient active substrate in the mixture to oxidize all the NADH (0.15mM) completely at the high rate of FP1. It was thus possible to confirm that the enzyme is present as a complex without NADH during this phase of the reaction and at the same time to determine the number of active sites on the enzyme molecule by using a novel titration method, which has subsequently been applied to alcohol dehydrogenase by Shore *et al.* (1977).

When solutions of malate dehydrogenase containing excess NADH are rapidly mixed with a solution of the fluoro substrate, the concentration of NADH that should be present immediately after mixing can be estimated (see Fig. 12). The observed [NADH] immediately after mixing was less than that expected. The concentration of NADH that was rapidly oxidized during the mixing time of the apparatus (B, Fig. 12) was approximately equal to the concentration of malate dehydrogenase subunits. It was, however, difficult to be precise about the concentration of rapidly oxidized NADH, since the measurement depends on taking the difference between blank experiments without substrate and the

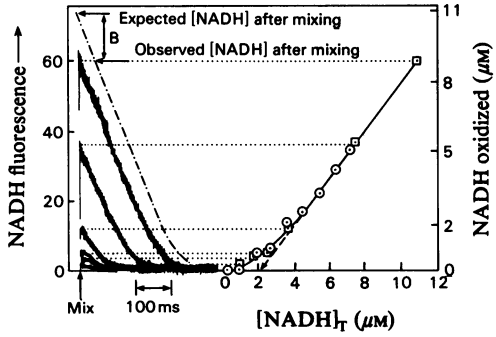


Fig. 12. *Kinetic titration of the number of sites on malate dehydrogenase that can rapidly oxidize NADH*

Solutions of malate dehydrogenase [to give 2 μM (⊙) or 2.16 μM (□) of mol.wt. 35000 after mixing] and NADH (to give concentrations shown as $[NADH]_T$ after mixing) were mixed with an equal volume of 16mM-3-fluoro-oxaloacetate in 50mM-H_3PO_4 adjusted to pH 6 with 10M-NaOH. The subsequent oxidation of NADH was observed from the decrease in NADH fluorescence (the six traces at the left of the Figure) or from the decrease in A_{340} [curves not shown, but observed amplitude of A_{340} decrease ($\Delta A_{340}/\varepsilon_M \times 10^6$) (⊙) given on the right]. The reactions observed after the dead-time of the apparatus are traced such that their end points (when all the NADH is oxidized) coincide and the observed amplitude of the decrease in [NADH] is plotted at the right of the Figure against the total concentration of NADH in the mixture $[NADH]_T$. The line –·– describes the expected decrease in NADH fluorescence if all of the added 11.1 μM-NADH was oxidized during the reaction time observed. Notice that an amount B of NADH is missing immediately after mixing has been oxidized at a rate (>1000s^{-1}) too fast to observe. The line ---- extrapolates to the $[NADH]_T$ axis and indicates that 2.2 μM-NADH is rapidly oxidized at a rate much higher than the steady state. The line —— is a theoretical curve drawn assuming [malate dehydrogenase active sites] = 2.1 μM and $K_{MDH-NADH} = 0.3\,\mu$M.

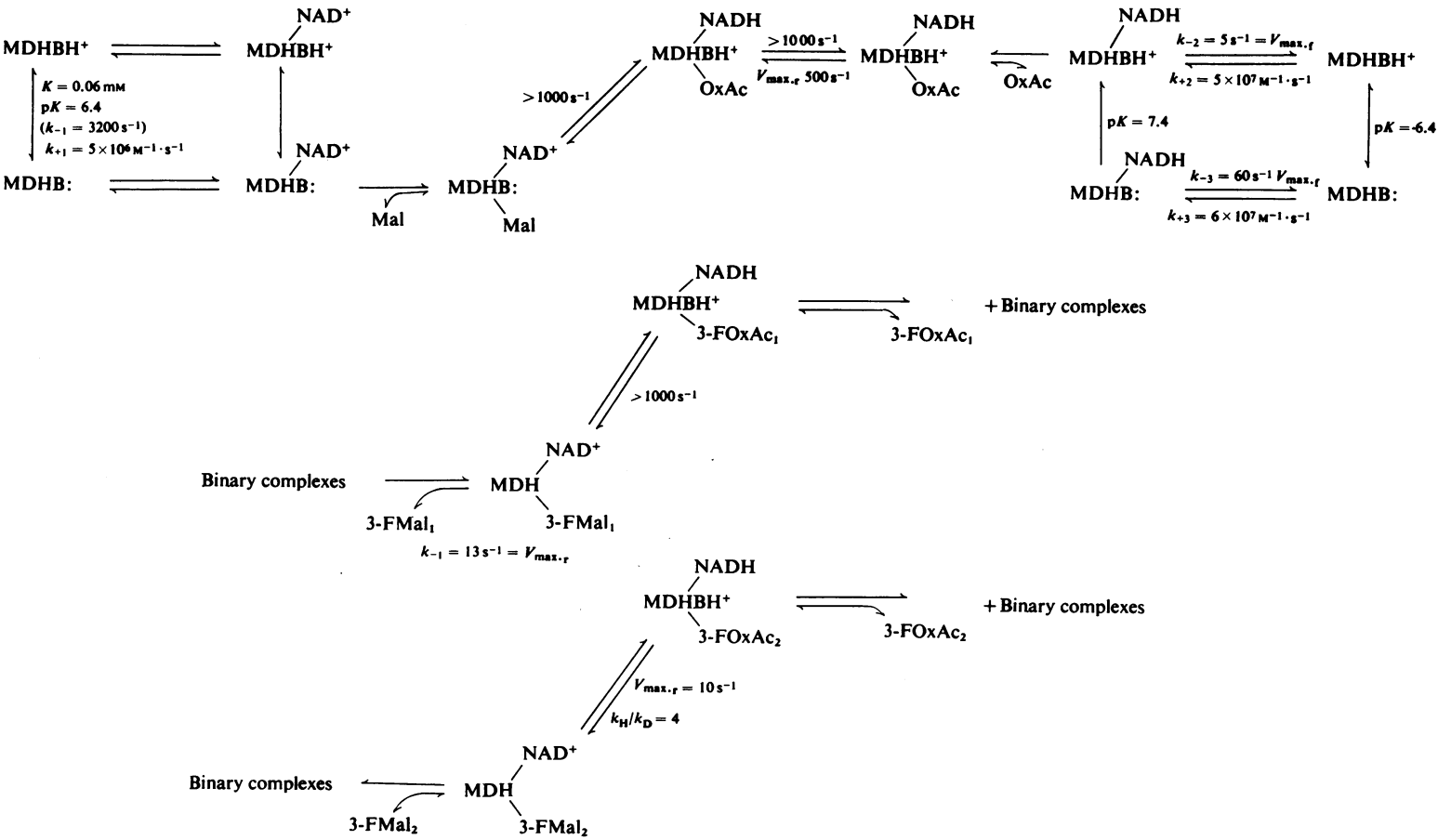
starting point with complete reaction mixtures. Slight variations in baselines made it difficult to decide whether 1 or 2 molecules of NADH were rapidly oxidized per malate dehydrogenase dimer. However, it was simple to measure the amplitude of the observed decrease in [NADH], largely because the end points of the reactions are well defined and provide an internal reference. Fig. 12 shows that the amplitude of the observed reaction, whether monitored by NADH fluorescence or by A_{340}, decreases as the concentration of NADH after mixing is decreased, and extrapolates to zero when there are still 2 equivalents of NADH added per malate dehydrogenase dimer. We thus conclude that malate dehydrogenase can bind two molecules of NADH and that these two molecules can be rapidly oxidized in an 'instant burst' by the fluoro substrate. After the initial burst, the reaction is limited by the rate of dissociation of an enzyme–NAD^+–fluoro substrate compound. The advantage of this kinetic method of determining the number of sites that are active is that it is independent of any assumptions about the absorption coefficient of enzyme-bound cofactors or substrates.

Since both the bound molecules of NADH are oxidized by the rapid fluoro substrate within the dead-time of the apparatus, the rate of hydride transfer must be greater than 1000s^{-1}. Hydride transfer for the other fluoro substrate is only 10s^{-1}, that is at least 100 times slower. It would be unlikely that there is much difference between the redox potentials of free D- or L-3-fluoro-oxaloacetate, and thus the result implies that the environment around C-3 of the substrate is important in the transition state. Lactate dehydrogenase will accept a number of substituted pyruvates and it has been observed that the rate of the isomerization of the initially formed enzyme–NADH–keto substrate compound varies from 250s^{-1} (pyruvate) to 0.05s^{-1} (nitrophenylpyruvate), again with changed substituents at C-3.

The three kinetic experiments reported in this present paper (the rate and amplitude of the dissociation of NADH from MDH–NADH, the instant production of enzyme-bound NADH in the forward reaction and the quantity of the NADH oxidized in the first turnover with fluoro-oxaloacetate) all lead to the same conclusion, namely that the two sites on the malate dehydrogenase dimer are independent and can function simultaneously. This conclusion complements that reached from equilibrium experiments (Lodola *et al.*, 1978*b*) and is the same as that reached by Frieden & Fernandez-Souza (1975) from complete reaction profile experiments.

Conclusion

In Scheme 1 we propose a reaction mechanism for pig heart cytoplasmic malate dehydrogenase that accounts for the kinetic and equilibrium properties that we have measured, for both the natural and the fluoro substrates. The mechanism is qualitatively identical with that of pig heart lactate dehydrogenase. In both enzymes the forward reaction is limited by the rate of decomposition of the enzyme–NADH compound. In malate dehydrogenase this rate is slightly (3-fold) more dependent on the state of protonation of the essential histidine than in lactate dehydrogenase. In the reverse reactions the rate-limiting step is the rearrangement of an enzyme–NADH–keto substrate compound. In both enzymes hydride transfer with natural substrates is immeasurably fast. Both enzymes contain an essential histidine,



Scheme 1. *Partial reaction mechanisms for malate dehydrogenase acting on its natural substrates (malate and oxaloacetate) and the 3-fluoro substrates*
In all three mechanisms the steps involved in the formation of the binary enzyme–coenzyme complexes are identical. B: and BH^+ are two forms of the histidine residue at the active site of the enzyme. Evidence for the isomerization of the two ternary complexes MDH–NADH–oxaloacetate is given in the text. The state of protonation of the active ternary complexes has not been measured, but the keto acid complexes are shown protonated, by analogy with MDH–NAD–SO_3^-, and with lactate dehydrogenase. Abbreviations: Mal, malate; OxAc, oxaloacetate; FMal; fluoromalate; FOxAc, fluoro-oxaloacetate; $V_{max.f}$, $V_{max.}$ for the forward reaction malate → oxaloacetate; $V_{max.r}$, $V_{max.}$ for the reverse reaction.

and the ionization of that residue is responsible for interactions that can activate the oxidized coenzyme by a factor of 10^5 for the addition of SO_3^{2-}. Both enzymes transform substrates in an essentially compulsory-ordered binding process; in lactate dehydrogenase this order of binding is related to an extended loop of polypeptide chain that can entrap bound reduced coenzyme molecules, and malate dehydrogenase contains this loop of polypeptide chain also (the position of the loop in different malate dehydrogenase complexes is discussed in Weininger *et al.*, 1977). We conclude that the two dehydrogenases have homologous mechanisms. The very similar structures (evidence summarized in Rossmann *et al.*, 1975) and mechanisms of malate dehydrogenase and lactate dehydrogenase are certainly consistent with the two enzymes being products of divergent evolution from a common ancestor. The number and degree of the similarities between the two dehydrogenases would suggest that they are more akin to trypsin–chymotrypsin (usually accepted as being due to divergent evolution) than to subtilisin-chymotrypsin (where the similarities are only at the level of mechanism and are thought to be due to convergent evolution). Of course no experiment with present-day proteins could conclusively distinguish between the two evolutionary situations.

A. L. was supported by an S.R.C. studentship. D. M. P. and J. J. H. thank the S.R.C. for a project grant. Travel between Bristol and Detroit was funded by NATO grant RF 766 to J. D. S. and J. J. H.

References

Cassman, M. & Englard, S. (1966) *J. Biol. Chem.* **241**, 793–799

di Franco, A. & Iwatsubo, M. (1972) *Eur. J. Biochem.* **30**, 517–532

Englard, S., Siegel, L. & Breiger, H. H. (1960) *Biochem. Biophys. Res. Commun.* **3**, 323–327

Francis, A. P. & Coughlan, M. P. (1976) *Int. J. Biochem.* **7**, 59–65

Frieden, C. & Fernandez-Souza, J. (1975) *J. Biol. Chem.* **250**, 2106–2113

Hill, E. J., Tsernoglu, D., Webb, L. E. & Banaszak, L. J. (1972) *J. Mol. Biol.* **72**, 577–591

Holbrook, J. J. & Gutfreund, H. (1973) *FEBS Lett.* **31**, 157–169

Holbrook, J. J. & Stinson, R. A. (1973) *Biochem. J.* **131**, 739–748

Holbrook, J. J. & Wolfe, R. G. (1972) *Biochemistry* **11**, 2499–2502

Holbrook, J. J., Hardman, M. J., Parker, D. M., Yates, D. W. & Gutfreund, H. (1977) *FEBS Lett.* **78**, 46–48

Kitto, B. G. & Kaplan, N. O. (1966) *Biochemistry* **5**, 3966–3980

Kitto, B. G. & Lewis, R. G. (1967) *Biochim. Biophys. Acta* **139**, 1–15

Kun, E. (1963) *Enzymes 2nd Ed.* **7**, 149–160

Kun, E. Grassetti, D. R., Fanshier, D. W. & Featherstone, R. M. (1958) *Biochem. Pharmacol.* **1**, 207–212

Lodola, A. Spragg, S. P. & Holbrook, J. J. (1978*a*) *Biochem. J.* **169**, 511–588

Lodola, A., Parker, D. M., Jeck, R. & Holbrook, J. J. (1978*b*) *Biochem. J.* **173**, 597–605

Loewus, F. A., Tchen, T. T. & Vennesland, B. (1955) *J. Biol. Chem.* **212**, 787–800

Parker, D. M. & Holbrook, J. J. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 485–501, Walter de Gruyter and Co., Berlin and New York

Parker, D. M., Lodola, A. & Holbrook, J. J. (1978) *Biochem. J.* **173**, 959–967

Rao, S. T. & Rossmann, M. G. (1973) *J. Mol. Biol.* **76**, 241–256

Raval, D. N. & Wolfe, R. G. (1962) *Biochemistry* **1**, 263–269

Rossmann, M. G., Liljas, A., Branden, C.-I. & Banaszak, L. J. (1975) *Enzymes 3rd Ed.* **11a**, 61–102

Shore, J. D., Weidig, C. F., Lodola, A., Parker, D. M. & Holbrook, J. J. (1975) *Enzymes, Electron Transport Systems, FEBS Meet. Proc.* **40**, 63–73

Shore, J. D., Halvorsen, H. R. & Lucast, K. D. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed, pp. 416–425, Walter de Gruyter and Co., Berlin and New York

Silverstein, E. & Sulebele, G. (1969) *Biochemistry* **8**, 2543–2550

Stinson, R. A. & Holbrook, J. J. (1973) *Biochem. J.* **131**, 719–728

Weininger, M., Birktoft, J. J. & Banaszak, L. J. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 87–100, Walter de Gruyter and Co., Berlin and New York