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Malate Dehydrogenase. Kinetic Studies of Substrate Activation of Supernatant Enzyme by L-Malate†

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ABSTRACT: At pH 8.0 in 0.05 M Tris-acetate buffer at 25 °C, homogeneous supernatant malate dehydrogenase exhibits substrate activation by L-malate. The turnover number, Michaelis constant for L-malate, and Michaelis constant for NAD are: 0.46×10^4 min^{-1}, 0.036 mM, and 0.14 mM, respectively, for nonactivated enzyme and 1.1×10^4 min^{-1}, 0.2 mM, and 0.047 mM for the same series of constants in activated enzyme. Nonactivating behavior is observed at concentrations between 0.02 and 0.15 mM L-malate and activating behavior is observed between 0.15 and 0.5 mM L-malate. L-Malate activation is compared with similar activation of mitochondrial malate dehydrogenase. While it is not possible to exclude unequivocally all mechanisms, the data seem to be consistent with the occurrence of a fundamentally ordered bi bi mechanism, possibly involving activation through the allosteric binding of L-malate. It is concluded that the data are consistent with a form of the "reciprocating compulsory order mechanism" in which nonactivated enzyme reflects catalysis by one subunit and activated catalysis expresses the coordinated activity of two subunits. The allosteric interaction and the "reciprocating mechanism" are not mutually exclusive.

This study deals with two rather general problems in enzymology, the significance of identical subunits, and the role of multiple enzyme forms. Pig heart malate dehydrogenase is of interest in relationship to the above because it occurs in two forms, mitochondrial and extramitochondrial (supernatant), each of which is a dimer of structurally identical or very similar subunits. The experimental approach involves a detailed study of the non-Michaelian kinetic behavior known as substrate activation, in this case by L-malate, with the purpose of ascertaining the mechanism correctly explaining the observed behavior. This is a continuation of previously published studies attempting to explain anomalous kinetic behavior in the structure-function framework (Harada & Wolfe, 1968), and

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TABLE I: Comparison of Kinetic Constants, Activating and Nonactivating, for Supernatant and Mitochondrial Malate Dehydrogenases.

constant[a]	supernatant enzyme			mitochondrial[b] enzyme		
	nonact.[c]	act.[d]	act. factor	nonact.	activ.	act. factor
	(1)	(2)	(3)	(4)	(5)	(6)
(1) V ($min^{-1} \times 10^{-4}$)	0.46 ± 0.04	1.1 ± 0.1	2.4	2.35 ± 0.18	2.75 ± 0.02	1.2
(2) K_{mal} (mM)	0.036 ± 0.002	0.20 ± 0.06	5.6	1.1 ± 0.15	5.6 ± 0.7	5.1
(3) K_{NAD} (μM)	0.14 ± 0.05	0.047 ± 0.025	0.36	0.48 ± 0.08	0.056 ± 0.009	0.12
(4) K_{iOAA} (μM)	12.0 ± 6.0	61 ± 11	5.1	17 ± 4.5	18 ± 3.2	1.1
(5) K_{iNADH} (μM)	1.8 ± 0.3	3.4 ± 0.05	1.9	24 ± 2.5	20 ± 1.0	0.83

[a] The values of V, K_{Mal}, and K_{NAD} are zero order with respect to both coenzyme and substrate. The velocity V refers to the reaction with NAD and malate. [b] Data for the mitochondrial enzyme are taken from Telegdi et al. (1973). [c] "Nonactivating" refers to L-malate concentrations between 0.02 and 0.15 mM for the supernatant enzyme and between 2.5 and 30 mM for the mitochondrial enzyme. [d] "Activating" refers to L-malate concentrations between 0.5 and 10 mM for the supernatant enzyme and between 30 and 200 mM for the mitochondrial enzyme.

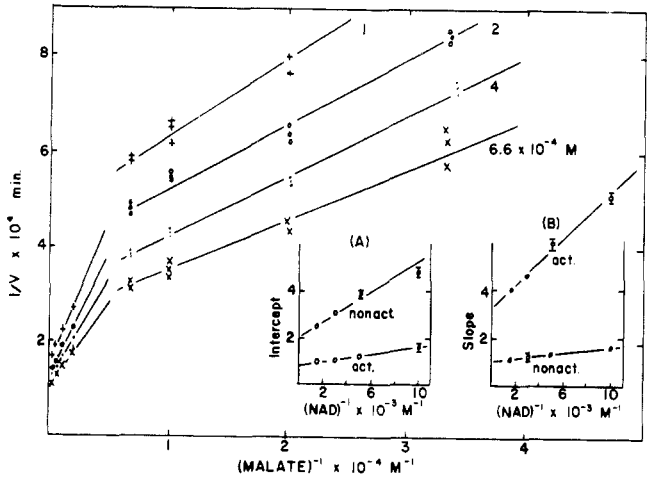


FIGURE 1: Substrate activation by L-malate. Reciprocal turnover number is plotted vs. reciprocal L-malate concentration with NAD concentration fixed as indicated by each line. The two inset graphs are replots of the intercepts (A) and slopes (B) of the primary double reciprocal plots. The vertical bars in the inset graphs represent the standard error. Vertical bars have been omitted when they are smaller than the diameter of the experimental point.

it completes a comparative study, of substrate activation of the mitochondrial enzyme by L-malate (Telegdi et al., 1973).

Within specified substrate and coenzyme concentration limits, both enzyme forms appear to have the simple ordered bi bi mechanism in which coenzyme is added first and coenzyme–product is released last. This mechanism is supported by initial rate data (Raval & Wolfe, 1962, 1963; Frieden & Fernandez-Souza, 1975) and isotope rate data (Schimerlik et al., 1975) as well as by equilibrium isotope rate data for the beef heart enzyme (Silverstein & Sulebele, 1969a,b). Outside of a specified range of substrate concentrations both enzymes show anomalous kinetic behavior, including activation or inhibition by substrates. The possibility that this anomalous behavior might relate to the occurrence of isoenzymes or dimeric enzyme structure prompted this and previously published (Harada & Wolfe, 1968; Telegdi et al., 1973) studies of malate dehydrogenase.

Experimental Procedure

Pig heart supernatant malate dehydrogenase was prepared by the method of Gerding & Wolfe (1969) modified slightly for large-scale isolation. Three enzyme peaks, of the same specific activity, appear in the last hydroxylapatite chromatography purification step. The second of the malate dehydrogenase peaks to elute was used in all kinetic experiments, although all three of these chromatographic peaks are activated by L-malate. The specific activity was 110 international units per milligram of protein (assuming $A_{280}^{1\%}$ equals 9.3) at pH 10, consistent with that observed routinely in isolating the enzyme. Enzyme of this specific activity has been shown to be homogeneous by ultracentrifugal, chromatographic, and electrophoretic criteria (Gerding & Wolfe, 1969). The preparations used in this study were homogeneous in urea gel electrophoresis in 8 M urea–7% polyacrylamide gel (pH 8.0 and 5.0), as indicated by a single protein band stained with Coomassie Blue.

All kinetic measurements were made at 25 °C in Tris buffer (pH 8.0), which was 0.05 M with respect to acetate. NAD (grade III), L-malic acid, and oxalacetic acid were obtained from the Sigma Chemical Co. and used without further purification. NADH, from the same source, was purified by DEAE-cellulose chromatography before use, as described by Mueggler et al. (1975). All solutions were degassed under vacuum and filtered twice through 0.22-μm Millipore filters in order to remove fine particles which interfere with the fluorescence assay.

The reaction rate was recorded with the use of a specially constructed filter fluorimeter similar to that described by Dalziel (1962). This highly sensitive fluorescence assay was necessary because of the very unfavorable equilibrium ($K_{eq}' = 10^{-4}$ for the oxidation of L-malate to oxalacetate at 25 °C and pH 8.0). Rates as low as 1 nmol per min could be measured accurately. At low substrate concentrations, rates were estimated from a tangent drawn to the recorded initial reaction profile. The enzyme concentration was adjusted to give very low rates at low substrate concentrations in order to facilitate more accurate initial rate measurements. The activity was measured in a 3.0-mL reaction volume and the reaction was started by the addition of 10 μL of appropriately diluted enzyme to the temperature-equilibrated reaction mixture. The stability of low concentration enzyme (0.2–10 nM) solutions was monitored carefully during the course of experiments with the use of a standard reference assay.

Michaelis constants, inhibition constants, and maximum velocities were evaluated from intercept and slope replots of reciprocal initial rate data, assuming an ordered bi bi mechanism (Segel, 1975). All data were treated statistically, similar to the method described by Cleland (1963). The exact substrate and coenzyme concentrations used will be apparent in the data.

Results

Figure 1 depicts data for a number of experiments in which reciprocal velocity is plotted against reciprocal L-malate concentration. These data show two approximately linear components, one in the 0.02–0.15 mM concentration range where L-malate is not activating, and the second in the 0.5–10

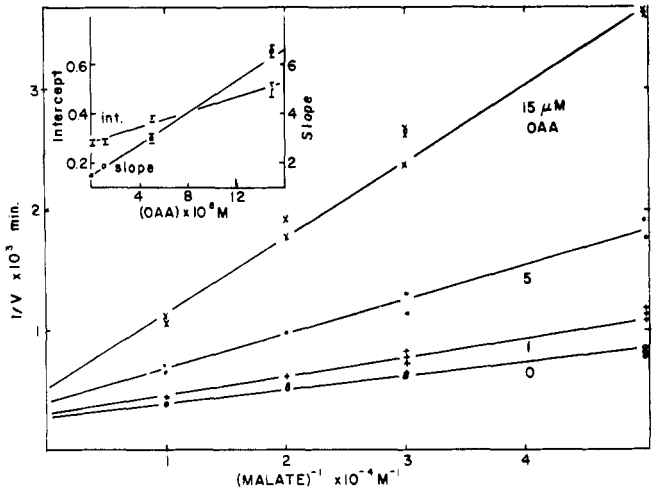


FIGURE 2: Product inhibition of oxalacetate with L-malate nonactivating. The NAD concentration was constant for all measurements at 1.6 mM. Reciprocal turnover number is plotted vs. reciprocal L-malate concentration at the oxalacetate concentration (μM) indicated by each line. The inset graph is a replot of slopes and intercepts of the primary double-reciprocal plots. Vertical bars, which represent the standard error, have been omitted when they are smaller than the diameter of the experimental points.

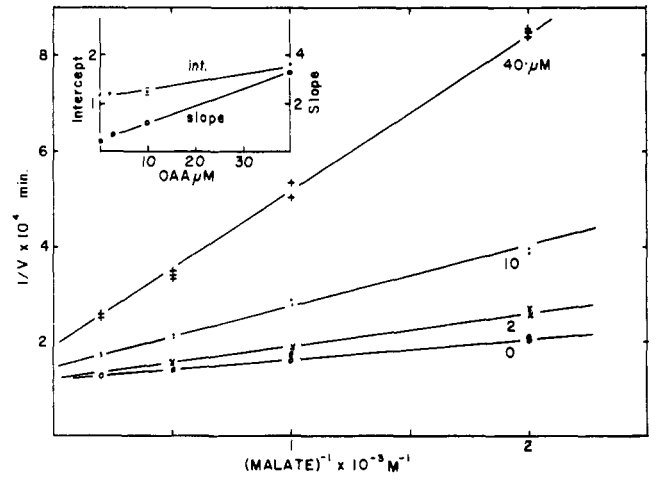


FIGURE 3: Product inhibition by oxalacetate with L-malate activating. The NAD concentration was constant at 1.6 mM in all measurements. Reciprocal turnover number is plotted vs. reciprocal L-malate concentration at the fixed oxalacetate concentration indicated by each line. The inset graph is a replot of the slopes of intercepts of the primary double-reciprocal plot data. See legend to Figure 2 for explanation of vertical bars in the inset graph.

mM concentration range where L-malate is an activator. Concentrations of L-malate greater than 10 mM were avoided because of substrate inhibition. The data shown in the insets of Figure 1, replots of intercepts (A) and slopes (B) of the primary plots, appear to be linear in both the nonactivating and activating L-malate concentration ranges. The maximum velocities and Michaelis constants derived from these data and the inhibition constants K_{iOAA} and K_{iNADH},[1] calculated assuming the ordered bi bi mechanism, are presented in Table I (columns 1 and 2). With the transition from nonactivated to

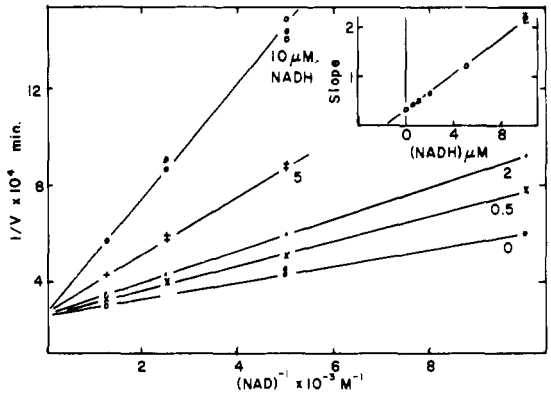


FIGURE 4: Product inhibition by NADH L-malate nonactivating and NAD varied in concentration. Reciprocal turnover number is plotted vs. reciprocal NAD concentration. The concentration of NADH was fixed as indicated by each line. The inset graph is a replot of the slopes of the primary double-reciprocal plots. See the legend to Figure 2 for an explanation of the vertical bars.

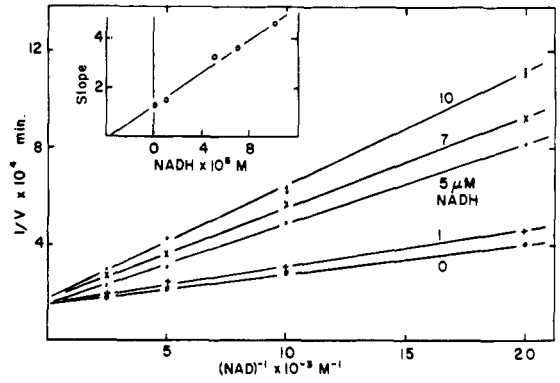


FIGURE 5: Product inhibition by NADH with L-malate activating (fixed at 10 mM) and NAD varied. Reciprocal turnover number is plotted vs. reciprocal NAD concentration. The NADH concentration (μM) is indicated by each line. The inset graph is a replot of the slopes of primary double-reciprocal plots.

activated character, the maximum velocity and Michaelis constants for L-malate increase twofold and fivefold, respectively. The Michaelis constant for NAD decreases about threefold with activation. Table I also compares data with that previously published for the mitochondrial enzyme (Telegdi et al., 1973).

Experiments, of the type depicted in Figure 1, were repeated at higher ionic strength (0.4 M NaCl and in 50 mM NaH_2PO_4). These gave qualitatively similar results with the transition to activating conditions, although there were quantitative changes in the kinetic constants. We interpret this to indicate that ionic strength was probably not the primary cause of the transition from nonactivating to activating enzyme behavior.

Figures 2 and 3 compare product inhibition by oxalacetate when L-malate is nonactivating and activating, respectively. It is apparent that oxalacetate is a noncompetitive inhibitor with respect to L-malate under both experimental conditions.

It is apparent in Figures 4 and 5 that product inhibition by NADH is competitive with respect to NAD when L-malate is activating or nonactivating. Because of the unfavorable equilibrium, it was not possible to determine initial rates accurately at L-malate concentrations below 0.01 mM. Kinetic constants, including the oxalacetate and NADH product inhibition constants, are summarized in Table I (columns 1 and 2). It is

[1] Abbreviations used: V_f and V_r, zero-order maximum velocities for the reaction with NAD-L-malate and NADH-oxalacetate, respectively; K_{OAA}, K_{Mal}, K_{NADH}, and K_{NAD}, Michaelis constants for oxalacetate, malate, NADH, and NAD; K_{iNADH} and K_{iNAD}, dissociation constants for NADH and NAD; K_{iOAA} is the dissociation constant for oxalacetate; OAA, oxalacetate; and Mal, L-malate.

TABLE II: Product Inhibition Patterns with L-Malate, Nonactivating or Activating.

substrate fixed	substrate varied	product inhibitor	Figure	inhibition patterns obsd	inhibition patterns pred[c]
(1) NAD	Mal (n)[a]	OAA	2	NC[b]	NC[b]
(2) NAD	Mal (a)[a]	OAA	3	NC	NC
(3) Mal (n)[a]	NAD	OAA	6 (A)	NC	NC
(4) Mal (a)[a]	NAD	OAA	6 (B)	NC	NC
(5) Mal (n)[a]	NAD	NADH	4	C[b]	C[b]
(6) Mal (a)[a]	NAD	NADH	5	C	C
(7) NAD	Mal (n)	NADH	6 (C)	NC	NC
(8) NAD	Mal (a)	NADH	6 (D)	NC	NC

[a] Nonactivating (n) or activating (a) L-malate concentrations. [b] Noncompetitive inhibition (NC); competitive inhibition (C). [c] The predicted patterns are those for an ordered bi bi mechanism with coenzyme-substrate binding first and coenzyme-product released last (Segel, 1975).

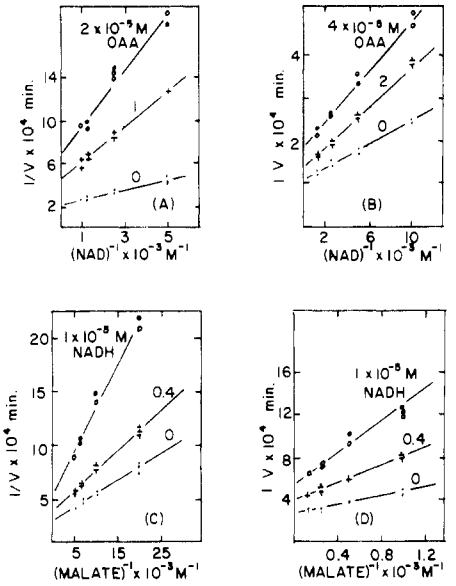


FIGURE 6: Product inhibition by oxalacetate or NADH. Reciprocal turnover number vs. reciprocal NAD of L-malate concentration. (A) Product inhibition by oxalacetate with L-malate nonactivating (100 μM) and NAD varied. (B) Product inhibition by oxalacetate with L-malate activating (10 mM) and NAD varied. (C) Product inhibition by NADH with L-malate nonactivating (50–100 μM) and NAD constant at 4 mM. (D) Product inhibition by NADH with L-malate activating (1–8 mM) and NAD constant at 1 mM.

apparent that the product inhibition constants K_{iOAA} and K_{iNADH} increase with the transition to activating conditions (see column 3, lines 4 and 5).

Figure 6 summarizes product inhibition data under activating and nonactivating conditions. Table II summarizes the product inhibition patterns (competitive or noncompetitive) with L-malate nonactivating and activating and presents a comparison of these patterns with those predicted for the ordered bi bi mechanism. The data are consistent with the persistence of the ordered bi bi mechanism following the transition from nonactivating to activating conditions, and other common alternative bireactant mechanisms are inconsistent with these results (see Discussion).

The value of the kinetic equilibrium constant was calculated with the use of constants listed in Table I and the following additional constants, determined through measurements of rates in the opposite reaction direction: $K_{OAA} = 3.1 \times 10^{-5}$ M, $K_{iNAD} = 4 \times 10^{-4}$ M, and $V_{NADH} = 3.6 \times 10^{4}$ min^{-1}. These data facilitate comparison of equilibrium constants derived from kinetic studies with those from equilibrium measurements through the Haldane relationship for the ordered bi bi mechanism.

Discussion

It follows from the data depicted in Figure 1 that the transition from nonactivated to activated kinetic behavior is apparently independent of NAD concentration. It can be shown by analysis of the theoretical treatment of random bi bi nonequilibrium mechanisms (Ferdinand, 1966), as well as random steady state mechanisms, that this transition should be coenzyme concentration dependent. This property of our data therefore argues against the occurrence of a random order mechanism, or an experimentally significant random steady-state mechanism. With the exception of the ordered bi bi mechanism, our product inhibition data are qualitatively inconsistent with all sequential bireactant mechanisms (both substrates bind before product release) known to us and described by Segel (1975, pages 653–655), including those involving the formation of abortive complexes.

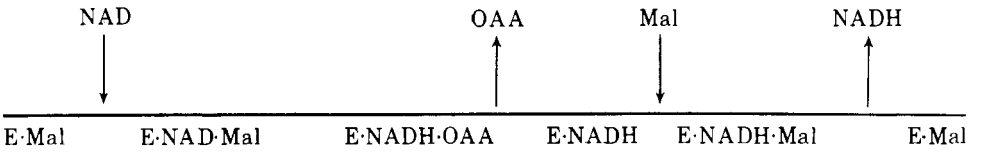
The product inhibition patterns summarized in Table II support the persistence of an ordered bi bi mechanism with the transition from nonactivating to activating conditions. The conclusion is consistent with the interpretation of full time course kinetic studies by Frieden & Fernandez-Souza (1975) and inconsistent with the initial rate kinetic studies of Cassman & Englard (1966).

As indicated in Table I, the kinetic constants change very significantly with the transition from nonactivating to activating behavior. Alternative explanations for this change in kinetic properties will now be considered.

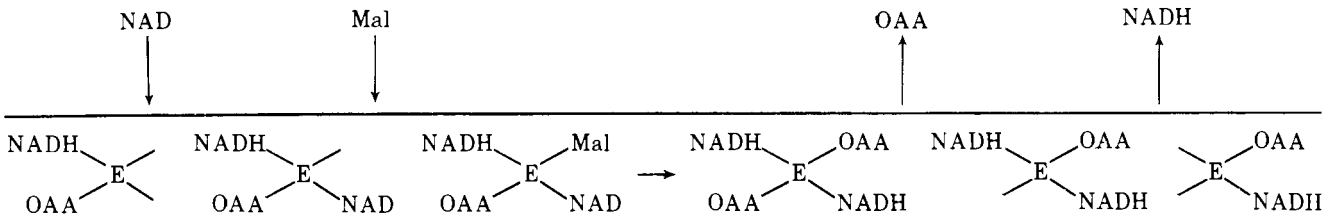
Since the enzyme is known to be a dimer (Devenyi et al., 1966; Bleile et al., 1977), an L-malate-mediated alteration of the monomer–dimer equilibrium might explain activation if the kinetic constants for the monomer and dimer differ. This possibility seems unlikely in that Bleile et al. (1977) have shown that the enzyme does not dissociate to a measurable extent, under conditions similar to those used in our experiments.

The presence of two enzymes, each having different values for their kinetic constants, might account for the biphasic nature of the data in Figure 1. The criteria for homogeneity of our enzyme have been discussed in the Experimental Procedure section of this paper. While isoenzymes of supernatant malate dehydrogenase can be demonstrated in gel electrophoresis (Kulick & Barnes, 1968), peptide maps of tryptic hydrolysates show half the number of peptides expected from the basic amino acid content per 70 000 daltons, consistent with the occurrence of two structurally very similar, if not identical, subunits. Experience in other laboratories (Glathaar et al., 1972; Lodola et al., 1978) suggests that the isoenzyme forms are an artifact of deamidation. In our hands the isoenzyme forms are partially resolved in three nearly completely separated enzyme peaks obtained in hydroxylapatite chromatography. All three of these chromatographically distinguishable

MECHANISM I



MECHANISM II



forms show substrate activation by L-malate. It is of possible significance that substrate activation is not observed when nonphysiological substrates such as hydroxymalonate or mesotartrate are substituted for L-malate. We conclude that it is unlikely that the biphasic data in Figure 1 is caused by the presence of two enzymes.

Previous studies of malate activation of the mitochondrial form of malate dehydrogenase were interpreted (Telegdi et al., 1973) in terms of activation through binding at an allosteric site. This followed the observation that substrate activation occurred at L-malate concentrations about 10 times higher than the value of the L-malate Michaelis constant for the activated enzyme. Since, in the case of the supernatant enzyme, L-malate activates at a concentration (0.2–0.3 mM) equal to the Michaelis constant (K_{mal} = 0.2 mM), no such difference is apparent. This, however, does not exclude the possibility that activation could occur through allosteric binding of L-malate.

Substrate activation might be explained by a mechanism, simplified as Mechanism I below, which was proposed by Dalziel & Dickinson (1966). In this mechanism substrates are bound nonsequentially, in that product (oxalacetate) is released before substrate (malate) is bound. The general form is that of the ping pong mechanism, sometimes associated with double displacement reactions and two enzyme forms. If the rate-dominating step, possibly the release of NADH, is faster in Mechanism I (activating) than it is in the ordered bi bi (nonactivating) mechanism, activation by L-malate might be explained. Equilibrium binding studies by Mueggler et al. (1975) are consistent with the formation of an E·NADH·Mal complex, but only at L-malate concentrations which are five-fold higher than the observed Michaelis constant when L-malate is activating. Mechanism I requires that product inhibition by oxalacetate be competitive with respect to L-malate; noncompetitive inhibition is in fact observed (Table II, line 2). Although a dead-end complex formed by product inhibitor oxalacetate might explain this type of inhibition, Mechanism I has no reaction intermediate capable of forming such a complex. This mechanism seems unlikely because the reciprocal plot patterns are not parallel and oxalacetate product inhibition is not as predicted.

It is possible to explain substrate activation in terms of the modified "reciprocating compulsory order mechanism" depicted in Mechanism II.

The original description of this mechanism (Harada & Wolfe, 1968, page 4136) anticipated the possibility that alternative representations of the mechanism were possible, a point apparently overlooked by Silverstein & Sulebele (1969b) in declaring the original mechanism invalid. Mechanism II differs from the originally published version in that the chemical transformation (hydride transfer) step has been restored to the point between substrate addition and product release. This modification is required by the equilibrium isotope rate experiments of Silverstein & Sulebele (1969a, 1969b), which indicate that the rate of coenzyme oxidation is strongly suppressed by high concentrations of L-malate and oxalacetate in the equilibrium system. It is also of interest that the equilibrium isotope rate experiments of Silverstein & Sulebele (1969a) support the occurrence of an ordered bi bi mechanism in supernatant enzyme from beef heart. Similar data apparently have not been published for the pig heart enzyme.

The "reciprocating mechanism" was suggested to explain the influence of substrate structure, comparing ketomalonate with oxalacetate, on the zero-order maximum velocity of mitochondrial enzyme by the presence of substrate or (product) on the enzyme complex immediately preceding the rate-determining step (NAD release). It was further proposed (Harada & Wolfe, 1968) that the two subunits were functionally interdependent, only one serving as catalyst at a given time, and two of the cycles depicted in Mechanism II are necessary to define the role of both subunits. The inferred subunit interaction presumably expresses negatively cooperative interactions between ligand binding sites. Such negatively cooperative interactions have been demonstrated in supernatant malate dehydrogenase by Mueggler et al. (1975) in equilibrium binding studies.

The rather sharp transition from nonactivating to activating behavior observed in the mitochondrial enzyme (Telegdi et al., 1973, Figure 2) is also apparent in data for the supernatant enzyme. Extrapolation of data from high (activating) malate concentrations in Figure 1 gives an intersection very close to the data points for the highest nonactivating concentration of L-malate. As discussed by Engel & Dalziel (1970), such sharp transitions can be explained by sufficiently strong negative interactions among the active sites.

If one assumes that at relatively low substrate concentrations the affinity of enzyme for L-malate is sufficient only to interact significantly with one higher affinity subunit, a half-active (nonactivated) enzyme might result. As the concentration of L-malate is increased to the range of the lower affinity binding constant on the second subunit, both subunits begin to function in the coordinated reciprocating manner. L-Malate activation would then reflect the transition from a half-active to a fully coordinated dimer in catalysis.

This model, proposing negatively cooperative interactions

between subunits, predicts that the dissociation constants for the various reacting ligands should increase with the induction of activating conditions. In the ultimate case, all four dissociation constants might increase, although thermodynamic constraints may limit changes to fewer than four constants. It is apparent from the data in Table I that the calculated dissociation constants for oxalacetate and NADH, K_{iOAA}, and K_{iNADH}, increase fivefold and twofold respectively with activation. Assuming Mechanism II, the calculated value of K_{iNAD} increases from 6.4×10^{-5} to 4.0×10^{-4} M with activating conditions even though the Michaelis constant for NAD decreases. Unfortunately, the calculation of the fourth dissociation constant, for L-malate, is not straightforward. It seems that three and possibly all four of the ligands bind less tightly with activation, consistent with the generation of negatively cooperative interactions between subunits with substrate activation.

The fact that nonactivated enzyme has a turnover number equal to half that of the activated enzyme (Table I) suggests that the subunit specific activity may remain unchanged but only one subunit is catalytically active in the nonactivated enzyme. The significant changes in experimentally determined values of kinetic constants with activation, however, argue against the simple "switching off" of one independent subunit parent thermodynamic advantage in catalysis, although such an advantage might lie obscured in intermediate steps not necessarily expressed in the rate-limiting step.

In theory, stopped-flow studies might be enlightening if they provide information on the pre-steady-state stoichiometry of the reaction. Unfortunately the steady-state rate of the catalyzed reaction is too rapid to facilitate resolution of the steady-state from the pre-steady-state rates. Our search for a slower reacting substrate, which might provide this physical condition, has invariably disclosed that nonphysiological substrates have more rate-dominating chemical conversion steps, precluding the possibility of determining the pre-steady-state stoichiometry.

The L-malate activation data for supernatant malate dehydrogenase can be compared with similar data for the mitochondrial enzyme in Table I. The difference between the two enzymes is not apparent in the "activation factor" (columns 3 and 6, Table I), which indicates the factor by which each of the kinetic constants is changed with the transition to conditions in which L-malate is a substrate activator. While only two constants, K_{Mal} and K_{NAD}, change significantly with activation in the mitochondrial enzyme, all of the kinetic constants listed in Table I for supernatant enzyme change. K_{Mal} and K_{NAD} change by about the same factor in both enzymes. In addition, the product inhibition constants (Table I, lines 4 and 5) increase with activation of the supernatant enzyme. The activation factor for K_{iNADH} (Table I, line 5, column 3) is about the same as that for maximum velocity (Table I, line 1, column 3), consistent with retention of the ordered bi bi mechanism with NADH-off as a rate-influencing step. This follows because the definition for maximum velocity and K_{iNADH}, assuming the ordered bi bi mechanism with NADH-off rate dominating, contain common rate constants. Activation of the mitochondrial enzyme relates primarily to the smaller K_{NAD} and saturation with NAD at lower concentrations, increasing the prevailing velocity. By comparison, the zero-order velocity is increased when the supernatant enzyme is activated by L-malate.

It was reported by Telegdi et al. (1973) that the mitochondrial enzyme shows activation similar to that reported with L-malate by the divalent anions sulfate and phosphate. No such activation by phosphate was observed in the supernatant enzyme, indicating that activation of the supernatant enzyme is more substrate specific.

Although significant differences are now apparent in the two enzymes, the relationship of these kinetic properties to either catalysis or enzyme regulation is not obvious.

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