Regulation of Mitochondrial Malate Dehydrogenase

EVIDENCE FOR AN ALLOSTERIC CITRATE-BINDING SITE*  

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The effect of citrate on the structure and function of porcine heart mitochondrial malate dehydrogenase (EC 1.1.1.37) has been characterized. The native dimeric form of this enzyme is specifically activated by citrate in the NAD⁺ → NADH direction and inhibited by citrate in the NADH → NAD⁺ direction. It is proposed that citrate is bound at a regulatory site that is distinct from the catalytic site of the enzyme. In binding to this regulatory site, citrate greatly reduces the binding of NAD⁺ as determined by fluorescence titration and "Hummel-Dreyer"-type experiments, but does not diminish the binding of NADH. As would be expected for an effector altering the equilibrium between two conformational forms of an enzyme, citrate favorably perturbs the equilibrium for the reaction in the direction of NAD⁺ reduction. Using [14C]citrate, the stoichiometry of citrate binding to mitochondrial malate dehydrogenase has been determined to be two equivalent sites per dimer, with a dissociation constant of 12.5 µM. In detailed kinetic studies, it has also been observed that activation by citrate abolishes (masks) the enzymatic activity induced by high concentrations of the substrate, L-malate. In addition, Hummel-Dreyer-type experiments indicate that less than a stoichiometric amount of NADH is bound to the enzyme under conditions of malate activation. These data are consistent with a previously suggested second "substrate" binding site proposed to explain the enzymatic activation observed at high concentrations of the substrate, L-malate (Telegdi, M., Wolfe, D. V., and Wolfe, R. G.; J. Biol. Chem. 248, 6484–6489). This allosteric site may exist only on the enzyme conformation capable of binding NAD⁺.

One important goal of enzymology is to attempt to understand the role of protein-protein interactions in metabolic pathways and to provide an understanding of the factors affecting these interactions. The present view of metabolic pathways, such as the tricarboxylic acid cycle, is one of a highly organized, multienzyme complex in which the enzymes and their substrates are to some degree compartmentalized (1, 2). Varying degrees of physical interaction between different enzymes have been proposed (3–5). Prominent among these enzymes is mitochondrial malate dehydrogenase. The unfavorable equilibrium of the reaction catalyzed by this enzyme [(L-malate)/[oxalacetate] > 10⁴] (6, 7) necessitates rapid removal of the product, oxalacetate, before the back reaction can occur. For this reason, interactions between mitochondrial malate dehydrogenase and citrate synthase or aspartate transaminase have been proposed as a means of efficiently utilizing or drawing off the oxalacetate produced by mitochondrial malate dehydrogenase (4, 8).

Physical interactions between different enzymes of a pathway can be envisaged as promoting directed substrate diffusion (9, 10) or inducing conformational changes that modulate enzymatic activity (11). Additionally, the vectorial nature of a metabolic pathway could be enhanced by selectively inhibiting a kinetically favored back reaction while still allowing the forward reaction to be catalyzed at a normal or enhanced rate. In this investigation, data are presented indicating that mitochondrial malate dehydrogenase is an enzyme under such regulatory control, with citrate acting as an effector molecule. Citrate specifically affects several structure-function relationships in mitochondrial malate dehydrogenase, including monomer-dimer equilibrium, protein conformation, and enzymatic activity. Since malate dehydrogenase is the only enzyme in the tricarboxylic acid cycle catalyzing a reaction with a highly unfavorable equilibrium, the regulation of this enzyme by citrate may play an important role in determining the level of tricarboxylic acid cycle activity in vivo.

Previous kinetic investigations of mitochondrial malate dehydrogenase have indicated that, at high concentrations of the substrates malate or oxalacetate, deviations from Michaelis-Menten kinetics are observed (12, 13). Under these conditions, malate was observed to cause an activation of enzymatic activity while oxalacetate acts to suppress or diminish enzymatic activity. Several mechanisms have been proposed to explain these observations; however, these proposals have not fully taken into account the conformational changes this dimeric enzyme may undergo or the possibility of substrates binding at noncatalytic sites, i.e. a regulatory site. In light of the effect of citrate upon the structure and function of mitochondrial malate dehydrogenase, it now appears possible to interpret both activation and inhibition data in relation to a regulatory site and/or conformational changes within the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Porcine heart mitochondrial malate dehydrogenase was purified from acetone powders as previously described by Gregory et al. (14). All other materials were purchased from Sigma Chemical Co. with the exceptions of [1,5-[14C]citric acid and Scintiverse II scintillation mixture, which were obtained from Amersham and Fisher Scientific, respectively.

Enzymatic Assays—The standard assay medium for measurement of mitochondrial malate dehydrogenase activity consisted of 50 mM Tris-HCl, pH 8.1, 25 °C. Substrate concentrations, unless otherwise noted, were 0.2 mM NADH and 0.25 mM oxalacetate in the NADH → NAD⁺ assays, with 10.0 mM malate and 5.0 mM NAD⁺ in NAD⁺ → NADH assays. Total assay volume was 3.0 ml. The change in absorbance was measured at 340 nm on a Gilford 250 spectrophotom-
eter. Protein concentrations were determined spectrophotometrically at 280 nm using the extinction coefficient $E_{280} = 2.53$.

**Fluorescence Titrations**—NADH fluorescence in the presence or absence of enzyme and citrate was measured using a SLM Instruments S800 fluorimeter in the photon-counting mode, with the sample holder thermostatted at 20°C. Excitation and emission wavelengths were 350 and 420 nm, respectively. Initial enzyme concentration was 10 μM in a total volume of 2.0 ml. All NADH stock solutions were prepared immediately prior to use in 50 mM Tris-Cl buffer, pH 8.1, containing the appropriate concentration of citrate. NADH concentrations were determined spectrophotometrically at 340 nm using the extinction coefficient $E = 6200 \text{ M}^{-1} \text{cm}^{-1}$.

**Steady State Kinetics**—The effect of citrate on enzymatic activity was determined using the basic assay system described above. Stock solutions of each substrate were prepared at the highest concentration to be used in each experiment and dilutions were made for assays at lower substrate concentrations. Enzymatic calculations were performed by use of a UNC microcomputer and the data were plotted in Lineweaver-Burk form with least squares analysis.

**Measurement of Ligand Binding by Gel Filtration**—Hummel-Dreyer ligand-binding experiments (15, 16) were performed at 4°C on a column (36 × 0.8 cm) of Sephadex G-25 equilibrated with 50 mM Tris-Cl, pH 8.1. Enzyme and ligand concentrations were as noted elsewhere. The column was monitored for NADH or NAD$^+$ by absorbance at 340 or 260 nm ($E = 17,000 \text{ M}^{-1} \text{cm}^{-1}$ at 260 nm), or by monitoring of $[^{14}C]$citrate.

**Equilibrium Dialysis**—Equilibrium dialysis was performed at pH 7.0, 7.5, and 8.1, in 25 mM Tris-Cl, in 1-ml cell compartments separated by a dialysis membrane of $M_r = 12,000$ cutoff. Enzyme concentration was 2 mM in a total volume of 0.5-0.6 ml side. The specific radioactivity of the citrate used was 333.33 μCi mmol$^{-1}$. Equal aliquots of $[^{14}C]$citrate were initially added to each side of the membrane such that equilibrium was obtained after 24–30 h at 4°C. Duplicate aliquots were assayed for radioactivity in 5 ml of scintillation mixture using a Nuclear Chicago liquid scintillation counter. The data were analyzed in Scatchard form.

**Molecular Weight Determinations**—Molecular weight determinations were performed on a standardized Sephacryl S-200 column measuring 2.5 by 82 cm, at either pH 5 or 7.5 in 50 mM sodium phosphate buffer. Sample load was usually 1-2 mg of mitochondrial malate dehydrogenase in a total volume of 2 ml. Elution volume was ascertainment by determining the absorbance at 220 nm or by the enzymatic activity of each fraction.

Molecular weight determinations were also performed on a standardized Sephadex G-100 column measuring 2.5 by 88 cm, equilibrated at pH 6.8 in 25 mM Tris-Cl. The sample (2 ml) containing 1 μM mitochondrial malate dehydrogenase was loaded onto the column. This is a concentration under which the enzyme exists as monomers. The column was monitored for NADH or NAD$^+$ by absorbance at 340 or 260 nm ($E = 17,000 \text{ M}^{-1} \text{cm}^{-1}$ at 260 nm), or by monitoring of $[^{14}C]$citrate.

**Equilibrium Perturbation**—The forward and reverse reactions catalyzed by mitochondrial malate dehydrogenase were allowed to approach their equilibrium position, i.e. no further change in absorbance at 340 nm. Once equilibrium was established, citrate was added to the assay mixture (final citrate concentration was 15 mM) and the change in absorbance at 340 nm was followed. Initial assay conditions were 5 mM malate and 4.5 mM NAD$^+$ or 5 mM oxalacetate and 4.5 mM NADH in pH 8.1 Tris-Cl buffer. Initial assay volume was 3 ml.

**RESULTS AND DISCUSSION**

Fig. 1 represents the effect of increasing concentrations of citrate on the enzymatic activity of mitochondrial and cytoplasmic malate dehydrogenase. The data are presented as the percentage of enzymatic activity observed in the absence of citrate. As can be seen, there is a marked difference in the effect of citrate on the mitochondrial enzyme, depending upon the direction of the enzymatic assay. In the mitochondrial enzyme, enzymatic activity is enhanced in the NAD$^+$ → NADH assays and diminished in the NADH → NAD$^+$ assays in the presence of citrate. Under these same assay conditions, nominal effects on mitochondrial malate dehydrogenase activity were observed for isocitrate, aspartate, glutamate, and succinate. By contrast, the activity in both assay directions is diminished for the cytoplasmic malate dehydrogenase in the presence of citrate, although not to the same extent as the inhibition observed with the mitochondrial enzyme.

It appears unlikely that citrate influences the enzyme by binding at the catalytic site, since mitochondrial malate dehydrogenase activity increases in the NAD$^+$ → NADH assay direction and decreases in the NADH → NAD$^+$ assay direction. These results, however, can be interpreted as suggesting that citrate binds at a secondary or regulatory site whose role is to influence an equilibrium between two conformations of the enzyme. Each conformation would preferentially bind either the reduced or the oxidized form of nicotinamide adenine dinucleotide, with citrate apparently binding only to that conformation of the enzyme that also binds NAD$^+$. Data supporting this scheme are represented in the Hummel-Dreyer (15, 16) ligand-binding results summarized in Table I. Mitochondrial malate dehydrogenase is capable of binding 2 citrate molecules/dimer. However, increasing the concentrations of malate or oxalacetate decrease the ability of the enzyme to bind citrate. Although citrate and NAD$^+$ both exhibit simultaneous binding to mitochondrial malate dehydrogenase (total binding $n = 3.9$), citrate and NADH appear mutually exclusive in their binding (total binding $n = 2.1$). In addition, concentrations of malate which cause apparent activation of the enzyme (>$20$ mM) also decrease NADH binding to the enzyme.

The effect of citrate on NADH binding was also examined by fluorescence titration experiments (Fig. 2). By plotting the percentage of saturation (measured by the change in fluorescence intensity between bound and free NADH) as a function of the NADH/mitochondrial malate dehydrogenase ratio, the fluorescence titration curve yields a value of $≈2$ NADH molecules bound/dimer of mitochondrial malate dehydrogenase in the absence of citrate. However, the addition of citrate makes extrapolations no longer possible due to the poorly defined linear portions of the curve, resulting from an apparent increase in the binding constant of NADH. The NADH/mitochondrial malate dehydrogenase-binding ratios in the presence of citrate appear much greater than 2, indicating increased "competition" between citrate and NADH for the appropriate binding form of the enzyme. Since Hummel-Dreyer ligand-binding data also indicate that less NADH can bind to mitochondrial malate dehydrogenase with citrate-pres-
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TABLE I

<table>
<thead>
<tr>
<th>Citrate</th>
<th>Ligand</th>
<th>[Citrate]/[Malate dehydrogenase]</th>
<th>[Ligand]/[Malate dehydrogenase]</th>
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<tr>
<td>mM</td>
<td>mM</td>
<td></td>
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<tr>
<td>0.5</td>
<td>0</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>0.4</td>
<td>Malate (2)</td>
<td>1.21</td>
<td>ND</td>
</tr>
<tr>
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<td>Malate (50)</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>0.4</td>
<td>OAA (2)</td>
<td>1.26</td>
<td>ND</td>
</tr>
<tr>
<td>0.4</td>
<td>OAA (26)</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>0.4</td>
<td>NAD⁺ (0.0325)</td>
<td>1.75</td>
<td>2.15</td>
</tr>
<tr>
<td>0</td>
<td>NADH (0.032)</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>NADH (0.032)</td>
<td>0.87</td>
<td>1.2</td>
</tr>
<tr>
<td>0</td>
<td>NADH (0.032) +</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malate (40)</td>
<td></td>
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</tr>
</tbody>
</table>

Enzymatic activity increases in the presence of citrate, and enzymatic activation at the higher concentrations of malate (>20 mM) is abolished or "masked." These kinetic data are consistent with a previously proposed second substrate site suggested as a means to accommodate the activation observed at these higher concentrations of malate (17). In Fig. 4B, where NAD⁺ is the varied substrate, increasing citrate concentrations increase enzymatic activity without altering the apparent $K_m$ for NAD⁺. It therefore appears unlikely that NAD⁺ and citrate are competing for the same site on the enzyme.

At noninhibitory oxalacetate concentrations, citrate and oxalacetate appear to be noncompetitive (Fig. 5A). However, the apparent decrease in NADH binding by citrate observed in fluorescence titration experiments is not due to NADH simply binding in a manner undetectable by this particular technique.

From equilibrium dialysis experiments performed with [¹⁴C] citrate, the stoichiometry of citrate binding has been determined to be two equivalent sites per enzyme dimer (Fig. 3). This result is in agreement with data obtained in Hummel-Dreyer-type experiments (Table I). The apparent dissociation constant varies from 12.5 μM at pH 7.0 to 75 μM at pH 8.1 (data not shown). These data suggest that a limited number of specific sites are available to which citrate may bind, with a dissociation constant in the range of metabolite concentrations expected in vivo for respiring mitochondria.

In kinetic studies, the alterations in kinetic parameters observed in the presence of citrate support the model presented for citrate regulation of mitochondrial malate dehydrogenase activity. Fig. 4A represents the effect of citrate on enzymatic activity as a function of malate concentration.
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**Fig. 5.** Effect of citrate on steady state kinetics. Mitochondrial malate dehydrogenase (m-MDH) was assayed in the direction of NADH oxidation. A, NADH concentrations were 0.2 mM. Citrate concentrations were 0 (●), 3 (■), and 10 mM (▲). B, oxalacetate (OAA) concentrations were 0.25 mM. Citrate concentrations were 0 (●), 2 (■), and 5 mM (▲).

**Fig. 6.** Activity as a function of citrate concentration at inhibitory levels of oxalacetate. The concentration of NADH was 0.2 mM. The oxalacetate concentrations employed were 5 (●), 10 (■), and 20 mM (▲). The decrease in oxalacetate concentration in these initial velocity measurements was negligible. m-MDH, mitochondrial malate dehydrogenase.

if inhibitory concentrations of oxalacetate are used (>1.0 mM), citrate and oxalacetate apparently compete for the same site on the enzyme. Fig. 6 represents a modified Yonetani-Theorell plot (18), used in determining the interaction of two different inhibitors with an enzyme. Since the inhibitor oxalacetate is also a substrate, \( V_i \) (and not \( V/V_i \)) was plotted versus citrate concentrations at varying oxalacetate concentrations. The \( V_i \) versus [citrate] lines obtained are parallel; therefore, the interaction constant (\( k \)) for these two inhibitors is \( \infty \). According to this procedure, when \( k = \infty \), mutual exclusion is demonstrated in which the binding of the second inhibitor prevents binding of the first inhibitor, presumably due to the fact that the two inhibitors are not capable of simultaneously occupying the same site on the enzyme. This type of competition was also suggested by the data in Table I. With NADH as the varied substrate (Fig. 5B), citrate and NADH appear to compete with each other. This anomalous inhibition pattern has also been observed by Cennamo et al. (19). From the kinetic data previously presented, however, it is unlikely that NADH and citrate are competing for the same site on the enzyme. The steady state kinetic data are consistent, however, with the conclusion that citrate binds to an enzyme conformation that also binds NAD\(^+\), and that citrate does not bind to a conformation that binds NADH. Citrate and NADH would thus appear competitive in steady state kinetics (Fig. 5B) as both would be competing for different sites existing on mutually exclusive conformations of the enzyme.

The existence of two binding conformations of malate dehydrogenase is further supported by the data shown in Fig. 7. If citrate does alter the equilibrium between two conformations of an enzyme catalyzing two opposing reactions, citrate might also perturb the apparent equilibrium position for the two reactions. This result was indeed observed. Upon addition of citrate to a solution of mitochondrial malate dehydrogenase and its substrates at equilibrium (i.e., no further change occurring in absorbance at 340 nm), a new equilibrium becomes established in which the overall reduction of NAD\(^+\) is enhanced, regardless of the manner of direction from which the original equilibrium was approached.

This new apparent equilibrium position observed for the forward and reverse reactions does not reflect a changed equilibrium constant for the substrates free in solution, but does indicate an altered equilibrium constant for reactants and products on the surface of the enzyme (20). Thes, the change in apparent equilibrium constant for the enzyme-catalyzed reaction can be interpreted as resulting from citrate perturbing the relative proportion of two enzyme conformations, each capable of preferentially binding a different form of cofactor and substrate. This change is expressed in altered relative velocities for the two reaction directions. This proposed mechanism of inhibition and activation of mitochondrial malate dehydrogenase by citrate appears to be a special
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Case of a perturbable “oscillating enzyme,” initially proposed by Jencks (21), in which the force sufficient to define two distinct enzyme conformations is supplied, or augmented, by citrate. Even though the forward and reverse reactions are preferentially catalyzed on different conformations, the principle of microscopic reversibility (22) is not violated. An opposing reaction may still be catalyzed on the less favorable enzyme conformation for that reaction, although to a lesser extent than if the opposing reaction were catalyzed on a subunit in the preferred conformation for that reaction. Thus, microscopic reversibility still applies to each subunit conformation, even though the overall macroscopic reaction pathway preferentially occurs on different enzyme conformations for each opposing reaction. Citrate apparently changes the relative proportions of the two enzyme conformations.

In gel filtration experiments performed under conditions favoring monomer formation of mitochondrial malate dehydrogenase (pH 5) (23, 24), citrate, oxalacetate, and malate were observed to induce partial (half-way) redimerization of the subunits. Isocitrate, fumarate, succinate, aspartate, and glutamate, however, were found to be ineffective in facilitating redimerization. Mitochondrial malate dehydrogenase also exhibits a concentration-dependent dissociation (23). When the enzyme is applied to a G-100 column at pH 6.8 under conditions where it already exists as monomers, citrate and malate induce full redimerization (Fig. 8). Unlike monomers at pH 6.0, monomers at pH 6.8 do not need to undergo a gross conformational change (i.e., a proline cis/trans-isomerization) prior to reactivation upon redimerization (25). Therefore, the inflection points observed in Fig. 8 are indicative of each ligand’s Kd. The Kd for citrate (~10 μM) obtained from column chromatography is in close agreement with the 12.5 μM Kd obtained from equilibrium dialysis experiments (see above). If a compulsory ordered mechanism is required for substrate addition (26), redimerization by substrates cannot be a result of their binding at the active site, since the cofactors, NADH and NAD⁺, were absent in these experiments. These data, therefore, are in agreement with a binding site on mitochondrial malate dehydrogenase to which citrate, as well as malate and oxalacetate, may bind if present in sufficient concentration.

In previous studies from this laboratory involving immobilization of mitochondrial malate dehydrogenase on Sepharose beads (27), different conformations of mitochondrial malate dehydrogenase were suggested. Of particular interest is the observation concerning the significantly greater decrease in observed enzymatic activity determined for immobilized enzyme assayed in the NADH → NAD⁺ assay as compared to that for enzymatic activity observed in the NAD⁺ → NADH assay direction. These data could be interpreted as suggesting a conformationally restricted resin-bound enzyme which favors NAD⁺ reduction. This form of the enzyme would then be similar in conformation to the proposed citrate-binding form. In other studies, different binding conformations have also been suggested by the differential effects of lipoic acid, palmitoyl-CoA, and other long chain fatty acids on mitochondrial malate dehydrogenase activity (28-31). In each of these instances, the effect on enzymatic activity is much greater, by several orders of magnitude, on one direction of enzymatic activity as compared to the other.

Any possible rationale for the effects observed with citrate on mitochondrial malate dehydrogenase must take into account the unfavorable equilibrium for the reaction mitochondrial malate dehydrogenase catalyzes (6, 7). Citrate regulation of mitochondrial malate dehydrogenase enzymatic activity could favorably perturb the reaction equilibrium, thereby increasing the overall level of tricarboxylic acid cycle activity. Additionally, the precursor for oxalacetate, malate, is readily transported across the mitochondrial membrane (32), whereas oxalacetate is not. Thus, the depletion of malate from the mitochondria and the lowering of subsequent tricarboxylic acid cycle intermediates can be at least partially controlled by regulation of the enzymatic activity of mitochondrial malate dehydrogenase, thereby ensuring an adequate supply of tricarboxylic acid cycle intermediates when needed.

In addition to the effect of citrate on malate dehydrogenase activity, it has been reported that citrate inhibits citrate synthase by competing with oxalacetate (Ki = 1.6 mM) (33). Therefore, under certain conditions, citrate may simultaneously promote oxalacetate production by the action of malate dehydrogenase and inhibit its utilization by citrate synthase. This effect could result in a “channeling” of oxalacetate out of the tricarboxylic acid cycle and into the malate-aspartate shuttle for utilization by aspartate aminotransferase. Recent estimates of the intramitochondrial citrate concentration have been obtained by various rapid cell separation techniques (34, 35). The citrate concentrations observed ranged from 1.72 to 8.75 mM, suggesting that the in vivo concentration of citrate is well within the range of the observed in vitro effects of citrate on malate dehydrogenase activity. In addition, it is probable that the localized citrate concentration in respiring mitochondria may even be slightly higher than the values reported above due to microcompartmentation within the mitochondria. Thus, the observed in vitro effects of citrate on malate dehydrogenase may well play a role in metabolic regulation in vivo.

It has previously been observed that the release of coenzyme A is the probable rate-limiting step for the forward reaction catalyzed by mitochondrial malate dehydrogenase (26, 36, 37). One possible molecular basis for the observed rate enhancement in the NAD⁺ → NADH direction by citrate would be a facilitated release of NADH from the enzyme. If citrate binding favors an enzyme conformation that binds NADH less favorably, then release of NADH as a rate-limiting step would become less restrictive. This would be a major advantage for a conformationally oscillating-type enzyme, which could be differentially perturbed depending on cellular metabolic conditions. It appears likely that the observed malate activation of mitochondrial malate dehydrogenase may also function through the proposed citrate regulatory site by a similar mechanism. The effects of citrate and malate activation were not determined to be additive (Fig. 4A), as might be expected if citrate and malate activation of mitochondrial malate dehydrogenase occurred by different mechanisms. An alterna-

FIG. 8. Effect of citrate on the concentration-dependent dissociation of mitochondrial malate dehydrogenase. Malate dehydrogenase (1.0 μM) was applied to a Sephacryl G-100 column equilibrated with ligand at the concentrations indicated: ○, citrate; □, L-malate.
tive explanation for oxalacetate inhibition via a "dead-end" complex formation with NAD" (38) is also possible, since, at inhibitory concentrations of oxalate, oxalacetate and citrate appear to compete for the same binding site (Fig. 6). In the scheme presented here, oxalacetate would be binding to a regulatory site on a conformation not conducive to oxalacetate catalysis, thus causing inhibition for that assay direction. This scheme, however, does not preclude additional mechanisms of inhibition by oxalacetate, such as the one proposed by Bernstein et al. (13), involving a binary complex between the enzyme and the enol form of oxalacetate.

Telegdi et al. (17), in originally proposing a second site for malate activation, observed that NADH was noncompetitive with malate under nonactivating conditions. They also concluded that NADH was noncompetitive with malate under activating conditions, even though the differences in the 1/v intercept for the Lineweaver-Burk plots were all within experimental error. This conclusion was reached primarily because no simple bieactant product inhibition pattern could adequately explain the apparent competitive inhibition pattern between NADH and malate. However, it now appears that the kinetic pattern these authors observed under conditions in which malate activates the enzyme may be better understood if interpreted according to the scheme presented here for citrate activation and inhibition of mitochondrial malate dehydrogenase. Their results may indeed indicate a competition between malate and NADH, not for the same conformation of mitochondrial malate dehydrogenase shown in Fig. 9. Even though the kinetic pattern these authors observed under conditions of activating malate would appear at activating concentrations would be binding at the citrate binding site, but for two conformations of the enzyme. Malate at activating concentrations would be binding at the citrate regulatory site, a site existing only on that conformation of the enzyme that binds NAD". Thus, product inhibition by NADH under conditions of activating malate would appear as classical competitive inhibition.

In summary, fluorometric titrations, steady state kinetics, equilibrium perturbation, and ligand-binding experiments all support the proposed model for citrate binding to mitochondrial malate dehydrogenase shown in Fig. 9. Even though the two conformations are represented as dimers, it is not meant to be implied that citrate binding to the enzyme is a concerted process. In this model, citrate and NAD" both bind to the same conformation of mitochondrial malate dehydrogenase and may do so simultaneously. Citrate and NADH, however, preferentially bind to mutually exclusive enzyme conformations. The binding of citrate perturbs the equilibrium between two conformations of the enzyme, allowing both activation and inhibition, depending upon the assay direction. Previously obtained anomalous kinetic data may be explained by this model, including malate activation and oxalacetate inhibition. In preliminary studies, similar citrate activation and inhibition patterns have been observed for porcine liver and bovine heart mitochondrial malate dehydrogenase.

Recently, Beeckmans and Kanarek (8) have observed, by use of protein immobilization techniques, mitochondrial malate dehydrogenase interactions with specific enzymes of the tricarboxylic acid cycle and the malate-aspartate shuttle. Their data suggest that a redistribution of enzymes involved in complexes with mitochondrial malate dehydrogenase can occur. It is therefore possible that the stoichiometry of these enzyme complexes might change in response to changes in cellular conditions. Experiments are currently in progress in this laboratory to determine the role citrate and other metabolites might play in regulating the type of mitochondrial malate dehydrogenase complexes that may be formed under various metabolic conditions. The interesting possibility exists for the shunting of intermediates in and out of pathways as a consequence of changes induced in enzyme complexes by the metabolic state of the cell.

REFERENCES

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