Temperature-Jump Study of the Interaction of Malate Dehydrogenase with Reduced Nicotinamide–Adenine Dinucleotide and D-Malate*

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ABSTRACT: A temperature-jump study, with fluorescence detection, was made of the interaction of pig-heart mitochondrial malate dehydrogenase with NADH and D-malate in 0.05 M sodium phosphate–0.054 M NaCl at pH 8, 25°. A relaxation effect was observed in the presence of enzyme and NADH which can be associated with an isomerization of the enzyme–NADH complex formed by a relatively rapid bimolecular reaction. The relaxation time and the amplitude of this relaxation effect increased upon addition of 0.03 M, 0.12 M, and 0.22 M D-malate. At a concentration of 0.22 M D-malate, where the enzyme is essentially saturated with D-malate, the relaxation effect can be identified with an isomerization of the enzyme–NADH–D-malate complex following a relatively fast bimolecular reaction of enzyme–D-malate complex and NADH. A second, slower, relaxation process was also seen in the presence of enzyme, NADH, and D-malate. This process can be associated with an interconversion between two forms of the enzyme; the relaxation time for this process is increased by both NADH and D-malate. The corresponding rate and dissociation constants for the overall mechanism are calculated and the mechanistic implications of the results are discussed.

The mechanism of action of mitochondrial malate dehydrogenase has been extensively studied. Although the enzyme is membrane bound in the mitochondria, it can be readily solubilized. The properties of the soluble and membrane-bound enzyme have been compared (Munkres and Woodward, 1966), but the details of the membrane-enzyme interaction are not known. The soluble enzyme has a molecular weight of 70,000 (Thorne and Kaplan, 1963), and comprehensive steady-state kinetic studies have been done by Wolfe and coworkers (*cf.* Harada and Wolfe, 1968, and references therein). More recently equilibrium isotope exchange measurements have been used to investigate the mechanism of action of this enzyme (Silverstein and Sulebele, 1969). The predominant mechanism involves a ternary complex, with an ordered binding sequence, NADH and NAD being the first substrates bound.

The binding of malate dehydrogenase with NADH and of D-malate with the enzyme–NADH complex produces a substantial fluorescence difference spectrum (Theorell and Langan, 1960; Pfleiderer and Hohnholz, 1960). Although L-malate forms a similar ternary complex, an appreciable fluorescence spectral change does not occur (Theorell and Langan, 1960; Thorne and Kaplan, 1963). Of course, unlike its enantiomer, D-malate is not oxidized by the enzyme in the presence of NAD.

In this work, the results of a temperature-jump study, with fluorescence detection, of the interaction of pig heart mitochondrial malate dehydrogenase with NADH and D-malate are presented. A temperature-jump study of the interaction of NADH and commercial enzyme has been reported (Czerlinski and Schreck, 1964), but the enzyme used was quite impure. Two relaxation processes have been observed. One of them can be identified with an isomerization of the enzyme–NADH complex formed by a relatively rapid bimolecular reaction. The rate constants associated with this isomerization are considerably decreased when D-malate is also bound to the enzyme. The second, slower, relaxation process is observed in the presence of enzyme, NADH, and D-malate. This process can be associated with an interconversion between two forms of the enzyme; the relaxation time for this process is increased by both NADH and D-malate. The mechanistic implications of these findings are discussed.

Experimental Section

The mitochondrial malate dehydrogenase was prepared from fresh pig hearts using the ammonium sulfate and zinc–ethanol fractionation method of Wolfe and Neilands (1956), followed by column chromatography on Amberlite CG-50 (100–200 mesh) (Thorne, 1962; Harada and Wolfe, 1968). The purified enzyme had a specific activity of 1050 units/mg of protein (1968 Seravac Catalog). Enzyme concentrations were determined with a Zeiss PMQII spectrophotometer, using an extinction coefficient of 0.305 cm^2/mg at 280 nm (Harada and Wolfe, 1968). The 280 nm/260 nm absorbance ratio was 1.6 (literature value, 1.6–1.7; Thorne, 1962). The enzyme was stored as a suspension in 70% ammonium sulfate at 4°. Aliquots of this suspension were used in the temperature-jump experiments after centrifugation of the enzyme and overnight dialysis (at 4°) against pH 7 sodium phosphate buffer.

NADH and D-malic acid were purchased from Sigma and Aldrich, respectively. All other reagents were the best available commercial grade.

The temperature-jump apparatus with fluorescence detection is described in detail elsewhere (del Rosario, 1970). This apparatus has a total volume of about 0.3 ml and utilizes two conical lenses at right angles to each other. A small mirror

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received August 12, 1970.* This work was supported by a grant from the National Institutes of Health (GM 13292).

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is imbedded in the cell opposite to and 0.7 cm from each window in order to double the effective path lengths. An exciting wavelength of 340 nm from a 200-W Hanovia xenon–mercury arc lamp was isolated with a Bausch and Lomb grating monochromator. The emission wavelength, centered at about 450 nm, was isolated from the exciting wavelength by means of a Corning C.S. 0-51 ultraviolet transmitting clear filter. The changes in fluorescence were detected with an EMI 9635QB photomultiplier tube. Temperature jumps of 7.7° were applied to the solutions by discharge of a 0.2-μF capacitor which had been charged with 10,000 V. The resolution time of the apparatus is approximately 10 μsec.

All solutions used in the temperature-jump experiments were 0.050 M in sodium phosphate and 0.054 M in NaCl. They were prepared from degassed, deionized, distilled water. The pH of the solutions was adjusted to 8.00 with NaOH and HCl using a Radiometer Model 26 pH meter. Solutions of enzyme, NADH, and D-malate were prepared just prior to use and stored at 4°. In all cases the temperature jump applied to the solutions was from 17.3° to 25°. The relaxation times were computed from logarithmic plots of the relaxation amplitude *vs.* time. Each data point reported represents the average obtained from a mimimum of six oscilloscope traces.

Results and Treatment of Data

A relaxation effect occurring only in the presence of mitochondrial malate dehydrogenase and NADH was observed at pH 8, 25°. A typical oscilloscope trace is shown in Figure 1 (top). The concentration dependence of the reciprocal relaxation time, $1/\tau_1$, is shown in Figure 2. The reciprocal relaxation time tends to reach a limiting value at high concentrations, and therefore the associated chemical process must involve an intramolecular transformation. A simple mechanism consistent with the data is

$$E + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ER \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'R \qquad (1)$$

where E, R, ER, and E'R are enzyme, NADH, and enzyme–NADH complexes, respectively. If the first step is assumed to equilibrate much faster than the second step, the smallest reciprocal relaxation time is (*cf.* Hammes and Schimmel, 1970, for a general discussion of methods for calculating relaxation times)

$$1/\tau_1 = k_{-2} + \frac{k_2}{1 + k_{-1}/(k_1[(\bar{E}) + (\bar{R})])} \qquad (2)$$

where $(\bar{E})$ and $(\bar{R})$ are the equilibrium concentrations of enzyme active sites and NADH, respectively. These concentrations were calculated using an equilibrium dissociation constant of 2 μM for the enzyme–NADH complex (literature values: 5 μM, pH 8 and 25°, Raval and Wolfe, 1962; 1 μM, pH 7.15 and 23.5°, Theorell and Langan, 1960). This value of the dissociation constant was found to provide a good fit of the experimental data to eq 2. A PDP-9 computer was programmed to search for the "best" values of the variables k_{-1}/k_1, k_2, and k_{-2} by an iterative procedure; these best values minimized the squares of the standard deviation of the experimental data from eq 2. The best values of the rate constants are given in Table I. These rate constants, which were used to calculate the uppermost curve in Figure 2 according to eq 2, have an estimated uncertainty of ±25%.

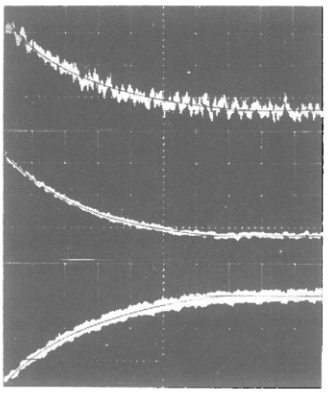

FIGURE 1: Oscilloscope traces of typical relaxation effects: 0.05 M sodium phosphate–0.054 M NaCl, pH 8, 25°. Top trace: the horizontal scale is 0.5 msec per large division and the vertical scale is in arbitrary units of fluorescence intensity. The solution had a total malate dehydrogenase active site concentration of 7.9 μM and total NADH concentration of 16.2 μM (D-malate absent). Middle trace: the horizontal scale is 2 msec per large division and the vertical scale is in arbitrary units of fluorescence intensity. Total concentrations of enzyme active sites, NADH, and D-malate, were 8.0 μM, 10.0 μM, and 0.070 M, respectively. Bottom trace: The horizontal scale is 20 msec per large division and the vertical scale is in arbitrary units of fluorescence intensity. The total concentrations of enzyme active sites, NADH, and D-malate were 8.0 μM, 10.0 μM, and 0.070 M, respectively.

An alternative, slightly more complex, interpretation of the data is to assume that both steps in eq 1 equilibrate at comparable rates. In this case, the two coupled relaxation times must be calculated and used to analyze the data (*cf.* Hammes and Schimmel, 1970). The analytical expression for the slower relaxation time quantitatively fits the data with $k_1 = 5.4 \times 10^7$ M^{-1} sec^{-1}, $k_{-1} = 170$ sec^{-1}, $k_2 = 450$ sec^{-1}, and $k_{-2} = 775$ sec^{-1}. With this interpretation, at low concentrations the relaxation time is essentially associated with the bimolecular reaction coupled to a relatively fast isomerization

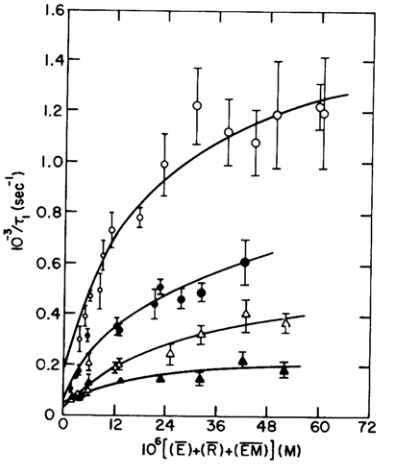

FIGURE 2: A plot of the reciprocal relaxation time *vs.* the sum of the equilibrium concentrations of E, R, and EM for the faster relaxation process associated with interaction of malate dehydrogenase with NADH and D-malate at the following concentrations of D-malate: 0.00 M (○), 0.03 M (●), 0.12 M (△), and 0.22 M (▲); 0.05 M sodium phosphate–0.054 M NaCl, pH 8, 25°. The uppermost curve was calculated from eq 2 using $k_{-1}/k_1 = 0.21 \times 10^{-4}$ M, $k_2 = 1.5 \times 10^3$ sec^{-1}, and $k_{-2} = 0.16 \times 10^3$ sec^{-1}. The three lowest curves were calculated from eq 6 using the appropriate kinetic parameters given in Tables I and II.

TABLE I: Rate and Dissociation Constants for the Interaction of Malate Dehydrogenase with NADH and D-Malate.[a]

$10^4 k_{-1}/k_1$ (M)	0.21	$10^{-2}(k_5 + k_{-5})$ (sec^{-1})	0.55
$10^4 k_{-3}/k_3$ (M)	0.21	$10^{-2}(k_6 + k_{-6})$ (sec^{-1})	0.17
$10^{-3} k_2$ (sec^{-1})	1.5	$10^3 K_{RM}$ (M)	22
$10^{-2} k_{-2}$ (sec^{-1})	1.6	$10^3 K_{RM}'$ (M)	22
$10^{-3} k_4$ (sec^{-1})	0.24		
$10^{-2} k_{-4}$ (sec^{-1})	0.25		

[a] 25°, 0.050 M sodium phosphate–0.054 M NaCl, pH 8.0.

TABLE II: Kinetic Parameters for the Interaction of Malate Dehydrogenase with NADH and D-Malate.[a]

D-Malate (M)	$10^{-2}(k_{-2}^* + k_{-4}^*)$ (sec^{-1})	$10^{-3}(k_2^* + 10_4^*)$ (sec^{-1})	$10^4 K_1^*$ (M)	$10^4 K_1$ (M)	$10^6 K_R'$ (M)
0.00	1.60	1.50	0.21	0.21	2.0
0.03	0.64	0.72	0.18	0.18	1.7
0.12	0.50	0.59	0.38	0.38	3.6
0.22	0.54	0.23	0.27	0.27	2.6

[a] 25°, 0.050 M sodium phosphate–0.054 M NaCl, pH 8.0.

step. However, the bimolecular reaction becomes faster as $[(\bar{E}) + (\bar{R})]$ becomes larger until the relaxation time essentially characterizes the isomerization step coupled to a relatively fast bimolecular reaction. These two alternative interpretations are equally consistent with the data, although it should be mentioned that the relaxation time associated with the "coupled" mechanism has one additional parameter.

Addition of sodium D-malate to a solution of malate dehydrogenase and NADH causes an increase in the relaxation time, τ_1, and in the amplitude of the relaxation effect mentioned above, and the appearance of a second, slower, relaxation effect with an amplitude change opposite in sign to the first. Typical relaxation effects for these two cases are included in Figure 1. Both relaxation times increase with increasing concentration of D-malate up to about 0.2 M D-malate; above this concentration, the relaxation times are essentially independent of D-malate concentration. The concentration dependence of $1/\tau_1$ at various concentrations of D-malate (0.0–0.22 M) is shown in Figure 2.

At a given concentration of D-malate, the concentration dependence of the reciprocal relaxation time (Figure 2) is qualitatively similar, namely the reciprocal relaxation time increases as the concentration of NADH increases and reaches a limiting value at high concentrations. This suggests a simple extension of the mechanism of eq 1.

$$\begin{array}{ccccc} E + R & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ER & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & E'R \\ \pm M \Big\uparrow K_M & & \pm M \Big\uparrow K_{RM} & & \pm M \Big\uparrow K_{RM}' \\ EM + R & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} & ERM & \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} & E'RM \end{array} \qquad (3)$$

Here M, EM, ERM, and E'RM are D-malate, enzyme–D-malate complex, and enzyme–NADH–D-malate complexes, respectively. The lower case k's are rate constants and the capitals are equilibrium dissociation constants. If the vertical steps in eq 3 for binding and dissociation of D-malate and the bimolecular reactions are assumed to equilibrate much faster than the isomerization steps, the smallest reciprocal relaxation time, $1/\tau_1$, is

$$1/\tau_1 = k_{-2}/[1 + (\bar{M})/K_{RM}'] + k_{-4}/[1 + K_{RM}'/(\bar{M})] + \frac{k_2/[1 + (\bar{M})/K_{RM}]}{1 + \dfrac{K_1[1 + (\bar{M})/K_M]}{[(\bar{E}) + (\bar{R}) + (\bar{EM})][1 + (\bar{M})/K_{RM}]}} + \frac{k_4/[1 + K_{RM}/(\bar{M})]}{1 + \dfrac{K_1[1 + (\bar{M})/K_M]}{[(\bar{E}) + (\bar{R}) + (\bar{EM})][1 + (\bar{M})/K_{RM}]}} \qquad (4)$$

where the bars designate equilibrium concentrations. Because of the relatively high concentration of D-malate, the assumption was also made that its concentration remained constant. The equilibrium concentrations were calculated from the equation

$$K_R' = \frac{[(\bar{E}) + (\bar{EM})](\bar{R})}{[(\bar{ER}) + (\bar{ERM}) + (\bar{E'R}) + \bar{E'RM})]} = \frac{[(E_0) - x][(R_0) - x]}{x} = \frac{K_R[1 + (\bar{M})/K_M]}{1 + (\bar{M})/K_{RM}^{\ddagger}} \qquad (5)$$

Here (E_0) and (R_0) are the initial concentrations of enzyme active sites and NADH, respectively. K_R is the equilibrium dissociation constant of the enzyme–NADH complex, $K_{RM}^{\ddagger}$ is the overall equilibrium dissociation constant of D-malate from the enzyme–NADH–D-malate complex, and x is the sum of all enzyme–NADH species. Values for K_R of 2.0 μM, 2.8 μM, and 3.3 μM were used at 0.03 M, 0.12 M, and 0.22 M D-malate concentrations, respectively. These values of K_R have been corrected for the effect of ionic strength (Theorell and Langan, 1960). The values of K_M and $K_{RM}^{\ddagger}$ were taken to be 22.3×10^{-3} M and 8.9×10^{-3} M, respectively (Thorne and Kaplan, 1963).

Equation 4 can be rewritten as

$$1/\tau_1 = (k_{-2}^* + k_{-4}^*) + \frac{(k_2^* + k_4^*)}{1 + \dfrac{K_1^*}{[(\bar{E}) + (\bar{R}) + (\bar{EM})]}} \qquad (6)$$

where the starred parameters vary with D-malate concentration according to the relations

$$k_{-2}^* = k_{-2}/[1 + (\bar{M})/K_{RM}']; \; k_{-4}^* = k_{-4}/(1 + K_{RM}'/(\bar{M})]$$
$$k_2^* = k_2/[1 + (\bar{M})/K_{RM}]; \; k_4^* = k_4/[1 + K_{RM}/(\bar{M})] \qquad (7)$$
$$K_1^* = K_1[1 + (\bar{M})/K_M]/[1 + (\bar{M})/K_{RM}]$$

Equation 6 is of the same form as eq 2 and reduces to the latter equation in the absence of D-malate. An iterative computer program similar to that used to fit the enzyme–NADH data to eq 2 was used to analyze the enzyme–NADH–D-malate data, at each D-malate concentration, according to eq 6. The best values of the parameters $(k_{-2}^* + k_{-4}^*)$, $(k_2^* + k_4^*)$, and K_1^* are given in Table II. These parameters were used to calculate the three lowest curves in Figure 2 according to eq 6. A second iterative computation was done to find the best values of k_4, k_{-4}, and K_{RM} in eq 7 using the values

of ($k_{-2}^* + k_{-4}^*$) and ($k_2^* + k_4^*$) in Table II and those of k_2 and k_{-2} in Table I. The final values of the unstarred parameters as well as of k_{-3}/k_3 and K_{RM}', which are equal to $k_{-1} \cdot K_{RM}/(k_1 K_M)$ and $k_2 k_{-4} K_{RM}/(k_{-2} k_4)$, respectively, by detailed balance, are also given in Table I. Although the parameters in Tables I and II gave the best fit of the data by a least-squares criteria, they are not precisely defined by the data: some of the parameters for nonzero concentrations of malate could be varied a factor of two, while still providing a satisfactory fit of the data in Figure 2. Values of the overall dissociation constant of NADH, K_R', from the enzyme–NADH and enzyme–NADH–D-malate complexes in eq 3 were calculated from the relationship

$$K_R' = \frac{K_1[1 + (\bar{M})/K_M]}{1 + k_2/k_{-2} + [(\bar{M})/K_{RM}][1 + k_4/k_{-4}]} \quad (8)$$

and are also given in Table II.

The concentration dependence of the relaxation time in the presence of D-malate can also be analyzed in terms of the coupled mechanism previously discussed, where the bimolecular reactions between NADH and enzymic species equilibrate at a rate similar to that of the isomerization processes. In this case, $k_1^* + k_3^*$ ($\sim 5 \times 10^7$ M^{-1} sec^{-1}) and $k_2^* + k_4^*$ ($\sim$400 sec^{-1}) are roughly independent of the concentration of D-malate, whereas $k_{-1}^* + k_{-3}^*$ increases (to $\sim$1500 sec^{-1}) and $k_{-2}^* + k_{-4}^*$ decreases (to $\sim$50 sec^{-1}). The starred rate constants are defined in a manner analogous to that previously used. The dependence of the rate constants on the concentration of D-malate is not as well fit as with the simpler analysis, although the "coupled" mechanism cannot be completely excluded.

The reciprocal relaxation time associated with the slow process that is observed only in the presence of enzyme, NADH, and D-malate decreases with increasing D-malate concentration up to about 0.2 M malate, and with increasing NADH concentration up to about 20 μM NADH, as shown in Figure 3. This relaxation process was not visible at NADH concentrations of 40 μM and 60 μM or below about 3 μM, at 0.03–0.22 M D-malate. This rather unusual concentration dependence is characteristic of a mechanism where the species binding D-malate exist in two interconvertible states, with the rate of interconversion being slower when D-malate is bound. In this present case, this mechanism can be written as

$$\begin{array}{ccc} (E,ER) & \underset{\text{fast}}{\overset{\pm M}{\rightleftharpoons}} & (EM,ERM) \\ k_5 \downarrow \uparrow k_{-5} & & k_6 \downarrow \uparrow k_{-6} \\ (E'',E''R) & \underset{\text{fast}}{\overset{\pm M}{\rightleftharpoons}} & (E''M,E''RM) \end{array} \quad (9)$$

where the two chemical species inside the parentheses are assumed to be in rapid equilibrium, the unprimed species are identical with those in eq 3, and the doubly primed species are isomeric forms of the unprimed species. If this mechanism is combined with that of eq 3, eq 9 must be represented in three dimensions where the upper part, $(E,ER) \overset{\pm M}{\rightleftharpoons} (EM,ERM)$, represents the left "square" of eq 3 and the lower part represents a similar square involving isomeric species. These two squares would be joined by the vertical steps in eq 9 to give a cube. However, this representation merely serves to make the formal mechanism unnecessarily complex. Therefore the analysis will be given in terms of eq 9; the analysis for the complete mechanism is virtually identical but more complex.

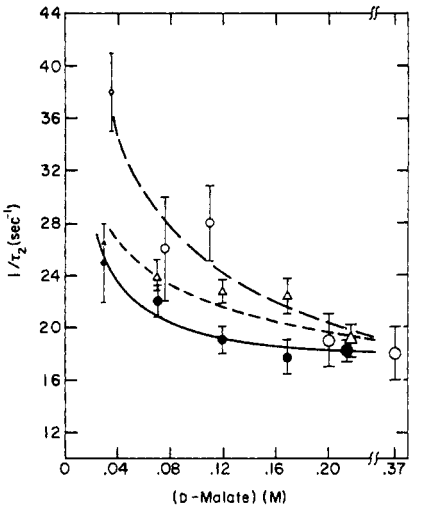

FIGURE 3: A plot of the reciprocal relaxation time *vs.* the concentration of D-malate for the slower relaxation process associated with the interaction of malate dehydrogenase with NADH and D-malate at the following total concentrations of NADH: 4.5 μM (○), 10.0 μM (△), and 20.0 μM (●); 0.05 M sodium phosphate–0.054 M NaCl, pH 8, 25°. The solid curve was calculated from eq 10 using ($k_5 + k_{-5}$) = 55 sec^{-1}, ($k_6 + k_{-6}$) = 17 sec^{-1}, and K_M^* = 8.9 $\times$ 10^{-3} M. The dashed curves are approximations of theoretical curves based on eq 10 as described in the text.

If the steps for binding and dissociation of D-malate in eq 9 are assumed to be very fast, the reciprocal relaxation time, $1/\tau_2$, for the second relaxation effect is given by

$$1/\tau_2 = \frac{k_5 + k_{-5}}{1 + (\bar{M})/K_M^*} + \frac{k_6 + k_{-6}}{1 + K_M^*/(\bar{M})} \quad (10)$$

Here K_M^* is the overall dissociation constant of D-malate from the enzyme–malate and enzyme–NADH–malate complexes. In general, K_M^* will be a function of the NADH, D-malate, and enzyme concentrations. For the results denoted by closed circles in Figure 3 the initial concentrations of enzyme active sites and NADH were 8.0 μM and 20.0 μM, respectively. At these concentrations the enzyme is at least 90% saturated with NADH, so that K_M^* is essentially equal to $K_{RM}‡$. An iterative computer program was used to calculate the best values of $k_5 + k_{-5}$ and $k_6 + k_{-6}$ under these conditions with $K_{RM}‡$ = 8.9 $\times$ 10^{-3} M. The values obtained are given in Table I and were used to calculate the solid curve in Figure 3. A quantitative fit of eq 10 to the data in Figure 3 corresponding to initial NADH concentrations of 10.0 μM and 4.5 μM and initial enzyme active site concentrations of 8.0 μM and 9.0 μM, respectively, is not presented because of the difficulty in determining the appropriate value of K_M^* for each set of points. (Since K_M^* is a function of the NADH, D-malate, and enzyme concentrations, in general it would be different for every point.) However, by using reasonable approximations for K_M^*, curves could be calculated according to eq 10 which closely follow the dashed curves in Figure 3. Thus, the mechanism of eq 9 gives a very good fit of the data.

Discussion

As shown in the top curve of Figure 2, the reciprocal relaxation time, $1/\tau_1$, for the relaxation process observed with malate dehydrogenase and NADH reaches a limiting value

at high values of the sum of the equilibrium concentrations of enzyme and NADH. This is not in accord with the results of Czerlinski and Schreck (1964), who did not observe the plateau of $1/\tau_1$, and therefore interpreted their results in terms of a different mechanism than that postulated here. A possible explanation for this discrepancy is that the apparatus used in this work was apparently more sensitive than that of Czerlinski and Schreck (1964), and the enzyme used was considerably purer.

The mechanism of eq 1 fits the data very well; furthermore a two-step binding mechanism has been reported for many other enzyme–substrate reactions, with similar rates being observed for the isomerization process (Hammes, 1968a,b). Whether or not kinetic coupling between the two steps is of importance cannot be ascertained. The simpler analysis, which assumes the bimolecular step equilibrates much faster than the isomerization step, provides a better fit of all the data, and in any event the simplest possible analysis is always preferable. However, the magnitudes of the rate constants are similar in both cases. (If the bimolecular step is assumed to equilibrate relatively rapidly, it can be estimated that $k_1^* + k_3^* > 10^8$ M^{-1} sec^{-1}.)

The analysis given of the data obtained in the presence of D-malate is clearly only an approximation. Coupling between the two relaxation processes has not been explicitly considered. Furthermore, a precise synthesis of the mechanisms of eq 3 and 9 would give a spectrum of relaxation times, and the analysis presented here is certainly not an exact solution of such a general mechanism. However, the simplified approach used here does give an adequate description of the data. An additional difficulty in interpreting the results is that the ionic strength of the solution changes appreciably at the high concentrations of D-malate used. Previously Theorell and Langan (1960) have reported that the dissociation constant of NADH for the enzyme–NADH–D-malate complex increases significantly at high concentrations of D-malate. The increase in K_1 at high concentrations of D-malate (Table II) may be a reflection of this effect. The value of $k_2^* + k_4^*$ also appears to decrease more than anticipated at the highest D-malate concentration. Nevertheless, the kinetic parameters seem to be self-consistent, and the values of K_R' calculated from them, 1.7–3.6 μM, are in reasonable agreement with those interpolated from published data, 1.1–1.4 μM (Theorell and Langan, 1960). The value of K_{RM} obtained from the kinetic analysis is 22×10^{-3} M, compared with a value of 9×10^{-3} M obtained by equilibrium methods (Thorne and Kaplan, 1963). These discrepancies could be due to the high concentrations of D-malate or to the simplified analysis used. Because of these difficulties, kinetic parameters should be regarded as semiquantitative only, *i.e.*, correct to within about a factor of 2.

The above discussion has emphasized the difficulties and shortcomings of this investigation. However, the essential characteristics of the mechanism which are firmly established by the data should not be overlooked. The concentration dependence of τ_1 clearly indicates an intramolecular process is occurring, and the simplest mechanism involves a conformational change or isomerization of the enzyme–NADH complex. The rate of this conformational change is slowed down by the presence of D-malate. On the other hand, the concentration dependence of τ_2 requires a different explanation, and the simplest one is that a conformational equilibrium exists between enzyme species prior to the binding of D-malate and NADH. The rate associated with this process is slowed down by both NADH and D-malate. This latter process can only be observed in the presence of enzyme, NADH, and D-malate, which suggests NADH is not directly involved, *i.e.*, the effect of NADH on the rate probably occurs indirectly by altering the binding constant for D-malate with the enzyme. These findings again emphasize the many conformational states which can be of importance in enzyme–substrate interactions.

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