Ligand-Induced Asymmetry between Active Sites of Cytoplasmic Malate Dehydrogenase: A Chemical Modification Study†

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ABSTRACT: The iodoacetate-dependent and iodoacetamide-dependent inhibition of cytoplasmic malate dehydrogenase (s-MDH) has been examined. We have confirmed previous reports that iodoacetate inhibits this dimeric enzyme by modifying a single active site methionine per s-MDH subunit. Time courses for the inactivation of the solution-state enzyme with both reagents indicate each s-MDH subunit is modified with equal rapidity in the absence of substrate or cofactor. However, the subunits react with distinctly different rates in the presence of cofactor or cofactor/substrate combinations, indicating some conformational asymmetry between subunits occurs when these ligands are bound. This is consistent with solution-state s-MDH behaving as a cooperative enzyme. Apo and holo crystalline s-MDH are also inhibited by iodoacetic acid. However, subunits of the crystalline enzyme are inhibited with different rates in the presence or absence of active site ligands. This suggests subunit conformations of the dimeric enzyme are not identical in crystalline s-MDH preparations regardless of ligand binding. Furthermore, by the criterion of inhibition rate constants, subunit conformations of the crystalline enzyme are not rigid but are perturbed by ligand binding. Comparisons of inactivation time courses for solution- and crystalline-state s-MDH suggest crystalline s-MDH exhibits at least some of the subunit asymmetry associated with the solution-state enzyme.

Mechanistic studies of enzyme function are often based on interrelationships drawn between detailed structural information obtained by X-ray diffraction analysis of crystalline enzymes and functional properties determined in enzyme solutions. While in some instances these structure–function correlations appear direct, in others they are obscure. With cytoplasmic malate dehydrogenase (s-MDH)[1] structure–function relationships involving crystal-phase and solution-phase data are clouded by differences between kinetic and ligand binding characteristics anticipated on the basis of crystallographic structures and those actually observed in solutions.

s-MDH is one of two enzymes that comprise the cytoplasmic portion of the malate–aspartate shuttle, a pathway important for the net transfer of NADH into and NAD out of the mitochondrion as well as for the coordination of glycolytic and citric acid cycle fluxes (Safer, 1975). This dimeric enzyme, which catalyzes the interconversion of oxaloacetate (OAA) and malate with NADH/NAD as cofactors, is composed of chemically identical 35 000-dalton subunits, which are thought to function independently (Banaszak & Bradshaw, 1975). X-ray diffraction studies of pig heart s-MDH crystallized in the presence of NAD, however, show that the enzyme's subunits are not conformationally identical (Tsernoglou et al., 1971; Webb et al., 1973; Birktoft et al., 1982; Birktoft & Banaszak, 1983). Differences may arise from adventitious interactions between s-MDH molecules in the crystal lattice (Weininger et al., 1977), or they may reflect functionally significant subunit asymmetry expressed during the enzyme's normal catalytic function.

Under appropriate conditions non-Michaelis–Menton behavior has been repeatedly observed in the substrate kinetics of s-MDH (Banaszak & Bradshaw, 1975). This behavior could indicate a number of processes. Allosteric or cooperative interactions may be operative (Mueggler & Wolfe, 1978), consistent with the subunit asymmetry observed in the crystalline-state enzyme. Alternatively, secondary substrate and/or product interactions that require neither subunit asymmetry nor protein conformational changes may occur and could produce the kinetic anomalies (Segel, 1975; Frieden & Fernandez-Sousa, 1975; Bernstein et al., 1978). Apparently conflicting results from cofactor and substrate binding studies also have been reported. On the basis of NADH fluorescence titrations, pressure dialysis experiments, and circular dichroism studies, Wolfe and co-workers concluded that in the presence of L-malate NADH binds to s-MDH in a negatively cooperative manner and that a protein conformational change accompanies ternary complex formation (Mueggler et al., 1975; Eberhart & Wolfe, 1975; McLoughlin, personal communication). Other reports, however, dispute this view. Utilizing fluorescence properties of NADH to monitor its binding to s-MDH, Lodola and co-workers (Lodola et al., 1978b) found no cooperativity either in the presence or in the absence of malate. Further, on the basis of both fluorescence and calorimetric studies, Johnson and Rupley (1979) concluded s-MDH does not bind cofactor cooperatively.

A novel approach for examining cooperativity is used in the study reported here. If a dimeric enzyme such as s-MDH has identical and noninteracting active sites, then these sites should

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[1] Abbreviations: BDCOH, [4,4′-bis(dimethylamino)diphenyl]-carbinol; CAPS, (cyclohexylamino)propanesulfonic acid; DTP, dimethyl 3,3′-dithiobis(propionimidate); DTT, dithiothreitol; Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IAA, iodoacetic acid; IAM, iodoacetamide; MAL, malate; NEM, *N*-ethylmaleimide; OAA, oxaloacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); s-MDH, cytoplasmic malate dehydrogenase.

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remain identical when substrate and/or cofactor binds. However, if the enzyme is cooperative, cofactor and/or substrate binding may induce conformational differences between active sites. Conformational differences can be probed by studying time courses for the irreversible inactivation of the enzyme by a reagent that reacts in the vicinity of the enzyme's active sites. When subunits are conformationally different, they may inactivate with dissimilar rates. Using a chemical modification approach, we have probed solution-state s-MDH active sites. Further, we have compared these results with results from similar experiments performed on the crystalline-state enzyme.

Materials and Methods

Materials. Dithiothreitol (DTT), iodoacetic acid (IAA), iodoacetamide (IAM), [4,4′-bis(dimethylamino)diphenyl]-carbinol (BDCOH), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), *N*-ethylmaleimide (NEM), CAPS buffer, HEPES buffer, PIPES buffer, NAD (type III), and NADH (type III) were purchased from Sigma Chemical Co. Dimethyl 3,3′-dithiobis(propionimidate) (DTP), phenyl isothiocyanate, amino acid standards, and constant-boiling HCl were obtained from Pierce Chemical Co. Formic acid, hydrogen peroxide, Gold Label triethylamine, HPLC-grade acetonitrile, and sodium tetrathionate were obtained from Aldrich Chemical Co. Remaining chemicals used in these studies were of reagent grade or better.

Amino Acid Analysis. Amino acid analyses of acid-hydrolyzed native and IAA-modified s-MDH were performed with a phenyl isothiocyanate precolumn derivatization method and HPLC chromatographic method of Bidlingmeyer and co-workers (Bidlingmeyer et al., 1984). For quantification of the extent of methionine modification, s-MDH was treated with performic acid as described by Hirs (1967) prior to hydrolysis to free amino acids. This procedure converts unreacted methionine to its corresponding sulfone, a species that is stable during hydrolysis and amino acid analysis (Glazer, 1975).

Enzyme Preparation, Crystallization, and Cross-Linking. Porcine heart s-MDH was prepared, purified, crystallized from ammonium sulfate buffers, and cross-linked with the DTT-cleavable cross-linker DTP as previously described (Zimmerle & Alter, 1983; Banaszak et al., 1971). Both apo crystals, grown in the absence of NAD, and holo crystals, grown in the presence of 2 mM NAD, were prepared. The crystals used here were approximately 20 μm × 10 μm × 0.5 μm. During cross-linking reactions, enzyme activity was protected by addition of 5 mM NAD. Crystals were extensively dialyzed to remove NAD prior to IAA inactivation experiments. As judged by the production of NADH in the presence of malate, both crystal types were devoid of NAD. Light microscopy indicated manipulations during cross-linking, washing, and ligand additions did not alter the crystals' appearance. Further, modification of lysines with DTP or introduction of cross-links had little or no effect on the crystalline enzyme, as judged by the criterion of enzymatic activity (Zimmerle & Alter, 1983).

Several characteristics of our final crystalline preparations appear different than those of preparations grown under very similar conditions by Banaszak's group (Glatthaar et al., 1972). They find one molecule of NAD per s-MDH remains if either apo or holo crystalline s-MDH preparations are soaked in NAD solutions and then washed extensively with NAD-free buffer. We find that after extensive washing our apo and holo crystals have no bound NAD. The reason for these differences is not clear. However, the crystalline preparations used in this study and those used by Glatthaar and co-workers differ widely in size and were treated differently after they are grown. Glatthaar and co-workers soaked crystals for prolonged periods (40 h) with NAD in concentrated ammonium sulfate solutions. Further, they washed NAD-treated/containing crystals with concentrated ammonium sulfate solutions. In contrast, prior to cross-linking the ammonium sulfate buffer in which our crystals were grown was replaced by sodium citrate. The crystals were then washed with sodium citrate–NAD (25 mM) for about 1–2 min prior to reacting with the DTP. The reaction time was short (about 2–5 min), and the crystals were washed extensively with concentrated ammonium sulfate and then with 50 mM HEPES solutions. Routinely the crystals were stored in the HEPES solution.

Inactivation of s-MDH. Crystalline- and solution-state s-MDH preparations were routinely inactivated by reaction with either 250 mM IAA or 250 mM IAM buffered by 50 mM HEPES, pH 8.0 at 37 °C. Protein concentrations were usually 1 mg/mL. When protecting agents were used (100 mM for the substrates OAA and malate and 25 mM for the cofactors NAD and NADH), they were added directly to the reaction mixture. Reactions were routinely initiated by addition of the modifying agent to the reaction mixture; however, the same results were obtained if the reaction was initiated by the addition of s-MDH. Inactivation mixtures involving crystalline s-MDH were constantly stirred to ensure a uniform distribution of enzyme throughout the solution during inactivations. At various times microliter aliquots of the reaction mixture were quenched by adding them to 1 mL of 0.3 M DTT buffered with 50 mM PIPES, pH 6, or 0.3 M histidine. In the DTT quenching mixture, cross-links stabilizing s-MDH crystals were broken while unreacted IAA was quenched. Thus crystalline enzyme preparations dissolved in this step. When histidine was used to quench the reaction in crystalline preparations, low concentrations of DTT (50 mM) were then added to break cross-links. The same results were obtained regardless of the procedure used. Activities of quenched s-MDH preparations were determined in 1-mL steady-state assays containing 0.25 mM NADH, 2 mM OAA, and 50 mM HEPES at pH 8.0 and 25 °C. Changes in NADH concentrations were quantitated spectrophotometrically at 340 nm using an extinction coefficient of 6300 M^{-1} cm^{-1} (McComb et al., 1976). Microliter quantities of quenched enzyme solutions were routinely used in activity assays. In the process of quenching and assaying, s-MDH was diluted 10^3–10^5-fold, making the components of the inactivation mixture very dilute in assay solutions. Neither DTT nor histidine affected s-MDH activity at concentrations as high as 0.3 M.

The inactivation of s-MDH by several putative sulfhydryl reagents was examined. Experimental conditions were 1 mg/mL s-MDH, pH 8.0, 37 °C, and either 7 mM sodium tetrathionate, 7 mM Ellman's reagent, 10 mM NEM, or 0.3 M β-mercaptoethanol. For reactions with BDCOH the reaction conditions were 1 mg/mL s-MDH, 50 mM sodium acetate, pH 5.2, 37 °C, and 1 mM BDCOH. Reactions were quenched with DTT and immediately assayed as just described.

Analysis of Inactivation Time Courses. Kinetic time courses were conveniently plotted as log activity, A, versus time, t, since for simple first-order processes these plots are linear. Though plots of some time courses (obtained in the absence of protecting agents) approached linearity, others were distinctly biphasic (Figure 1). Therefore, a more complex analysis was necessary. We assumed that each subunit of s-MDH was inactivated in a first-order process and that the inactivation rate constants as well as the percentage of activity associated with inactivation of each subunit need not be equal.

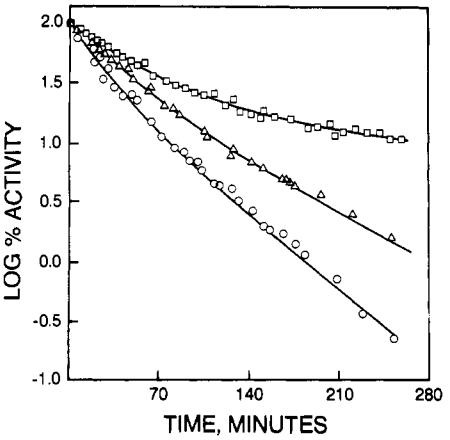

FIGURE 1: Inactivation of solution-state s-MDH in the absence of added ligands (○), in the presence of 25 mM NAD (△), and in the presence of 25 mM NADH (□). IAA reaction conditions as well as enzyme assay procedures are described under Materials and Methods. Lines represent the best fit of experimental points to the sequential model for s-MDH inactivation (Materials and Methods). Kinetic constants derived from time courses such as these are summarized in Table I.

For the case of a dimeric enzyme with identical and cooperative subunits, inactivation of one subunit per dimer will decrease activity to the same level (not necessarily 50%) regardless of the subunit modified. Remaining activity will be lost when the second subunit is modified, making the inactivation appear to proceed as two sequential first-order processes. The mathematical model for this case is given by eq 1 (Ca-

$$A_t/A_0 = 100S_1 + \mathrm{HA}(2k_f)(S_1 - S_2)/(k_s - 2k_f) \quad (1)$$

pellos & Bielshi, 1972), where S_1 and S_2 are given respectively by

$$S_1 = \exp(-2k_f t) \qquad S_2 = \exp(-k_s t) \quad (2)$$

HA is the fraction of maximal activity characteristic of a singly modified dimeric enzyme, and k_f and k_s are first-order rate constants for the inactivation of the first and second subunits of the enzyme, respectively. The sequential model just mentioned (eq 1) reduces to a *single first-order process only when $k_f = k_s$ and 50% of the activity is lost with modification of one of the dimeric enzyme's subunits.*

For the case of a dimeric enzyme with nonidentical and independent subunits, inactivation proceeds as two simultaneous first-order processes, each with a particular fraction of the total activity. The fraction of activity lost, then, depends on the subunit modified. A mathematical model for this case is given by eq 3, where A_t, A_0, t, HA, k_f, k_s, and S_2 have the

$$A_t/A_0 = (100 - \mathrm{HA})S_1 + (\mathrm{HA})S_2 \quad (3)$$

meanings previously described while $S_1 = \exp(-k_f t)$. *When $k_f = k_s$, the inactivation reduces to a single first-order process regardless of the percentage of activity lost with modification of each subunit.*

Data from crystalline and solution s-MDH preparations were fit to both models with a nonlinear least-squares regression routine utilizing the Marquardt algorithm of SAS (Statistical Analysis System). Results are presented as first-order plots.

RESULTS

Site of Iodoacetic Acid Modification. We have verified the reports of Leskovac (Leskovac & Pfleiderer, 1969; Leskovac, 1971) that modification of an active site methionyl residue by IAA inactivates s-MDH. Specifically, we have (1) correlated the loss of activity with modification of methionine, (2) examined the ability of other putative sulfhydryl reagents to inactive s-MDH, and (3) examined the pH dependence of the inactivation.

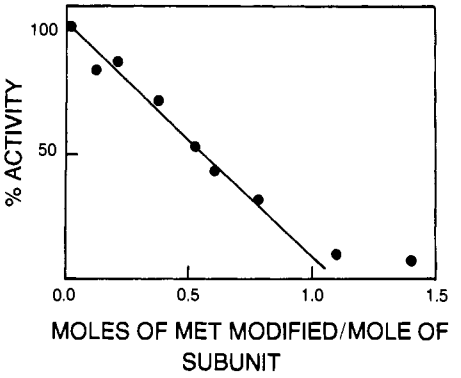

FIGURE 2: Correlation of loss of s-MDH activity with modification of methionine side chains. IAA reaction conditions as well as enzyme assay procedures are described under Materials and Methods. The extent of methionine modification was determined by amino acid analysis, also as described under Materials and Methods.

Though IAA is usually most reactive with cyteine, its modification of methionines, histidines, and lysines has been reported (Lundblad & Noyes, 1984; Glazer, 1975; Means & Feeney, 1971). Amino acid analyses were performed on s-MDH at various extents of IAA inactivation. Activity was completely lost when a single methionine per active site was modified (Figure 2). No cysteine, however, was modified during the time course as judged by either the production of (carboxymethyl)cysteine or loss of cysteines (within an accuracy of 0.3 residue per subunit based on eight determinations). Further, the histidine content did not change during the time course (within an accuracy of 0.3 residue per subunit based on nine determinations), and no lysine residues were modified (within an accuracy of 0.4 residue per subunit based on seven determinations). Similar results were obtained when the modification reaction was performed in the presence of 25 mM NADH. Under these conditions nonlinear inactivation profiles were found (Figure 1). Once again loss of activity correlated with modification of a single methionine (1.1 ± 0.2 residues per subunit). No (carboxymethyl)cysteine was produced, histidine residues were not lost (within an accuracy of 0.4 residue per subunit for six determinations), and no change in lysine content was observed (within an accuracy of 0.7 residue per subunit for ten determinations). In all, the good correlation between loss of activity and modification of a single methionine per s-MDH subunit argues that all activity is lost with the modification of that methionine.

To confirm that cysteine modification does not inhibit s-MDH, the effect of several sulfhydryl reagents on the enzyme's activity was examined. BDCOH was used since it has been shown to be a sensitive reagent for the quantitative determination of sulfhydryl residues in proteins (Rohrbach et al., 1973; Humphries et al., 1973) and rapidly inhibits mitochondrial MDH by reacting with a single sulfhydryl residue (Humphries & Harrison, 1974). Sodium tetrathionate and Ellman's reagent were chosen since they also can be used to quantitate sulfhydryls (Inglis & Liu, 1970; Anderson & Wetlaufer, 1975) but do not react with methionine, histidine, or lysine (Means & Feeney, 1971; Glazer et al., 1975). NEM, a widely used alkylating reagent that is reactive with sulfhydryl groups and to a smaller extent with lysine side chains, was also used. Finally, β-mercaptoethanol was used to determine whether disulfide bond formation was an important determinant. Under conditions in which these reagents normally extensively modify sulfhydryls (37 °C, 1 h, buffer and reagent concen-

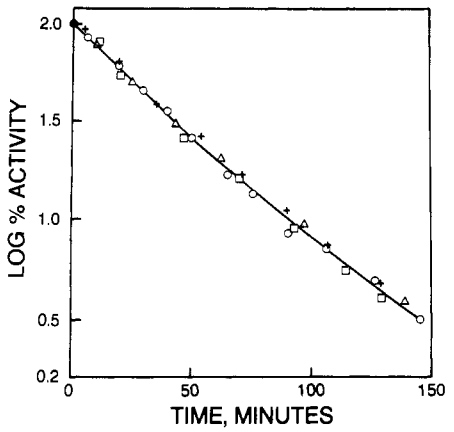

FIGURE 3: Inactivation of solution-state s-MDH at pH 5,4 (50 mM citrate buffer) (○), pH 6.6 (50 mM HEPES buffer) (△), pH 8.0 (50 mM HEPES buffer) (□), and pH (9.3) (50 mM CAPS buffer) (+). Remaining IAA reaction conditions as well as enzyme assay procedures are described under Materials and Methods. The line is the best fit of experimental points to a sequential model for inactivation (Materials and Methods). Inactivation rate constants describing the line are 20.6 $\times 10^{-3}$ min^{-1} and 20.1 $\times 10^{-3}$ min^{-1} for k_f and k_s, respectively.

trations noted under Materials and Methods), 99 ± 2%, 100 ± 4%, 95 ± 7%, 94 ± 7%, and 99 ± 4% of s-MDH activity remains after treatment with BDCOH, sodium tetrathionate, Ellman's reagent, NEM, and β-mercaptoethanol, respectively. In contrast 24% of s-MDH activity remains after 1-h reaction with IAA under routine inactivation conditions (Figure 1; Materials and Methods). This indicates modification of any cysteine side chains by IAA does not affect s-MDH activity.

pH dependencies of s-MDH inactivation also suggest that Met but not Cys, His, or Lys modifications cause loss of activity. Since Cys, His, and Lys side chains should change ionization states in the pH range from 5.5 to 9.5, their reactivity with IAA should also change in this pH range. However, inactivation time courses remained constant over the pH range 5.4–9.3 (Figure 3), making it unlikely that these residues are involved in the inactivation. In contrast, Met's side chain does not ionize in this pH range. Therefore, the insensitivity of the inhibition time courses to pH (Figure 3) is consistent with methionine modification causing s-MDH inactivation.

IAA Inactivation of Solution-State, Apo Crystalline, and Holo Crystalline s-MDH. Representative time courses for the IAA inactivation of solution-state, apo crystalline, and holo crystalline s-MDH are shown in Figures 1 and 4. The effect of added substrate, cofactor, and an abortive substrate–cofactor pair on these time courses was examined. Though first-order plots for the inactivation of solution-state s-MDH approached linearity in the absence of cofactors and substrates, they were distinctly biphasic in their presence (Figure 1). In contrast, first-order plots for the inactivation of crystalline apo- and holo-s-MDH were distinctly biphasic in the absence as well as the presence of ligands (Figure 4). Like the solution-state enzyme, however, inactivation profiles of both crystal types were perturbed by added cofactor and/or substrate ligands. Time courses were analyzed by assuming subunits were inactivated in pseudo-first-order processes (Materials and Methods). This is consistent with our observation that inactivation half-times were independent of enzyme concentration over a concentration range of 5 nM to 1 μM (250 mM IAA, no protecting agents added) (results not shown). Both sequential and simultaneous mechanisms were considered, and results were generally well described by both mechanisms. On the basis of considerations described under Discussion, however, the sequential model was used to analyze results. So-

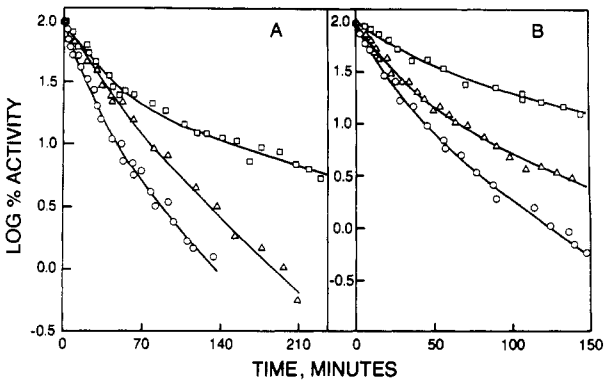

FIGURE 4: IAA inactivation of apo (panel A) and holo (panel B) crystalline s-MDH in the absence of added ligands (○), in the presence of 25 mM NAD (△), and in the presence of 25 mM NADH (□). IAA reaction conditions as well as enzyme assay procedures are described under Materials and Methods. Lines represent the best fit of experimental points to the sequential model for s-MDH inactivation (Materials and Methods). Kinetic constants derived from time courses such as these are summarized in Table I.

Table I: Rate Constants for Inactivation of s-MDH with Iodoacetic Acid[a]

enzyme preparation	ligand[b]	k_f ($\times 10^3$ min^{-1})[c]	k_s ($\times 10^3$ min^{-1})[c]	k_f/k_s[d]
solution	none	21.3 ± 4.9	20.4 ± 2.2	1.04
	MAL	22.7 ± 6.7	22.7 ± 2.4	1.00
	OAA	27.1 ± 8.7	18.4 ± 3.3	1.47
	NAD	19.1 ± 0.5	12.2 ± 0.1	1.57*
	NADH	20.2 ± 0.6	3.6 ± 0.1	5.61*
	NADH/MAL	25.4 ± 1.1	2.8 ± 0.1	9.07*
apocrystals	none	73.6 ± 7.3	23.7 ± 0.6	3.11*
	MAL	56.1 ± 9.2	23.2 ± 0.6	2.42*
	OAA	33.5 ± 3.4	17.1 ± 0.8	1.96*
	NAD	31.8 ± 3.9	17.8 ± 0.7	1.78*
	NADH	30.1 ± 1.9	5.9 ± 0.2	5.10*
	NADHs/MAL	24.5 ± 2.7	3.0 ± 0.4	8.16*
holocrystals	none	72.8 ± 6.6	25.8 ± 0.4	2.82*
	MAL	62.7 ± 12.5	20.2 ± 1.5	3.10*
	OAA	64.1 ± 4.6	18.4 ± 0.1	3.48*
	NAD	56.8 ± 7.9	16.0 ± 0.9	3.55*
	NADH	27.0 ± 3.0	4.8 ± 1.0	5.63*
	NADH/MAL	21.2 ± 1.8	2.4 ± 0.3	8.69*

[a] Experimental conditions were 1 mg/mL s-MDH, 250 mM iodoacetate, 50 mM HEPES, pH 8.0, and 37 °C. [b] Concentrations of added ligands were 100 mM for substrates (OAA or MAL) and 25 mM for cofactors. [c] Rate constants were obtained by fitting experimental data to the sequential inactivation model as described under Materials and Methods. Error is expressed as the standard error of the estimate. [d] An asterisk (*) indicates value significantly different from 1 at $P > 0.95$.

lution- and crystalline-state inactivations were well described by this mechanism. In Figures 1 and 4, for example, the points are experimental data, and the smooth lines are calculated with eq 1 (for sequential first-order processes) and parameter values obtained by the analysis described under Materials and Methods. In analyses the percentage of activity lost in the "first" and "second" phases of the inactivation and rate constants for each phase were adjustable parameters. Results from time courses measured in the presence of NADH or NADH plus malate clearly indicated approximately the same proportion of activity (75.7 ± 2.4% for solutions, 77.8 ± 5.6% for apo crystals, and 73.6 ± 3.7% for holo crystals) was lost in the first phase of the inactivation regardless of whether crystalline or solution preparation were used. The remaining activity was lost in the second phase. Therefore, the activity lost in the first phase was set at 75% in all analyses. This caused no degradation in the fit of the data and led to a more precise determination of values for rate constants. Pseudo-

Table II: Inactivation of Solution-State s-MDH with Iodoacetamide[a]

ligand[b]	k_f ($\times 10^3$ min^{-1})[c]	k_s ($\times 10^3$ min^{-1})[c]	k_f/k_s
none	30.3 ± 6.1	30.3 ± 4.2	1.00
MAL	22.3 ± 5.9	22.3 ± 2.4	1.00
NADH	20.1 ± 4.1	20.2 ± 3.2	1.00
NADH/MAL	28.1 ± 1.4	6.6 ± 0.3	4.26[d]

[a] Experimental conditions were 1 mg/mL s-MDH, 250 mM iodoacetamide, 50 mM HEPES, pH 8.0, and 37 °C. [b] Concentrations of added ligands were 100 mM for malate and 25 mM for NADH. [c] Rate constants were obtained by an iterative fitting of experimental data to the sequential inactivation model described under Materials and Methods. Error is expressed as standard error of the estimate. [d] Significantly different from 1 at $P > 0.95$.

first-order rate constants derived from analyses of time courses are summarized in Table I. The ratios of k_f (the rate constant of the first component of the inactivation) to k_s (the rate constant for the second component of the inactivation) are also shown in Table I. Starred entries are statistically different from unity at the 95% confidence level.

Substrate and cofactor concentrations used in the preceding inactivation protection studies (100 and 25 mM, respectively) were higher than the probable dissociation constants or K_m's for these molecules. Solution-state s-MDH K_D's or K_m's for NADH and NAD have been determined in several laboratories and are 10 μM or less and 5 mM or less, respectively (Johnson & Rupley, 1979; Lodola et al., 1978a; Mueggler & Wolfe, 1978; Frieden & Fernandez-Sousa, 1975). NADH's and malate's dissociation constants describing the formation of the nonproductive NADH plus malate complex are about 15 μM and 1.4–2.5 mM, respectively (Zimmerle, 1985). Substrate binding in the absence of cofactor is characterized by 2.0 mM and approximately 5 mM dissociation constants for OAA and malate, respectively (Tung and Alter, unpublished results; Lodola et al., 1978a). OAA and malate K_m's measured in solution-state kinetic studies are about 50 μM and 40–8000 μM, respectively. These were derived by assuming an ordered bi-bi mechanism with cofactor binding before substrate (Mueggler & Wolfe, 1978; Frieden & Fernandez-Sousa, 1975). Smaller parameter values are obtained if random cofactor and substrate binding is assumed (Zimmerle and Alter, unpublished results). Finally, NADH, NAD, OAA, and malate K_m's are less than 1, 10, 5, and 10 mM, respectively, for crystalline-phase s-MDH preparations (Zimmerle and Alter, unpublished results). Therefore, concentrations of substrates (100 mM) and cofactors (25 mM) used in protection experiments were at least 2.5-fold (and frequently more than 100-fold) higher than K_D's and/or K_m's of the respective ligands. Under these conditions active sites should be substantially or completely occupied.

Iodoacetamide Inactivation of Solution-State s-MDH. Time courses for the inactivation of solution-state s-MDH with the reagent iodoacetamide (IAM) were also determined. First-order plots of the inactivations were nearly linear in the absence of protecting agents as well as in the presence of NADH or malate. Adding NADH plus malate made first-order plots distinctly biphasic. Inactivations were analyzed with the "sequential" model as already described for IAA inactivations. Results again indicate 75% of the activity was lost in the first process and 25% in the second. Rate constants for IAM inactivation of s-MDH are summarized in Table II.

Discussion

IAA Reaction Site. Studies reported here indicate IAA modification of a single active site linked methionine per subunit of s-MDH is responsible for the enzyme's inhibition. This conclusion is based on several observations. First, there is a good correlation between modification of a single Met per subunit and loss of s-MDH activity (Figure 2) while other potentially reactive side chains are substantially unperturbed (Results). Second, though IAA normally reacts most rapidly with cysteine, s-MDH is resistant to inhibition by a variety of sulfhydryl reagents (Results) (Humphries et al., 1973). Finally, inactivation of s-MDH is insensitive to pH (Figure 3), which implies modification of amino acids with ionizable side chains (including Lys, His, and Cys) by IAA does not inactivate s-MDH (Glazer, 1975; Means & Feeney, 1971). Our conclusion is in agreement with Leskovac's reports that IAA inactivates s-MDH by reacting at an essential methionine residue (Leskovac & Pfleiderer, 1969; Leskovac, 1971). It is also consistent with reports that platinum compounds, which frequently bind to methionyl side chains, inhibit s-MDH and that the active site ligands NAD and NADH protect against Pt inhibition (Wade et al., 1973; Teggins & Friedman, 1974; Friedman & Teggins, 1974a,b).

The precise location of the reactive methionine is suggested by a combination of chemical modification, primary sequence, and X-ray diffraction information. Leskovac (1971) isolated a peptide from IAA-modified s-MDH. This contained the majority of covalently attached IAA and had an amino acid composition identical with that of a peptide fragment of s-MDH, which, on the basis of X-ray crystallography, is located in the active site region of s-MDH (Birktoft et al., 1987; Fang et al., 1984; Birktoft et al., 1982a). This fragment, which contains Met-89, is part of a peptide loop near the active site of s-MDH and is involved in cofactor binding (Birktoft & Banaszak, 1984). The location of the reactive Met is also consistent with X-ray crystallographic studies of Pt-derivatized s-MDH, which found Pt in the region of the Met-89-containing loop (Wade et al., 1973).

IAA Inactivation of Solution-State s-MDH. Two schemes for describing the inactivation profiles of solution-state s-MDH have been presented under Materials and Methods. The "sequential" scheme characteristic of a cooperative enzyme, however, is the one most consistent with all information. A body of evidence indicates s-MDH is composed of two chemically identical subunits (Birktoft et al., 1987; Banaszak & Bradshaw, 1975). Yet, inactivation time courses, which are nearly first order in the absence of active site ligands, become quite biphasic in the presence of NADH or NADH plus malate (Figure 1). This implies IAA reactivities of side chains effecting the enzyme's activity are very similar in the absence of these ligands but become quite dissimilar in their presence. Since inactivation studies (Table I) and analyses of modified s-MDH (Figure 2) indicate complete loss of activity correlates nicely with the modification of a single methionine per s-MDH subunit, we conclude that similarly reactive active site methionines in each subunit become differentially reactive in the presence of active site ligands. These results indicate conformational characteristics of s-MDH subunits become dissimilar in the presence of some active site ligands. Inasmuch as ligand-induced asymmetry between subunit conformations is a central feature of cooperativity as described by Koshland's sequential model (Koshland et al., 1966), we conclude s-MDH displays cooperative characteristics. It follows that the sequential model, which describes inactivation of a cooperative enzyme, is the most appropriate model.

One might anticipate linear first-order plots should result when inactivation rates at each subunit are equal. However, if the enzyme is inactivated in a sequential process, linear plots occur only when the activity lost by inactivation of the first and second subunit per dimeric enzyme is equal or, alterna-

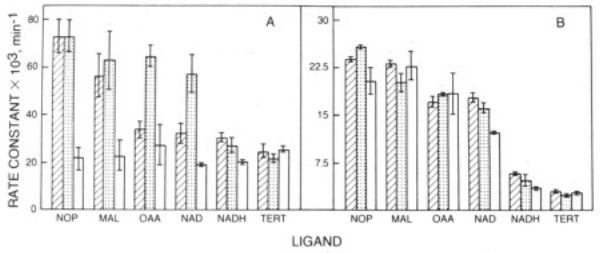

FIGURE 5: Rate constants for the fast (panel A) and slow (panel B) phases of the apo crystalline (crosshatched bars), holo crystalline (stippled bars), and solution-state (open bars) s-MDH inactivation time courses. NOP, MAL, OAA, NAD, and NADH correspond to no protecting agent, malate, oxaloacetate, NAD, and NADH, respectively, being present in the inactivation reaction mixtures. TERT corresponds to NADH and malate being present in the inactivation reaction mixtures. Error bars are standard errors of estimates generated in the analysis of time courses. Values plotted in this figure are taken from Table I, and concentrations of added protecting ligands are given in the footnotes of that table.

tively, if all activity is lost with modification of the first subunit (Materials and Methods). Our results indicate 75% rather than 50% or 100% of the enzyme's activity is lost with modification of the first subunit (Results). Thus, the slight curvature of inactivation time courses performed in the absence of ligands is consistent with active sites being identical and the inactivation following a sequential scheme (Figures 1 and 3). In fact, rate constants describing the inhibition at the first and second subunits of unliganded solution-state s-MDH are equal (Table I and Figure 3 legend).

Results from inactivation experiments performed with IAM, a reagent that inactivates s-MDH by modifying a single active site histidine per subunit (rather than a methionine) (Aspray et al., 1978), also indicate ligand binding induces asymmetry beween s-MDH subunits. It is likely that this histidine is in or near the substrate binding site (Birktoft et al., 1987; Birktoft & Banaszak, 1983). Though both subunits have the same conformation in the absence of ligands or in the presence of either NADH or malate, as judged by inactivation rates, they become distinctly different in the presence of NADH plus malate (Table II). NADH's inability to perturb modification of a histidine in the substrate binding site is not surprising in view of s-MDH's separate substrate and cofactor binding domains. Weak and/or perhaps improper binding accounts for the small effect malate by itself has on inactivation time courses.

Regardless of whether IAA or IAM is used to probe solution-state s-MDH active site conformations, the results suggest the same conclusion: subunit conformations are identical in the absence of ligands but become different in their presence. Though the magnitude of conformational differences between subunits cannot be deduced from results reported here, ratios of rate constants for the "fast" and "slow" processes (Table I) indicate NADH and NADH plus malate are quite proficient at inducing subunit asymmetry. These observations are in agreement with Wolfe and co-workers' contention that NADH binds cooperatively to solution-state s-MDH in the presence of malate (Meuggler et al., 1975).

IAA Inactivation of Crystalline s-MDH Preparations. IAA inactivation time courses indicate crystalline s-MDH has conformationally nonequivalent subunits in the absence as well as in the presence of active site ligands (Table I, last column). This is in good agreement with extensive X-ray diffraction studies of holo crystals, which have shown s-MDH subunits do not have identical conformations nor do they bind NAD equally (Glatthaar et al. 1972; Wade et al., 1973; Weininger et al., 1977; Birktoft et al., 1982). Interestingly, the apo crystals appear equally asymmetric by the criterion of IAA reactivity. At present, no molecular models from X-ray diffraction analysis of apo crystals have been reported.

Differences between inactivation time courses for apo and holo crystalline preparations are apparent (Table I and Figure 5: within each panel compare stippled with crosshatched bars for each ligand addition). They are almost entirely owing to dissimilarities between fast-phase rate constants for each crystal type (Figure 5A). The magnitudes of the differences vary depending on the active site ligand(s) added during the inactivation. The variations may reflect differing conformational responses to ligand binding, or they may reflect differences in the details of ligand interactions with rigid apo or holo crystalline enzyme structures. The former possibility seems most likely inasmuch as X-ray diffraction studies show that when incubated with NADH the crystal lattice arrangement of apo crystals changes to an arrangement characteristic of holo crystals (J. Birktoft, personal communication). The active site correlating with the slow-phase rate constants (Figure 5B) is similar in both crystal types regardless of whether ligands are added.

Comparison of IAA Inactivation Time Courses for Solution and Crystalline s-MDH. Despite differences in the time courses for inactivation of solution and crystalline s-MDH preparations (Figures 1 and 3), there are important similarities in these inactivations. By the criterion of IAA inactivation rate constants, the active site which is modified in the slow phase of inactivation time courses has similar conformations in apo crystalline, holo crystalline, and solution preparations (Figure 5B: compare open with stippled and crosshatched bars for each active site ligand addition). The active site which is modified in the fast phase of IAA inactivation time courses is conformationally different in solution and crystalline preparations either in the absence or in the presence of several active site ligands. However, this site becomes similar in all preparations when NADH or NADH plus malate is bound. In the presence of the latter ligands, s-MDH's conformation is not only similar in crystalline and solution preparations, but the enzyme's structure in all preparations seems to be characterized by conformational differences between each of its active sites.

In conclusion, our studies of the reactivities of s-MDH site residues seem most consistent with the view that s-MDH is a cooperative dimeric enzyme in aqueous solutions. Our results also indicate both apo and holo crystalline s-MDH preparations contain s-MDH dimers with nonidentical subunits. Further, in the presence of NADH or NADH plus malate, the conformations of s-MDH in all crystalline and solution preparations are very similar. It may be that some or many aspects of the conformational asymmetry found in X-ray diffraction analysis of holo-s-MDH crystals (Birktoft et al., 1987; Birktoft & Banaszak, 1983; Weininger et al., 1977) reflect chracteristics of structures that develop in the normal functioning of this enzyme.

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