Cooperativity in the Mechanism of Malate Dehydrogenase†

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ABSTRACT: Cooperativity in the catalytic mechanism of porcine cytoplasmic malate dehydrogenase (sMDH) has been a point of ongoing discussion. Though previous investigations revealed little evidence of cooperativity, chemical modification studies reported by this laboratory demonstrate that binding of cofactor or cofactor plus substrate causes the enzyme's subunits to become chemically nonidentical. Therefore, we have reexamined the enzyme's steady-state kinetic and ligand-binding properties. To aide in characterizing sMDH kinetics, activities of the native enzyme and of sMDH, which was partially inactivated by an active-site-specific reagent, were examined. As expected for a negatively cooperative enzyme, steady-state kinetics (at pH 8.0, the pH optimum of the enzyme) are characterized by concave Eadie–Hofstee plots. Further, qualitative as well as quantitative results from partially inactivated sMDH strongly support negative cooperativity and eliminate many alternative mechanisms. Finally, results from equilibrium binding experiments are consistent with cytoplasmic malate dehydrogenase binding NADH in a negatively cooperative manner. Together, these results indicate that cytoplasmic malate dehydrogenase acts as a negatively cooperative enzyme.

Oxaloacetate (OAA)[1] is an important cytoplasmic intermediate in a variety of metabolic pathways including the malate shuttle, gluconeogenesis, and fatty acid synthesis. Cytoplasmic malate dehydrogenase (sMDH) plays a major role in controlling the metabolite's concentration and is a principle or ancillary component of these metabolic sequences (Voet & Voet, 1990; Conover, 1987; Srere, 1987; Safer, 1975). Despite its central metabolic role, disagreement remains over the kinetic properties of this enzyme.

Several laboratories have examined binding and kinetic characteristics of porcine sMDH and reached varying conclusions. Early fluorescence measurements of NADH-binding and steady-state kinetic analyses suggest that the enzyme is cooperative (Meuggler & Wolfe, 1978; Meuggler et al., 1975; Frieden & Fernandez-Sousa, 1975). However, stopped-flow kinetic studies and calorimetric binding investigations indicate the enzyme binds cofactor in a noncooperative manner (Lodola et al., 1978a,b; Johnson & Rupley, 1979). Although an atomic model of the enzyme has been derived from X-ray crystallographic studies (Birktoft et al., 1987; Webb et al., 1973; Hill et al., 1972), this has not settled the question of cooperativity. The enzyme's active sites differ in their conformational details and do not bind NAD identically in the crystallized NAD–enzyme complex (Glatthaar et al., 1972). This would seem consistent with cooperativity. However, crystal packing places each of the enzyme's active sites in a different environment, one near a protein–protein contact region of the crystal lattice. It may be that crystal packing interactions rather than cooperativity cause the observed active-site differences (Weininger et al., 1977).

We have previously reported active-site chemical modification studies which suggest that sMDH interacts with NADH, NAD, or abortive cofactor–substrate pairs in a cooperative manner (Zimmerle et al., 1987). Here we report NADH binding studies and steady-state kinetic characterizations of native and partially inactivated sMDH. From these lines of investigation, we conclude that sMDH is negatively cooperative.

EXPERIMENTAL PROCEDURES

Materials. Dithiolthreitol (DTT), HEPES buffer, iodoacetic acid (IAA), NAD (type III), and NADH (type III) were purchased from Sigma Chemical Co. L-Malic acid (MAL) and oxaloacetate (OAA) were obtained from ICN Biochemicals Inc., while Blue Sepharose was obtained from Pharmacia LKB Biotechnology Inc. Other chemicals used in these studies were reagent grade or better.

Enzyme Preparation. Porcine heart sMDH was purified according to a modification of the procedure of Glatthaar et al. (1974), which included a Blue Sephadex affinity chromatography step (Zimmerle & Alter, 1983). The purified enzyme was homogeneous as judged by SDS and native PAGE analysis. Further, by the criteria of OAA inhibition and 4,4′-bis(dimethylamino)diphenylcarbinal inactivation, there was no motichondrial MDH contaminant (Bernstein et al., 1978; Humphries et al., 1973). Enzyme preparations had specific activities of 750–800 units (min^{-1}) mg^{-1} of protein when assayed in the direction of OAA reduction (0.25 mM NADH, 1 mM OAA, 50 mM HEPES, pH 8.0, and 25 °C). This compares favorably with other reported preparations (Johnson & Rupley, 1979; Lodola et al., 1978a; Glatthaar et al., 1974; Gerding & Wolfe, 1969).

Partially inactivated sMDH was prepared by reacting purified sMDH (1 mg/mL) with 250 mM iodoacetic acid in 50 mM HEPES buffer, pH 8.0, 37 °C, and quenching with 0.3 M dithiotheitol buffered in 50 mM PIPES, pH 6.0 (Zimmerle et al., 1987), after various incubation times. These enzyme preparations were exhaustively dialyzed versus the appropriate buffer prior to kinetic or equilibrium-binding analyses.

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[1] Abbreviations: DTT, dithiothreitol; IAA, iodoacetic acid; OAA, oxaloacetate; MAL, malate; sMDH, cytoplasmic malate dehydrogenase.

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Enzyme Assays. sMDH was assayed spectrophotometrically at 340 nm with use of a Cary 219 spectrophotometer equipped with a thermostated cuvette holder. Reactions were routinely run at pH 8.0, the enzyme's optimum pH for OAA reduction (Zimmerle & Alter, 1983), in 50 mM HEPES at 25 ± 0.5 °C. Rates were calculated with use of the NADH extinction coefficient of 6300 M^{-1} cm^{-1} at 340 nm (McComb et al., 1976).

Titration of sMDH with NADH and Malate. NADH and malate binding were monitored fluorometrically by using a Perkin-Elmer Model 512 double-beam spectrofluorometer equipped with a thermojacketed cuvette holder and cylindrical semimicro quartz cells (0.5-cm path length, 0.5-mL capacity). Solutions made 0.3–35 μM in sMDH active sites were titrated with microliter aliquots of buffer containing the ligand of interest. Binding-linked changes in NADH fluorescence (increases) were follwed at 415 and/or 440 nm while the sample was excited at 340 nm. Concomitant quenching of sMDH fluorescence was monitored at 360 nm while the sample was excited at 297 nm. Routinely, titrations were carried out in 50 mM HEPES buffer at pH 8.0 and 25 °C.

Analysis of Steady-State Kinetic Data. The general sequential interaction model of Koshland, Nemethy, and Filmer (1966) as described by Segel (1975) was used to analyze solution-phase sMDH activity. In this model, subunit interactions affecting substrate binding are quantitated by "interaction constants", which are equilibrium constants for the conversion of a subunit from a ligand-free conformation to the conformation it has when a particular ligand binds. Since the binding of a ligand is viewed as being linked to a subunit conformational change, the observed binding constant is expressed as a product of the appropriate interaction constant and the reciprocal of an intrinsic ligand dissociation constant. For a two-subunit enzyme which binds a substrate and a cofactor, such as sMDH, 10 independent interaction constants are required to describe subunit and ligand interactions. The precision of our experimental data did not justify an independent fitting of all constants. However, the data were fit by using a simplification of the model which incorporates *interaction between subunits* in the following way: cofactor binding sites on different subunits are assumed to interact only with each other, as are substrate binding sites. The values of the constants describing the interaction between subunits containing substrate and cofactor are taken to be the product of cofactor–cofactor and substrate–substrate interaction constants ("Cofac" and "Subs", respectively). Each interaction constant is the ratio of K_D values for binding at the first site on a dimer to binding at the second site. We assume that the subunit interaction is expressed entirely in the binding of the second molecule of each ligand. The product of the interaction constant and the reciprocal of the ligand's intrinsic dissociation constant, then, is the association constant for ligand binding to the second subunit. *Interactions between substrate and cofactor on the same subunit* are included in this model using a factor (α) which is the ratio of the substrate dissociation constant in the absence of cofactor to the constant when cofactor is already bound. (The same constant describes the change in cofactor affinity caused by substrate binding to the same subunit.) Again, the product of the constant and the reciprocal of the intrinsic dissociation constant is the association constant for substrate binding to a cofactor:subunit binary complex. Finally, a multiplier of V_M accounting for turnover number changes resulting from cooperative interactions, ACT, is also included. The equation used for this analysis is

$$V = \{V_M([E_2C'S'] + [E_2CS_2] + [E_2C_2S]) + 2(ACT)(V_M)([E_2C_2S_2])\}/\{1 + [E_2S] + [E_2C] + [E_2C'S'] + [E_2S_2] + [E_2CS] + [E_2CS_2] + [E_2C_2] + [E_2C_2S] + [E_2C_2S_2]\} \quad (1)$$

where the concentration of each form of the enzyme is represented by a group of upper case letters. E_2, C, and S represent the dimeric enzyme, cofactor, and substrate, respectively. Primes indicate that cofactor and substrate are on the same subunit, while ligand subscripts correspond to the number of respective molecules bound to the enzyme. The concentration of each form of the dimeric enzyme is calculated by using dissociation and interaction constants as briefly described here and more fully discussed by Zimmerle (1985) and Segel (1975). The concentration of a complex containing a cofactor and substrate on one subunit and a cofactor only on the other subunit, for example, is given by

$$[E_2C_2S] = [S][C]^2[E_2]\frac{\alpha(\text{Cofac})}{K_{\text{Cofac}}{}^2K_{\text{Subs}}}$$

where K_{Cofac} and K_{Subs} are intrinsic dissociation constants for cofactor and substrate binding, respectively.

Initial velocity data were fit with this model with use of a nonlinear least-squares program (Statistical Analysis System, SAS Institute Inc., Cary, NC) that calculates weighted least-squares estimates of the model's parameters. Results are plotted in Eadie–Hofstee coordinates.

Analysis of Fluorescence Titration. Since NADH added significantly to the absorbance of the enzyme solutions during titrations with this cofactor, inner-filter corrections were made as described by Lloyd (1981). Associated with these corrections, an effective path length for the spectrophotometer and cuvette was calculated from the concentration dependence of NADH fluorescence in standard solutions (Lloyd, 1981). That the calculated and actual path lengths were in good agreement suggests that the corrections were accurately made. After being corrected for inner-filter and dilution effects, fluorescence titration data were analyzed using the relationship

$$\text{FE} = \frac{2F_1K_c[C] + F_2K_c{}^2[C]^2K_{CC}}{1 + 2K_c[C] + K_c{}^2[C]^2K_{CC}} \quad (2)$$

where FE is the fluorescence change observed relative to the fluorescence of the unliganded protein plus free ligand. F_1 and F_2 are the maximum fluorescence enhancements (or quenching) associated with the singly and doubly ligated enzyme, K_c is the intrinsic association constant for the ligand, [C] is the concentration of the free ligand, and K_{CC} is the fold increase/decrease in the cofactor binding constant when one of the enzyme's cofactor binding sites is occupied. F_1 was routinely determined using the relationship

$$F_1 = [E]_T(\theta' - \theta) \quad (3)$$

where $[E]_T$ is the total concentration of enzyme sites, θ is the molar emissivity of free NADH determined from the concentration dependence of ligand fluorescence in the absence of sMDH, and θ' is the molar emissivity of the E:C complex determined from the initial slope of fluorescence versus total ligand ($[C]_T$) plots derived from sMDH titrations conducted under stoichiometric ligand-binding conditions ($[E]_T > 1/K_c \gg [C]_T$, data not shown). These plots also suggested that the emissivity per site was the same for E:C and E:C_2 complexes. This is also consistent with the observation that the maximum

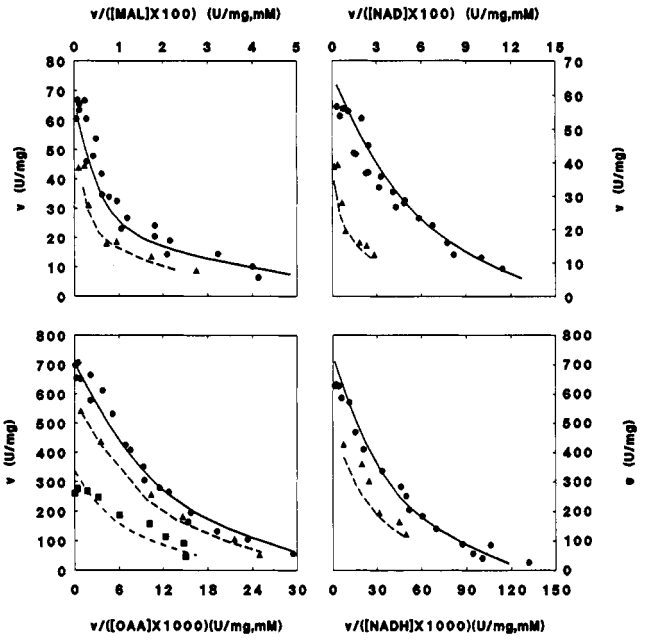

FIGURE 1: Representative Eadie–Hofstee plots of malate oxidation (upper panels) and oxaloacetate reduction (lower panels) by sMDH in 50 mM HEPES buffer, pH 8.0, 25 °C. In the right panels, NAD (upper) or NADH (lower) is varied at fixed concentrations of substrate; 0.5 and 10 mM malate (▲ and ●, respectively) in the upper panel, or 0.08 and 2.0 mM OAA (▲ and ●, respectively) in the lower panel. In the left panel, MAL (upper) or OAA (lower) is varied at fixed concentrations of cofactor; 0.39 and 2.0 mM NAD (▲ and ●, respectively) in the upper panel, or 0.01, 0.05, and 0.25 mM NADH (■, ▲, and ● respectively) in the lower panel. Symbols represent experimental data, while lines are calculated using the model presented in the Experimental Procedures section together with constants from Table I.

fluorescence change associated with ligand binding was equal to the change predicted by eq 3. F_2 for dimeric sMDH, therefore, is $2 \times F_1$. Free ligand concentrations used in eq 2 were determined using the relationship

$$[C] = [C]_T - \frac{F - [C]_T\theta}{\theta' - \theta'} \quad (4)$$

where F is the observed fluorescence at the total ligand concentration $[C]_T$ (Winer et al., 1959; Fromm, 1963).

For titrations with malate, free ligand concentrations were determined using

$$[\text{malate}] = [\text{malate}]_T - \frac{F}{F_{max}}[E]_T$$

where enzyme concentration is in terms of active sites, F_{max} is the maximum fluorescence change, and the subscript T denotes "total". F_{max} was estimated from the vertical axis intercept of $1/F$ versus $[\text{malate}]_T$ plots and/or by nonlinear least-squares regression analysis of the titration data.

RESULTS

Steady-State Kinetics. Steady-state kinetic profiles describing the sMDH-catalyzed oxidation of malate and reduction of oxaloacetate were determined. Representative examples, plotted in Eadie–Hofstee coordinates, are presented in Figure 1. Variations of cofactor and substrate concentrations (right and left panels, respectively) for malate oxidation (upper panels) and OAA reduction (lower panels) are shown. These profiles have pronounced curvature, indicating that the enzyme does not follow simple Michaelis–Menten kinetics. Kinetic data, the symbols in Figure 1, were well described by the cooperative kinetic model (eq 1) presented in the Experimental Procedures section (lines of Figure 1). Constants obtained by iteratively fitting experimental points with this model were used to calculate the lines in Figure 1 and are summarized in Table I.

Table I: Kinetic Constants for sMDH[a]

reaction	constant	value[b]	S.E.[c]
OAA reduction	K_{NADH}[d]	2.8	0.4
	K_{OAA} interaction	9.3	2.6
	α[e]	1.04	0.05
	Cofac[f]	0.3	0.08
	Subs[g]	0.2	0.04
	ACT[h]	2.0	0.07
	V_{max}	188.0	11.0
MAL oxidation	K_{NAD}	420.0	83.0
	K_{MAL} interaction	1000.0	210.0
	α	32.0	3.6
	Cofac	0.4	0.2
	Subs	0.1	0.06
	ACT	2.0	0.04
	V_{max}	17.3	0.8

[a] 50 mM HEPES, pH 8.0, 25 °C. Constants derived from fitting of experimental data as described in Experimental Procedures. [b] Dissociation constants are micromolar, while interaction and ACT parameters are dimensionless. V_{max} has the dimensions of μmol of cofactor/mg sMDH per min. [c] Standard error estimates of parameters generated by nonlinear least-squares program (Statistical Analysis Service, SAS Institute Inc., Cary, NC). [d] Dissociation constant for binding of the first molecule of the ligand indicated by the subscript. [e] Quotient formed by dividing the dissociation constant for binding in the absence of the companion ligand by the dissociation constant for binding when the companion ligand is bound. [f] Quotient formed by dividing the dissociation constant for the first cofactor molecule bound by the dissociation constant for the second. [g] Quotient formed by dividing the dissociation constant for the first substrate molecule bound by the dissociation constant for the second. [h] Ratio of V_{max} per subunit when both sites are occupied to V_{max} when only one site is occupied.

A rapid equilibrium rather than a steady-state kinetic mechanism was used in the analysis owing to its relative simplicity. The form of the equations in both cases is the same, though kinetic constants are apparent constants when steady-state conditions apply (Segel, 1975). Importantly, as long as cooperative interactions are faster than the reaction rate, cooperativity is well represented by the interaction parameters in Table I.

Detailed kinetic studies were also performed on sMDH preparations that were partially inhibited by iodoacetate, a reagent which binds covalently to the enzyme's active site (Zimmerle et al., 1987). Representative results are shown in Figure 2. Eadie–Hofstee plots of partially inactivated sMDH became progressively less curved as the degree of inhibition increased. Further, the same behavior was found whether reduction of oxaloacetate (left panel) or oxidation of malate (right panel) was examined. In all cases, the slope of kinetic profiles became similar to the portion of the Eadie–Hofstee plots at low partial saturation. Lines are calculated by using the constants in Table I and the model described in the Experimental Procedures section and by assuming that both active sites per sMDH dimer are equally reactive toward IAA.

NADH and Malate Binding. We have confirmed previous reports that NADH fluorescence is enhanced and the protein's fluorescence is quenched when this ligand binds to sMDH (Holbrook & Wolfe, 1972; Lodola et al., 1978b). Additional fluorescence changes occur when malate is added to the enzyme–cofactor complex. To characterize NADH binding, fluorescence intensities at various ligand concentrations were monitored and analyzed as described in the Experimental Procedures section.

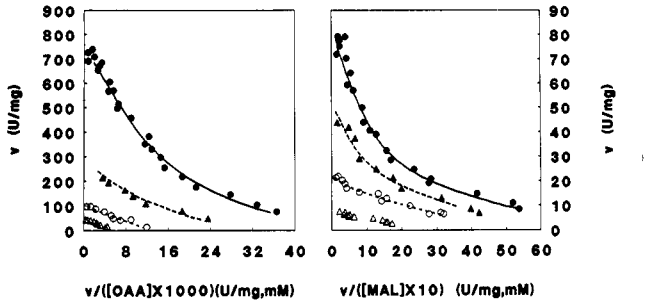

FIGURE 2: Representative Eadie–Hofstee plots of OAA reduction, left panel, and MAL oxidation, right panel, catalyzed by native (●) and, in the left panel, 52% (▲), 85% (○), and 92% (△) inactivated or, in the right panel, 34% (▲), 74% (○), and 92% (△) inactivated sMDH. In these profiles, cofactor concentrations were held constant at 0.25 mM NADH (left panel) or 2 mM NAD (right panel), while substrate concentrations were varied. Cytoplasmic MDH inactivations were carried out as described in the Experimental Procedures section. Assays were performed in 50 mM HEPES buffer, pH 8.0, 25 °C.

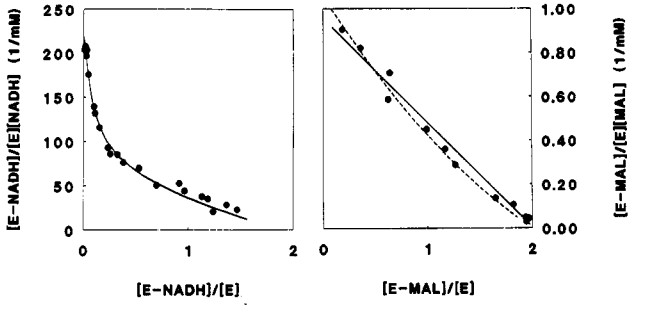

FIGURE 3: Representative Scatchard plots for NADH binding to sMDH (left panel) and malate binding to the sMDH–NADH complex (right panel). The line in the left-hand panel represents the best fit of experimental points (●) assuming that sMDH is negatively cooperative, while in the right panel, the best fits of experimental observations (●) to cooperative and noncooperative models are represented by the dashed and solid lines, respectively. The extent of binding was monitored by NADH fluorescence enhancement. Cytoplasmic MDH active-site concentrations were 0.46 μM in the NADH titration and 7.3 μM in the MAL titration. Binding assays were performed in 50 mM HEPES buffer, pH 8.0, 25 °C.

Representative examples of NADH and malate binding data are shown in Figure 3. Scatchard plots of NADH binding were curved regardless of whether quenching of sMDH fluorescence (left panel) or enhancement of NADH fluorescence (data not shown) was measured. This is consistent with NADH binding to sMDH in a negatively cooperative manner. NADH binding data were analyzed by using the cooperative binding model (eq 2) and methods described in the Experimental Procedures section. Constants derived from this analysis are summarized in Table II and were used to calculate the line in the left-hand panel of Figure 3. Scatchard plots for malate binding to the NADH:sMDH complex were equally well described by a cooperative model (eq 2) in which the value of the dissociation constant for the second malate molecule bound per sMDH dimer is 1.55-fold times the value of the first dissociation constant (dashed line) or by a model assuming that the malate sites are identical and noninteracting (solid line). Constants derived from the alternative analyses are summarized in Table II and were used to calculated the respective lines in the right-hand panel of Figure 3.

DISCUSSION

Here we report steady-state kinetic analyses and equilibrium binding studies which indicate that sMDH is a negatively cooperative enzyme.

Table II: Dissociation Constants for NADH and Malate Binding to sMDH[a]

ligand	K_D[b] high affinity	K_D[b] low affinity	$K_{D\,second}/K_{D\,first}$
NADH[c]	2.7 ± 0.3[d]	18.1 ± 1.1	6.7
NADH[e]	2.8 ± 0.6	17.0 ± 0.4	6.1
NADH (+ Malate)[c,f]	1.1 ± 0.1	15.7 ± 0.4	14.3
MAL (+ NADH)[c,g]	1400 ± 100	2200 ● 200	1.6
MAL (+ NADH)[c,g]	2100[h] ± 240		

[a] 50 mM HEPES, pH 8.0, 25 °C. [b] Dissociation constants are micromolar. These are deduced assuming that ligand binding is negatively cooperative (Experimental Procedures). [c] Measured from enhancement of NADH fluorescence monitored at 415 nm while the sample was excited at 340 nm. [d] Standard error estimates of parameters generated by nonlinear least-squares program (Statistical Analysis Service, SAS Institute Inc., Cary, NC). [e] Measured from quenching of sMDH fluorescence monitored at 360 nm while the sample was excited at 297 nm. [f] Titrations performed in the presence of 10 mM malate. [g] Titrations performed in the presence of 0.2 mM NADH. [h] Dissociation constant deduced assuming that MAL binds to identical and noninteracting active sites.

sMDH Kinetics. The curvature of its kinetic profiles (Figure 1) signifies that sMDH is not a simple Michaelis–Menten enzyme. Though curvature is consistent with previous reports of substrate activation in sMDH (Frieden & Fernandez-Soussa, 1975), it is not diagnostic of the molecular processes causing it (Segel, 1975). Possible molecular origins for nonlinearity might include (a) the presence of dissimilar active sites on the same or different proteins, (b) product or substrate inhibition, (c) multiple overlapping and productive substrate/cofactor binding active sites on each subunit, or (d) cooperative subunit interactions.

Several observations argue that nonlinearity does not result from possibility a. The presence of a single band in nondenaturing as well as SDS–PAGE analysis implies that our sMDH preparation contains identical or very similar polypeptide chains (Zimmerle, 1985). Further, as judged by specific activity, all catalytic centers of our preparation seem to be at least as active as those in any other reported preparation. Our enzyme's specific activity is equal to or greater than values characteristic of other preparations, regardless of whether they were used in studies reporting no cooperativity (Lodola et al., 1978a,b; Johnson & Rupley, 1979), in studies reporting cooperativity (Gerding & Wolfe, 1969; Crow et al., 1982), or in X-ray crystallographic analysis (Glattharr et al., 1974). Finally, previous studies from this laboratory have shown that under standard assay conditions, all active sites of the sMDH preparation are identical by the criterion of reactivity toward the active-site-specific reagent, iodoacetic acid. This reagent specifically modifies Met 98 of the enzyme's active site in a reaction whose rate is sensitive to changes in the active-site environment (Zimmerle & Alter, 1983; Zimmerle et al., 1987).

Kinetic studies of sMDH partially inhibited by IAA help to distinguish among possibilities b, c, and d (Figure 2). Parallel linearization of kinetic profiles and inhibition of the enzyme implies that product inhibition (in possibility b) cannot account for nonlinearity. If this were the case, then all reacted enzyme molecules would be completely inhibited and the remaining native enzyme would still have product inhibition, so Eadie–Hofstee plots would remain curved. The observation that activity is *completely* lost concomitant with linearization of the kinetic profiles argues against IAA modification eliminating alternative productive bind modes (possibility c), since in these cases we would expect substantial residual enzymatic activity.

The final suggestion (possibility d), that the curved kinetic profiles result from cooperative subunit interactions, is quite consistent with the kinetic behavior of partially inhibited sMDH. Inactivating the active site on either subunit of a negatively cooperative enzyme would eliminate cooperativity, making Eadie–Hofstee plots linear. Further, since IAA is not structurally similar to sMDH substrates or cofactors, its reaction should not mimic substrate or cofactor binding. Therefore, the "higher affinity" phase of the kinetic profile should remain since negatively cooperative interactions cannot be expressed in the inactivated companion subunit. Both of these expectations are fulfilled by results shown in Figure 2, where a shallower slope indicates "higher affinity". This fully supports the assertion that sMDH is negatively cooperative.

Since the plots in Figure 1 are concave rather than convex, regardless of whether the reaction is run in the direction of OAA reduction or MAL oxidation, negative rather than positive cooperativity is indicated (Segel, 1975). Steady-state kinetic profiles, therefore, were analyzed in terms of a negatively cooperative mechanism. Kinetic profiles of native (Figure 1) and partially inhibited (Figure 2) sMDH were well described by this mechanism. Constants deduced from this analysis indicate significant subunit interactions (Subs and Cofac in Table I). In fact, owing to cooperative interactions, there is an apparent 20-fold decrease in the tendency to form a ternary complex on a sMDH subunit once the companion subunit is fully ligated (Subs × Cofac). There is also an apparent 4-fold increase in the enzyme's V_M (2 fold increase per subunit (Table I, ACT)).

The results of our kinetic analyses are in qualitative agreement with several previous kinetic studies. At comparable ligand concentrations, the predominant binding order is cofactor then substrate, consistent with the ordered mechanism proposed by Silverstein and Sulebele (1969). We have found that binding affinities and V_M values in the direction of OAA reduction are greater than those in the direction of malate oxidation, consistent with the K_M and V_M values reported by Frieden and Fernandez-Soussa (1975) and Meuggler & Wolfe (1978).

Cofactor and Substrate Binding. Curved Scatchard plots of NADH binding to sMDH (Figure 3) also strongly suggest that the enzyme binds this cofactor in a negatively cooperative manner. Although this is consistent with our activity measurements (see above Discussion), alternative interpretations involving enzyme purity or complications associated with the fluorescence measurement are possible, if unlikely.

Since we see no indication of heterogeneity, it is unlikely that binding anomalies are owing to contaminating proteins (see Experimental Procedures and preceding Discussion). To eliminate the possibility that complications related to the fluorescence measurements might cause the nonlinearity (Figure 3), precautions were taken. Inner-filter corrections were made, and emissivites were routinely used in binding analyses to minimize errors from choosing titration endpoints (see Experimental Procedures). That the qualitative appearance and quantitative analysis of NADH binding curves is the same regardless of whether NADH fluorescence enhancement or indigenous protein fluorescence quenching is used to monitor NADH binding (Table II) supports our contention that curved Scatchard plots are not the result of technical complications associated with fluorescence measurements. Assuming that sMDH active sites are identical, these complications could account for our results only if the ratio of quenching of the protein's tryptophan fluorescence caused by binding the first as opposed to the second NADH is exactly equal to the ratio of enhancement of NADH's fluorescence when it binds at the first as opposed to the second site. Since the mechanisms of tryptophan quenching and NADH fluorescence enhancement are not identical, this seems a remote possibility.

NADH binding studies, then, are quite consistent with NADH binding to sMDH in a negatively cooperative manner, the first NADH per sMDH having a dissociation constant of 2.5–3.0 μM and the second about 6-fold weaker (Table II). These results are in reasonable agreement with steady-state kinetic results (Table I).

Directly comparable equilibrium and kinetic constants for malate's association were not obtained since MAL binding to the sMDH–NADH complex rather than the productive sMDH–NAD complex is measured by fluorescence titrations. However, from equilibrium measurements of MAL binding to sMDH–NADH, we find rather weak K_D values and very modest cooperative interaction. Though the agreement between the equilibrium and kinetic K_D values for MAL binding (1.4–2 (Table II) and 1 mM (Table I), respectively) is good, the magnitude of the cooperative interaction is much less in equilibrium (1–1.6-fold (Table II)) as opposed to kinetic measurements (10-fold (1/Subs, Table I)). The latter difference probably reflects significant variations between the conformations of a nonproductive and a productive enzyme–cofactor–substrate complex.

Negatively Cooperative sMDH. Results reported here are quite consistent with previously reported chemical modification studies from this laboratory (Zimmerle et al., 1987) and are at least qualitatively similar to results of previous studies from some other laboratories (Meuggler & Wolfe, 1978; Frieden & Fernandez-Sousa, 1975). Previous researchers ruled out formation of abortive enzyme–cofactor and/or substrate complexes or the existence of an activity-linked dimer–subunit equilibrium as possible reasons for sMDH's kinetic complexity (Frieden & Fernandez-Sousa, 1975; Frieden et al. 1978).

Binding studies that take advantage of the NADH's fluorescence or the cofactor's ability to quench endogenous sMDH fluorescence have been reported. However, investigators have reached differing conclusions concerning the cooperativity of sMDH. On the one hand, using fluorescence quenching and ultrafiltration, Meuggler and co-workers (Meuggler et al., 1975) reported results indicating sMDH is negatively cooperative. On the other hand, using stopped flow kinetic methods, Holbrook and colleagues (Lodola et al., 1978a,b) concluded that sMDH is not cooperative. The bulk of these stopped flow measurements were made at lower pH and/or higher ionic strength than used here. Similarly, based on equilibrium binding studies done at higher ionic strength than were used here (300 vs approximately 35 mM), Johnson and Rupley (1979) concluded NADH does not bind to sMDH cooperatively. This may suggest a pH-dependence or ionic strength dependence of cooperative behavior, a possibility currently under investigation in this laboratory. However, it is interesting to note that active-site concentrations comparable to or higher than the higher affinity K_D which we find were used in binding studies by both groups, possibly obscuring the high-affinity sMDH binding sites. Interestingly the K_D values these authors reported are similar to those of our lower affinity K_D values, approximately 7–11 vs 9.3 μM (K_D/Cofac) reported here for NADH. Finally, Johnson and Rupley also reported NAD binding studies concluding that the oxidized cofactor binds non cooperatively to sMDH. Though this is in apparent disagreement with our kinetic results, our studies indicate the

K_D values of high- and low-affinity NAD sites differ only modestly (about 2.5-fold).

In summary, we have examined steady-state kinetic and NADH binding properties of sMDH over a wide range of cofactor and substrate concentrations. Further, we have examined the functional behavior of partially inactivated sMDH. There is excellent qualitative and quantitative agreement between the results of these approaches. They indicate that sMDH displays negative cooperativity in its substrate kinetics and cofactor binding activities.

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