Crystallization-Induced Modification of Cytoplasmic Malate Dehydrogenase Structure and Function†

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ABSTRACT: In an effort to assess the effects of phase-state changes on protein conformations, we have compared several properties of cytoplasmic malate dehydrogenase in the crystalline and solution states. Two crystalline forms of the enzyme have been examined: one crystallized in the presence of nicotinamide adenine dinucleotide and the other in its absence. Though both forms catalyze cytoplasmic malate dehydrogenase's normal substrate conversions, they have specific activities 150–3000-fold less than the solution-state enzyme. These dramatic activity decreases cannot be accounted for by diffusion constraints imposed by the crystal lattice nor do they result from the manipulations necessary to crystallize or cross-link the enzyme. Further, crystal- and solution-state enzymes have different pH dependences of their enzymatic activities, have different sensitivities toward inactivation by the covalent inhibitor iodoacetate, and respond differently to nicotinamide adenine dinucleotide protection against this inactivation. Finally, crystals of the enzyme grown in the presence and absence of cofactor are also distinguishable from one another by using the same criteria. Taken together, these results suggest that crystallization perturbs the dynamics and, perhaps, the average conformation of cytoplasmic malate dehydrogenase.

Protein structure–function relationships are often developed by using information obtained from proteins in two different phase states. X-ray crystallography reveals the detailed three-dimensional structures of a protein in the crystalline phase while activity and chemical modification studies most commonly probe the functional parameters of the protein in the solution phase (Walsh, 1979; Makinen & Fink, 1977). Consequently, when developing structure–function relationships, it is important to establish the similarity of activity or macromolecular structure between phase states. Comparisons of enzyme activities in crystalline and solution states have produced variable results. Some enzymes are profoundly affected by crystallization (Kirsten et al., 1983; Kirsten & Christen, 1983; Bennett & Steitz, 1978; Spilburg et al., 1977; Alter et al., 1977; Kavinsky & Madsen, 1976; Theorell et al., 1966) while others are only slightly affected, if at all [Mozzarelli et al., 1979, 1982; Wilkinson & Rose, 1980; Vas et al., 1979; Berni et al., 1977; Bayne & Ottesen, 1976; for a review, see Makinen & Fink (1977)]. Therefore, it is necessary to establish whether crystallization perturbs enzymes on a case to case basis.

Previous studies of cytoplasmic malate dehydrogenase (s-MDH)[1] suggest crystallization perturbs the functional properties of this dimeric enzyme. For example, the cofactor NAD binds preferentially to one subunit when the enzyme is crystallized in its presence (holo crystals) (Glatthaar et al., 1972). In contrast, several studies of solution-state s-MDH indicate that the cofactor binding sites in this enzyme are identical and noninteracting in the presence or absence of substrate (Johnson & Rupley, 1979; Lodola et al., 1978). These differences could be explained if crystallization perturbs the structure and therefore the function of s-MDH. Thus, we have compared several properties of crystalline and solution-state s-MDH including the activity of the enzyme, the reactivity of the enzyme toward an irreversible inhibitor, iodoacetic acid, and the ability of the cofactor to protect against this inactivation. The studies reported here have a general significance in describing the effect of crystallization on protein structure. They also have a specific significance in confirming previous reports of crystalline s-MDH subunit asymmetry and in determining whether other functionally significant dynamic or structural modifications accompany crystallization.

Materials and Methods

Materials. CM-52 carboxymethylcellulose and DEAE-52 diethylaminoethylcellulose were obtained from Whatman, Inc. CL-6B Blue Sepharose was obtained from Pharmacia Fine Chemicals. Dimethyl 3,3′-dithiobis(propionimidate) (DTP) was obtained from Pierce Chemical Co. NAD (grade III) and NADH (grade III) were purchased from Sigma Chemical Co. and used without further purification.

Enzyme Purification. Pig heart cytoplasmic MDH was prepared by using a method similar to the procedure of Glatthaar and co-workers (Glatthaar et al., 1974). The gel filtration steps used by these investigators were replaced with a CL-6B Blue Sepharose affinity chromatography step. Protein in 5 mM potassium phosphate, pH 6.2, was loaded onto a CL-6B Blue Sepharose column equilibrated with the same buffer. After the column was washed (5 mM potassium phosphate, pH 6.2), s-MDH activity was eluted with 100 μM NADH. All chromatographic steps were performed at 4 °C.

On the basis of three observations, cytoplasmic MDH preparations were judged free of mitochondrial MDH contamination. First, the s-MDH preparations were not inhibited by 4,4′-bis(dimethylamino)diphenylcarbinol, an inhibitor of mitochondrial but not cytoplasmic MDH (Humphries et al., 1973). Second, s-MDH preparations exhibited no substrate inhibition at oxaloacetate concentrations of 1.5 mM while mitochondrial MDH is inhibited at these concentrations

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[1] Abbreviations: s-MDH, supernatant or cytoplasmic malate dehydrogenase; DTP, dimethyl 3,3′-dithiobis(propionimidate); DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAA, iodoacetic acid; OAA, oxaloacetic acid.

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(Bernstein et al., 1978). Finally, our preparations had a single active band in nondenaturing gel electrophoresis (pH 7.5, 7.5% polyacrylamide) that migrated with authentic s-MDH (Sigma Chemical Co.). Mitochondrial MDH has considerably less anodal mobility than s-MDH at this pH (C. T. Zimmerle and G. M. Alter, unpublished results; Hand et al., 1981).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (pH 8.0) of purified s-MDH produced a single Coomassie Blue staining band corresponding to a molecular weight of 35 000. When standard assay conditions were used, freshly purified preparations had specific activities of 110 units min^{-1} mg^{-1} when assayed in the direction of malate oxidation and 580–600 units min^{-1} mg^{-1} when assayed in the direction of oxaloacetate reduction. The specific activities are in good agreement with values of 100 units min^{-1} mg^{-1} for malate oxidation and 567 units min^{-1} mg^{-1} for oxaloacetate reduction reported by Lodola et al. (1978) and Glatthaar et al. (1974), respectively. On the basis of these observations, enzyme preparations were judged homogeneous.

Enzyme Crystallization and Cross-Linking. Apo and holo s-MDH crystals were grown from 60–65% saturated ammonium sulfate solutions as described by Banaszak and coworkers (Glatthaar et al., 1972; Banaszak et al., 1971). The crystals were similar in appearance to those described by these authors (as judged by light microscopy) and were cross-linked by using the reagent 3,3′-dithiobis(propionimidate) (DTP). Since this reagent reacts with ammonium sulfate, s-MDH crystals were repeatedly washed with 1.65 M sodium citrate immediately prior to the DTP reaction. The final wash, which contained 40 mM oxaloacetate and 100 mM NAD, was gradually adjusted to pH 9.0 by the addition of 4 M dibasic potassium phosphate. DTP (0.5 mg/mL, final concentration) was added to the crystalline suspension and the mixture rapidly stirred. The cross-linking reaction was quenched after 3 min by the addition of ammonium sulfate, pH 6.0, and the resulting cross-linked crystals were washed with 50 mM Hepes buffer, pH 8.0. Repeated washing removed residual soluble-phase enzyme as well as any remaining sodium citrate or ammonium sulfate.

Since DTP inactivates the solution-phase enzyme, presumably by modifying a catalytically essential lysine (Bleile et al., 1976), both oxaloacetate and NAD were added to the DTP reaction mixture. We found that this nonproductive tertiary complex fully protected solution-phase s-MDH during the time required to cross-link s-MDH crystals. The appearance of s-MDH crystals (as determined by light microscopy) was unchanged by the cross-linking procedure or by incubating cross-linked crystals for periods of up to 10 months in either 50 mM Hepes, pH 8.0, or distilled water. Further, no loss of crystal activity was observed after these incubations.

Protein Concentration and Activity. Concentrations of native s-MDH were determined spectrophotometrically by using $A^{1\%}_{1cm}$ = 15 at 280 nm (Lodola et al., 1978). Protein concentrations of cross-linked crystal suspensions were determined by using the Bio-Rad protein assay (Bio-Rad, Inc.) after cleaving crystal cross-links with 0.3 M dithiothreitol and allowing crystals to dissolve. Neither the cross-linking reaction nor DTT interfered with the Bio-Rad assay.

Standard assay conditions for measuring oxaloacetate reduction were 0.25 mM NADH, 1.0 mM oxaloacetate, and 50 mM Hepes, pH 8.0, 25 °C, while malate oxidation was assayed in solutions containing 2 mM NAD, 0.12 M malate, and 0.1 M glycine, pH 10.0, 25 °C. All rates were measured spectrophotometrically at 340 nm (E_{340} = 6300 M^{-1} cm^{-1} for NADH). When crystalline preparations were assayed, cuvettes were vigorously stirred to prevent any changes in absorbance due to crystal settling as well as to minimize diffusion resistance at the crystal–solution interface (Engasser, 1978). One enzyme unit is defined as the amount of enzyme which utilizes one micromole of NADH or NAD per minute.

Table I: Percent of Total Activity in the Supernatant after Successive Centrifugations of Apo Crystalline s-MDH

centri-fugation	native crystal	cross-linked crystal
1	18.3	35.0
2	13.3	2.1
3	16.0	0.1
4	13.8	0.008

Iodoacetate Inactivation of s-MDH. Crystalline or solution-phase s-MDH was inactivated in a reaction mixture containing 250 mM iodoacetate (IAA) and 50 mM Hepes, equilibrated at pH 8.0 and 37 °C. At different times, aliquots of this reaction mixture were quenched by the addition of 0.3 M histidine, pH 6.0, and the remaining activity was assayed by using standard assay conditions. When IAA inactivation of crystalline s-MDH was examined, reacted crystals were routinely dissolved, and then the resulting solution-state s-MDH activity was measured as already described.

Results

Crystalline preparations of s-MDH were characterized by an approximately 20-fold reduction in activity with respect to the solution-phase enzyme when both were assayed in 70% ammonium sulfate. Under these conditions, s-MDH did not precipitate from solution, and crystals appeared unchanged over a period of 1 h as judged by light microscopy. Two observations, however, suggested solution-state s-MDH contributed to the activity of these crystalline preparations, making an assessment of crystalline-phase activities unreliable. First, even after several successive washes and low-speed centrifugations, an appreciable portion of the activity remained in the supernatant, although all crystals sedimented. In fact, after each centrifugation, the supernatant contained a relatively constant amount of activity, suggesting that an equilibrium between crystalline (sedimentable) and solution-phase (nonsedimentable) s-MDH was established during each washing and centrifugation step (Table I). Second, crystals completely dissolved after 24 h when incubated in a 70% ammonium sulfate solution containing cofactor (10 mM NADH) and substrate (40 mM oxaloacetate).

Activity of Cross-Linked Crystalline s-MDH. To ensure that crystalline enzyme preparations were not contaminated by solution-phase enzyme, we cross-linked each crystalline preparation and removed soluble enzyme by repeated washings. Cross-linking was accomplished by using the cleavable amino group reactive cross-linking reagent DTP (Wang & Richards, 1975).

Under standard assay conditions (saturating levels of cofactor and substrate), the specific activities characterizing cross-linked apo and holo crystalline s-MDH were less than 1% of those found for the native solution-state enzyme (Table II). Regardless of crystal type, larger rate differences between phase states were found when measuring oxaloacetate reduction as opposed to malate oxidation. In addition, apo crystals exhibited approximately twice the activity of holo crystals, irrespective of the reaction measured.

The reversibility of DTP cross-links was used to demonstrate that lysine modification by DTP did not perturb s-MDH's catalytic efficiency. DTP cross-links contain a disulfide bond and so can be cleaved by the thiol reagent DTT. When crystals

Table II: Crystal- and Solution-Phase Activities of s-MDH

enzyme crystal type	substrate	sp act.[a] crystal[b]	dissolved crystal[c]	native s-MDH	inhibition[d]
apo	malate	0.7 ± 0.4	110 ± 6	110 ± 10	150
holo	malate	0.4 ± 0.2	110 ± 10	110 ± 10	300
apo	OAA	0.4 ± 0.2	580 ± 30	600 ± 30	1500
holo	OAA	0.2 ± 0.1	600 ± 25	600 ± 30	3000

[a] Assays as described under Materials and Methods. [b] Crystals cross-linked by DTP. [c] Cross-linked crystals dissolved by incubation with dithiothreitol as described under Materials and Methods. [d] Ratio of dissolved to cross-linked crystal specific activities.

were incubated in buffers containing 0.3 M DTT but lacking ammonium sulfate or sodium citrate (crystallizing agents), they completely dissolved within 2 min, and full activity characteristic of solution-state s-MDH was restored (Table II). This occurred regardless of whether substrate reduction or oxidation was measured, and irrespective of crystal type. Full restoration of activity upon crystal dissolution would not have occurred if DTP modified s-MDH activity.

By cleaving crystal cross-links in buffers containing as much ammonium sulfate or sodium citrate as was used to grow and maintain un-cross-linked crystals, we demonstrated cross-links, per se, had little or no effect on crystalline s-MDH activity. Under these conditions of high ionic strength and 0.3 M DTT, cross-links were again broken within 2 min since after this length of incubation crystals dissolved immediately when transferred to low ionic strength buffers lacking DTT. Yet, less than a 2-fold increase in the crystalline s-MDH specific activity was observed after a longer (5 min) incubation when assays were performed in high ionic strength buffers. The presence or absence of cross-links within the crystal matrix, then, did not significantly alter the activity. In all likelihood, the small activity increase found at 5 min resulted from trace amounts of crystalline protein returning to the solution state.

Additional evidence indicating cross-links had little or no effect on activity came from redissolved cross-linked s-MDH crystals. These preparations had multiple bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis that corresponded to multiples of the s-MDH subunit molecular weight. In fact, 15–20% of the protein was in bands with molecular weights greater than the subunit molecular weight. However, since the specific activities of the redissolved cross-linked crystals were identical with those of native, solution-phase s-MDH, the presence of cross-linked species apparently had little effect on the active-site function of the redissolved enzyme. Incomplete cleaving of all cross-links in our crystalline preparations is consistent with previous characterizations of DTP-cross-linked proteins (Fahien et al., 1978).

High concentrations of salts used in the crystallization and cross-linking of s-MDH had only a modest effect on enzyme activity. In the direction of oxaloacetate reduction, the activity of solution-state s-MDH was decreased 6-fold by 70% saturated ammonium sulfate and 2-fold by 1.6 M sodium citrate. In the direction of malate oxidation, activity was decreased 8-fold by 70% ammonium sulfate while it was unaffected by 1.6 M sodium citrate (Table II). Further, crystal-phase activities were unperturbed by these salt concentrations.

Effect of Diffusion on Crystalline Activity. Crystals used in kinetic studies reported here had thicknesses of 0.5 ± 0.3 μm, or substrate diffusion paths of about 0.25 μm. These were 3–12 times larger than the critical thickness calculated as described by Makinen & Fink (1977) for apo s-MDH. The critical thickness is the thickness up to which diffusion constraints reduce the activity of a crystalline enzyme to about 93% of its diffusion-free activity. Therefore, diffusion could have perturbed the activity of crystalline s-MDH. However, the crystal thickness required to cause as low an activity as was actually observed (Table II) was 30–120 μm according to the equations of Makinen and Fink. This was substantially greater than the crystal's actual thickness (0.5 μm) and suggested diffusion constraints alone could not account for the crystal's low activity.

Theories of the kinetics of immobilized and crystallized enzymes predict that when diffusion limits the rate of an enzyme's reaction, its activity will have distinctive properties. The activity should depend on the size of the enzyme's crystals and be proportional to the square root of the enzyme's active-site concentration within the crystals (Sundarum et al., 1970; Katchalski et al., 1971; Makinen & Fink, 1977; Laidler & Bunting, 1980) (see the Appendix). Activities of diffusion-free enzymes should be independent of crystal size and directly proportional to the enzyme concentration within the crystals. These predictions have been experimentally verified for crystalline enzymes by other investigators [i.e., see Spilburg et al. (1977) and Kirsten & Christen (1983)]. Both criteria were used to assess the role of diffusion on crystalline s-MDH activity. In these experiments, apo crystals were examined with oxaloacetate as a substrate. This combination of crystal type and substrate had the greatest activity (Table II) and so was most susceptible to diffusion effects (Engasser, 1978).

Batches of small (20 ± 10 μm × 10 ± 3 μm × 0.5 ± 0.3 μm) and large (150 ± 40 μm × 25 ± 5 μm × 3 ± 5.0 μm) crystals were fractionated from cross-linked crystal preparations by low-speed centrifugation, and their specific activities were measured. Despite an approximately 6-fold difference in the crystal's smallest dimension, the specific activities of both crystalline preparations were identical. This suggests that diffusion did not limit the reaction rate of the s-MDH crystals.

To examine the dependence of crystalline-state activity on active-site concentration, partially inactivated crystals were examined. Though the concentration of enzyme molecules in s-MDH crystals is fixed by the protein packing scheme characteristic of the crystal, the native active-site concentration in crystals was varied by inactivating a portion of these active sites with iodoacetate (Materials and Methods). Leskovac (1971; Leskovac & Pfleiderer, 1969) has reported that IAA inactivates s-MDH by reacting with an active-site methionine residue. The rate of iodoacetate inactivation of s-MDH was slow ($t_{1/2}$ = 10 min) with respect to the time expected to diffuse iodoacetate into the small crystals (<<1 s) (Figure 4) (Laidler & Bunting, 1973), ensuring that uniform inactivation of active sites occurred throughout the crystals. Crystals with differing concentrations of functional active sites were produced by quenching aliquots of the reaction mixture at various times. Specific activities of partially inactivated crystals were then measured in the crystal state (Materials and Methods), and the extent of active-site inactivation was determined by cleaving crystal cross-links and measuring the activity of the redissolved enzyme. Representative results are shown in Figure 1. They demonstrate a direct dependence of the crystal-phase activity on the activity of the redissolved enzyme, i.e., the concentration of functional active sites. (A square root dependence, expected if the reaction rate was dominated by diffusion, is also shown in Figure 1). Diffusion, then, did not have the controlling effect on the activity of the crystalline enzyme.

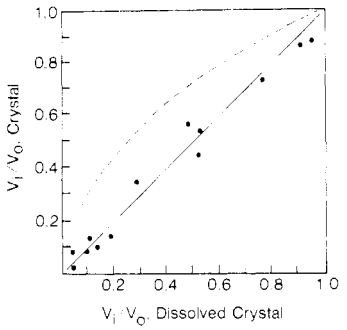

FIGURE 1: Dependence of apo crystalline s-MDH activity on native active-site concentration. V_0 and V_i are the initial rates for the enzyme-catalyzed reduction of OAA by unreacted and partially inactive s-MDH, respectively, for both crystalline (horizontal axis) and dissolved crystalline (vertical axis) enzyme. The solid line is drawn through experimental points while the dashed line represents a square-root dependence of crystal-phase activity on solution-state activity that would occur if diffusion limited the crystalline enzyme's activity (see Appendix).

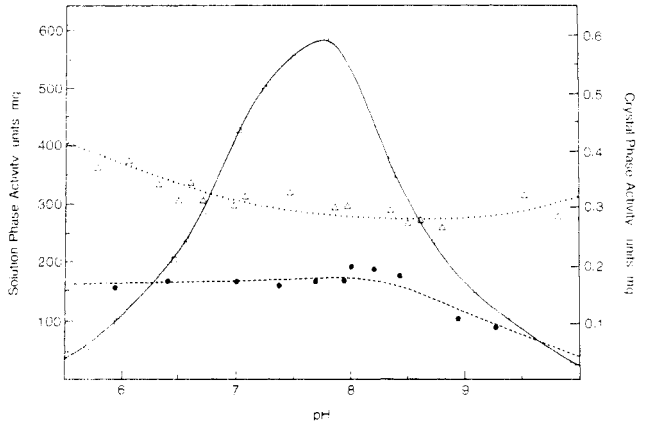

FIGURE 2: pH dependence of OAA reduction by solution-state (□), apo crystalline (△), and holo crystalline (●) s-MDH. Enzymatic activity was assayed as described under Materials and Methods by using 50 mM acetate buffer (pH 5.5–6.5), 50 mM Hepes buffer (pH 6.2–9.0), or 100 mM glycine buffer (pH 8.8–10.0). Each data point represents an average of three determinations.

In an effort to identify properties which may contribute to the low crystal-phase activity of this enzyme, we examined the pH dependence of the crystalline enzyme as well as the solution-state enzyme and probed the s-MDH active site in each state with an irreversible inhibitor, iodoacetic acid.

pH Dependence of s-MDH Activity. The pH dependence for the reduction of OAA catalyzed by solution-state s-MDH was bell shaped with a maximum at pH 7.8 (Figure 2). This agrees with previously reported pH profiles (Leskovac & Pfeiderer, 1969). Crystallization of s-MDH dramatically reduced the pH dependence of this reaction (Figure 2). Further, over the pH range examined, the crystalline-phase enzyme at its most active pH was still less active than its solution-phase counterpart at its least active pH.

When enzymatic activity was measured in the direction of malate oxidation, pK_a's of about 8.0, 9.5, and 9.9 characterized pH profiles of solution-state, apo crystalline, and holo crystalline s-MDH, respectively (Figure 3). Although both types of s-MDH crystal had greater pH dependences when using malate rather than oxaloacetate as substrate, their specific activities were still consistently low compared to the solution-state enzyme.

Regardless of the substrate or crystal type used, activities rose dramatically and then fell at pHs greater than 11.5 or less than 3.0 (data not shown). This probably corresponded

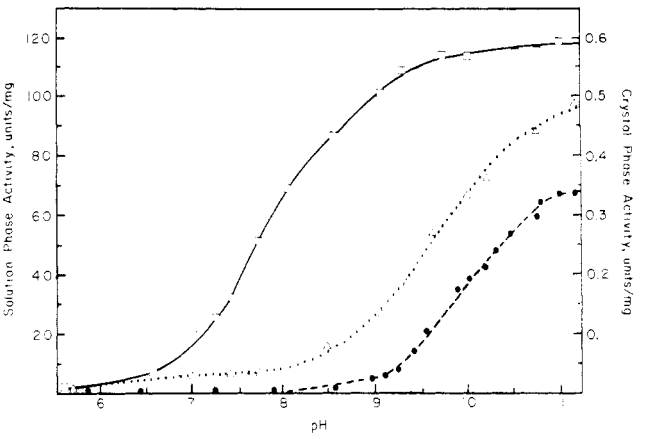

FIGURE 3: pH dependence of malate oxidation by solution-state (□), apo crystalline (△), and holo crystalline (●) s-MDH. Enzymatic activity was assayed as described under Materials and Methods by using 50 mM acetate buffer (pH 5.5–6.5), 50 mM Hepes buffer (pH 6.2–9.0), or 100 mM glycine buffer (pH 8.8–11.2). Each data point represents an average of three determinations.

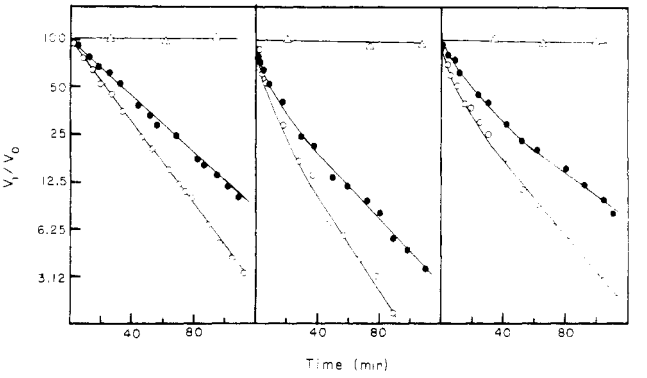

FIGURE 4: Time courses for iodoacetate inactivation of solution-state (left-hand panel), holo crystalline (center panel), and apo crystalline (right-hand panel) s-MDH in the presence (●) and absence (○) of 25 mM NAD. The rate of inactivation is represented by the ratio of the velocity of inactivated samples (V_i) to unmodified samples as a function of time. Time courses for the inactivation of s-MDH in the absence of IAA or NAD are also shown (△). Enzyme assays were measured by using OAA as a substrate. Conditions for IAA inactivation, crystal dissolution, and enzymatic assays are described under Materials and Methods.

to slight dissolution of crystals followed by enzyme denaturation at these pH extremes. In addition, the activity of apo crystals was always greater than that of holo crystals whether OAA reduction or malate oxidation was measured (Figures 2 and 3).

Iodoacetate Inactivation. In agreement with previous studies (Leskovac & Pfleiderer, 1969), we found solution-phase s-MDH was inactivated by IAA in a pseudo-first-order process (Figure 4). The time courses for the IAA inactivation of apo and holo crystalline s-MDH preparations were more complex, with 50% of the crystalline-state activity being lost in an initial rapid phase. The remaining activity was lost in a slower phase with rates similar to the pseudo-first-order rate of solution-state sMDH inactivation. In all enzyme preparations, inactivation by IAA was followed until less than 1% of the orginal activity remained. Thus, although the inactivation processes were different between phase states, all enzyme molecules were totally inactivated by iodoacetate.

Differences between crystal and solution phases were apparent when iodoacetate inactivation took place in the presence of high (25 mM) NAD concentrations. Again, a pseudo-first-order rate characterized the solution-state enzyme, while biphasic profiles were seen for the crystalline-state enzyme.

Table III: Crystallization-Linked Changes in s-MDH Activity

factors which may reduce activity	x-fold reduction in activity: apo crystal	x-fold reduction in activity: holo crystals
modification of lysines	none	none
introduction of cross-links	2	2
sterically blocked active sites	none	none
incompetent NADH binding sites	none	2
crystallization medium		
ammonium sulfate	6[a]	6[a]
sodium citrate	2[a]	2[a]
diffusion		
Katchalski theory	2-6[b]	
Engasser calcn	none[b]	
crystallization, obsd	1500	3000

[a] Effect on solution-state enzyme, measured in the direction of oxaloacetate reduction. [b] Calculated as described in the Appendix for oxaloacetate reduction in apo crystals.

The pseudo-first-order rate for solution-state enzyme and also the slower rates for both types of crystalline enzyme were approximately 2-fold less than those for their respective counterparts in the absence of NAD protection. Differences between apo and holo crystals were also apparent. Not only was the initial phase of IAA inactivation faster in the holo than apo crystals but also NAD-mediated protection during the rapid phase was less effective for holo than apo crystals (Figure 4).

Discussion

Enzymatic activity is a convenient and discerning criterion for judging the similarity of enzyme structures in crystalline and solution states. Since activity is usually directly affected by both the flexibility and average conformation of an enzyme, not only the similarity of the average protein structure(s) in both states but also the similarity of the enzyme dynamics in catalytically important transitions (Welch et al., 1982) is tested. However, in order to confidently determine activities which characterize crystalline-state enzyme conformations, the effect of manipulations required to obtain and cross-link crystals on enzyme activity, as well as the importance of diffusion constraints imposed by the crystal lattice, must be either quantitatively assessed or dismissed.

As the results summarized in Table III show, manipulations used to grow and cross-link crystals as well as packing characteristic of the s-MDH in crystals cannot account for the low activity of the crystalline enzyme. Neither the monofunctional derivatization of lysine residues by DTP nor the bifunctional cross-linking by DTP reduces enzymatic activity significantly. Any blocking of active sites as a result of enzyme packing in crystals must be minimal since all activity in crystalline s-MDH is susceptible to iodoacetate inactivation. It also appears that no more than half of the crystalline enzyme's active sites are deficient in binding dinucleotide since all the activity in apo crystals and at least half the activity of holo crystals can be protected by NAD. These protection experiments suggest that as many as half of the enzyme's active sites could be inactive owing to crystal packing or asymmetry of the dimeric enzyme's subunits in the crystal lattice (Glatthaar et al., 1972). Even if this is the case, a 2-fold instead of a 150–3000-fold reduction in activity would be expected. The ammonium sulfate or sodium citrate concentration necessary to crystallize s-MDH or to maintain un-cross-linked crystals decreases the activity of solution-state s-MDH by, at the most, only about $^1/_{25}$th the amount crystallization does. Further, these salts have a very small effect on the activity of the crystallized enzymes and are undoubtedly removed from the crystals during the extensive washing procedure (Materials and Methods) prior to routine crystal assays.

Both theoretical and experimental approaches indicate that diffusion of small molecules into and out of the crystal matrix probably does not control the measured activity of crystalline-state s-MDH (Table III). Vallee and co-workers (Spilburg et al., 1977; Alter et al., 1977) have shown the theory developed by Katchalski et al. (1971) to describe the effect of diffusion on the reaction kinetics of immobilized enzymes also accurately describes diffusion's role in the kinetics of crystalline Michaelis–Menten enzymes. Equivalent formulations have been developed by other investigators (Laidler & Bunting, 1980; Makinen & Fink, 1977; Engasser & Horvath, 1973). All theories conclude diffusion limitation is most severe at low substrate concentrations but can be overcome by using saturating substrate levels.

These formulations can also quantitatively estimate the effect of diffusion on reaction rates in the severest case, that of low substrate concentration (Katchalski's method), or under the conditions actually employed (Engasser's method). Using Katchalski's theory and assuming the inherent kinetic properties of s-MDH are identical in the crystalline and solution states, we note that the calculated effect of diffusion is small, accounting for a 2–6-fold reduction of enzymatic activity (see the Appendix). The actual extent of diffusion limitation must be much smaller since high concentrations of substrate and cofactor were used in the studies reported here. In fact, Engasser's method, which uses measured crystalline-state activities, suggests no diffusion limitation occurred under the conditions we used (see the Appendix). More importantly, the experimental results are consistent with diffusion not controlling the specific activity of the crystalline enzyme. The specific activity of s-MDH crystals is independent of crystal size, in the size range used here (Results), and directly dependent on the concentration of native active sites in crystals (Figure 1). These results would only occur if diffusion does not limit the rate of the reactions catalyzed by crystalline s-MDH.

The specific activities of solution- and crystalline-state s-MDH reported here (Table II) are properly termed V_M's since saturating levels of substrate and cofactor were used in our standard assays. The crystallization-linked activity changes therefore must be related to reduced crystalline s-MDH V_M's. Dramatic V_M reductions have been associated with crystallization-induced activity changes in several other systems (Kirsten et al., 1983; Spilburg et al., 1977; Alter et al., 1977; Kavinsky & Madsen, 1976). In at least one of them, carboxypeptidase A, the decreased crystalline V_M's may be correlated with an inhibition of conformational transitions in enzyme crystals (Scheule et al., 1980; Harrison et al., 1975). Since a conformational change associated with NADH binding has been suggested in solution-state s-MDH (Lodola et al., 1978), it is tempting to draw an analogy with carboxypeptidase A. The low activity of crystalline s-MDH may reflect the inhibition of a conformational change linked with nucleotide binding. This change in the enzyme's dynamics could result from constraints imposed by protein–protein interactions associated with the crystal lattice. In solution-state s-MDH, cofactor dissociation from the binary enzyme–cofactor complex appears to be the rate-limiting step (Banaszak & Bradshaw, 1975). If the same reaction path is followed in crystalline s-MDH, then perhaps an earlier step in the path, the cofactor-linked conformation change, becomes rate limiting. This implies that characteristics of the rate-limiting step should

change with crystallization and that the conformations of s-MDH subunits containing bound cofactor are different from the conformations of subunits lacking this molecule.

Differences in the pH profiles of crystal- and solution-state enzymes (Figures 2 and 3) are consistent with crystallization perturbing the rate-limiting step. Crystallization-linked shifts in the pH profiles for malate oxidation (Figure 3) might be explained by a shift in the pK_a of a catalytically important group or a pH shift in the local environment of the crystalline enzyme relative to the bulk solution. This, however, cannot account for the pH dependence of oxaloacetate reduction (Figure 2). It is also unlikely that this pH dependence is a diffusion artifact imposed by the crystal lattice. Engasser & Horvath (1974) pointed out that dampened pH profiles could result if an immobilized enzyme is inadequately buffered or if hydrogen ion diffusion through the crystal matrix is slow. In view of the appreciable buffer concentration used throughout each profile (50–100 mM), the large solvent pores in s-MDH crystals (Hill et al., 1972), and the substantial solvent content of s-MDH crystals (Banaszak et al., 1971), insufficient buffering or restricted diffusion of hydrogen and buffer ions seems unlikely. The pH dependences suggest that a different rate-limiting step is probed in crystals than in solution at least in the direction of OAA reduction.

That apo and holo crystals have detectably different conformations is demonstrated by studies of NAD protection against IAA-mediated inactivation of s-MDH. The cofactor protects apo but not holo s-MDH crystals in the rapid phase of iodoacetic acid inactivation (Figure 4). These observations cannot be readily explained by differences in molecular packing between crystal types. X-ray crystallographic results indicate packing in apo and holo crystals is very similar (Weininger et al., 1977; Banaszak et al., 1971; Tsernoglou et al., 1972). Further, Weininger and co-workers (Weininger et al., 1977) report that neither active site of s-MDH in holo crystals appears to be sterically impeded by the crystal lattice. Together these observations imply conformational differences within each enzyme molecule are responsible for differences in NAD protection.

The crystallization-linked reduction in s-MDH activity may, in part, be the result of differences in how s-MDH proceeds through similar conformational states in crystalline and solution states. However, additional evidence suggests conformational differences (perhaps subtle) between s-MDH in these states also occur.

Though s-MDH conformations in apo and holo crystals are different by the criterion of IAA inactivation, neither crystalline conformation is identical with the solution-state conformation. On the one hand, linear time courses characterize IAA inactivation of solution-state s-MDH. They indicate inactivation proceeds as a single pseudo-first-order process whether the enzyme is protected by NAD or not (Figure 4). This implies subunit conformations are identical and is consistent with several studies indicating the cofactor binds to all solution-state s-MDH active sites identically (Johnson & Rupley, 1979; Lodola et al., 1978). On the other hand, biphasic time courses characterize the inactivation of crystalline s-MDH in the presence and absence of NAD. They suggest two separate processes occur during IAA inactivation. We cannot rigorously exclude the possibility that the two processes result from the modification of two catalytically linked residues in identical s-MDH subunits. However, this seems unlikely since not only would the reaction at each residue have to decrease activity 50% but also the reaction rates would have to be different in crystals but identical in solution. A more likely explanation is that the two inactivation rates correspond to inactivation at each of two nonidentical subunits in crystalline s-MDH. Dissimilarity between subunits seems to be a property unique to the crystalline forms of the enzyme. Interestingly, another report of crystallization-induced subunit asymmetry involving the enzyme aspartate aminotransferase has recently appeared (Kirsten et al., 1983).

Dissimilar inactivation rates for subunits of crystalline s-MDH could reflect either differences between subunit conformations or differences between subunit environments within the crystal. In view of previous reports, the former possibility is most likely. Banaszak and co-workers have shown that asymmetry between subunit conformations occurs in the holo crystalline enzyme by using the criteria of X-ray diffraction analysis, *p*-(hydroxymercuri)benzenesulfonate reactivity, and NAD binding (Birktoft & Banaszak, 1983; Birktoft et al., 1982; Weininger et al., 1977; Glatthaar et al., 1972; Tsernoglou et al., 1972). Further, asymmetric binding of NAD to apo crystals has been reported (Glatthaar et al., 1972). Our results are consistent with these reports and indicate that asymmetry between subunits is present not only in holo crystals but also in apo crystals even in the absence of cofactor or substrate.

The relationship between the detailed conformation of crystalline and solution-state s-MDH must not be direct. The enzyme structures in apo and holo crystals may be related to conformations occurring in the reaction pathway of solution-state s-MDH, the dynamics of structural interconversions being primarily responsible for the crystal's low activities. However, IAA inactivation studies suggest that neither crystalline enzyme form is identical with the solution-state enzyme's conformation. Both changes in the dynamics of catalytically important transitions and perturbations of average conformations (relative so solution-state s-MDH), then, may contribute to the low activity associated with the crystalline enzyme. The identification of interactions responsible for these effects will be valuable for interpreting the structural basis of this enzyme's activity.

Appendix

Several investigators have described the effect that diffusion has on the activity of immobilized and crystalline enzymes (Sundaram et al., 1970; Katchalski et al., 1971; Makinen & Fink, 1977; Engasser, 1978; Laidler & Bunting, 1980). The treatments of Katchalski and of Makinen and Fink are used in the first part of the Appendix. In the second part, the treatments of Katchalski and of Engasser are used.

Effect of Crystal Size and Active-Site Concentration on the Activity of Diffusion-Limited Crystals. The effect of diffusion on the activity of crystalline and immobilized enzymes has been expressed in terms of an effectiveness factor (F in Katchalski's treatment, e in that of Makinen and Fink) given by

$$F = e = \frac{V}{V_0} = \frac{\tanh(\gamma l)}{\gamma l} \tag{1}$$

where V is the observed activity of the crystalline enzyme, V_0 is the activity of an equal concentration of enzyme under diffusion-free conditions, l is the crystal thickness, determined from crystal dimensions, and γ is given by

$$\gamma = \frac{1}{2}\left(\frac{k_{cat}[E]}{K_M D_0'}\right)^{1/2} \tag{2}$$

where k_{cat} and K_M are pertinent steady-state kinetic parameters, [E] is the active-site concentration, and D_0' is the diffusion coefficient describing substrate diffusion within crystals.

Makinen and Fink [eq 7–9 of Makinen & Fink (1977)] combined parameters from eq 2 to define a critical thickness for the crystal (λ_c). This is simply related to γ: $\gamma = 1/(2\lambda_c)$.

Since the activity of Michaelis–Menten enzymes which are not affected by diffusion is directly proportional to enzyme concentration, eq 1 can be rearranged to reflect that proportionality:

$$V = \frac{\tanh(\gamma l)}{\gamma l} V_0 \qquad V \propto \frac{\tanh(\gamma l)}{\gamma l}[E] \qquad (3)$$

Both criteria used to test for diffusion limitation, the dependence of activity on crystal size and on active-site concentration (within the crystal), follow from this relation. Equation 3 explicitly shows the activity's dependence on l, the crystal thickness. Since γ is a function of enzyme concentration within crystals (eq 2), it must be considered when determining the dependence of activity on active-site concentration. If γl is 2.0 or greater (the crystal thickness is 4 times the critical thickness), tanh (γl) approaches very nearly its limiting value of 1, and eq 3 simplifies. This occurs if the effectiveness factor (F) is about 0.5 or less (Laidler & Bunting, 1980). Under these conditions, eq 3 becomes, to a good approximation, $V \propto [E]/(\gamma l)$ or in Makinen and Fink's notation, $V \propto [E]/[l/(2\lambda_c)]$. Since l is constant (for a particular size of crystal) and γ depends on $[E]^{1/2}$ (eq 2), V's dependence on enzyme concentration is given by

$$V \propto \frac{[E]}{(1/2)[k_{cat}/(K_M D_0')]^{1/2}[E]^{1/2}} \qquad V \propto [E]^{1/2} \qquad (4)$$

Then, V will be proportional to $[E]^{1/2}$ or, equivalently, to $[E]\lambda_c$.

Quantitative Estimation of Diffusion Effects in s-MDH Crystals. To estimate the degree to which diffusion constraints might reduce the activity of crystalline s-MDH, methods described by Engasser (1978) and Katchalski et al. (1971) were used. The two-substrate nature of the s-MDH reaction was accounted for by successive evaluation of diffusion effects on each substrate (Engasser, 1978). The method of Katchalski et al. (1971) was applied by using solution-state kinetic parameters for the enzyme and physiochemical properties of s-MDH crystals. In contrast, the Engasser method (1978) was used with measured activities of crystalline s-MDH as well as s-MDH crystal physiochemical properties. Parameters used in these calculations are summarized in Table IV. Both methods for estimating diffusion limitation use effectiveness factors, F (eq 1).

External diffusion, which describes the rate at which substrates move from bulk solution to the crystal surface, was not considered in our application of the Katchalski method. However, it was included in the estimation using the Engasser method.

The method of Katchalski et al. (1971) was applied as described by Spilburg et al. (1977). The effectiveness factor was calculated according to eq 1 and 2. The diffusion coefficient, D_0', was estimated. A value for NADH diffusion through a glutaraldehyde-cross-linked bovine serum albumin membrane (Millis & Wingard, 1981) was taken as the bulk diffusion coefficient for NADH and oxaloacetate in solution (Table IV). This was probably a low estimate since the membrane must, to some extent, impede NADH's diffusion. The bulk diffusion coefficient was corrected for simple area restriction owing to the size of the substrate or cofactor, and the size of crystal channels (Renkin, 1954). Pore and substrate or cofactor dimensions were determined from crystallographic data and extended substrate dimensions, respectively (Table IV). In the case of NADH, this almost certainly overestimated the molecule's size since dinucleotides normally fold in solution, reducing their maximum dimension by a factor of 2 (McDonald et al., 1972). Therefore, the calculated value of D_0' was probably smaller than the actual value, leading to an overestimate of diffusion limitation. Using this value of D_0', effectiveness factors for NADH and oxaloacetate were 0.2 and 0.65, respectively. If the diffusion coefficient for NADH diffusion in glutaraldehyde-cross-linked bovine serum albumin membranes was taken as representative of cofactor and substrate diffusion in s-MDH crystals, eq 1 yielded effectiveness factors of 0.44 and 0.8 for NADH and oxaloacetate, respectively. Diffusion, then, can account for only a 2–6-fold decrease in enzymatic activity.

Table IV: Values of Parameters Used To Estimate Diffusion's Effect on the Observed Crystalline s-MDH Activity

constant	value
crystal active-site concn (mM)	18.6[a]
crystal activity (V_M/volume) (μmol s^{-1} cm^{-3})	6510
solution k_{cat} (s^{-1})	350
solution K_M(NADH) (μM)	22
solution K_M(OAA) (μM)	140
crystal-phase diffusion constant ($\times 10^{-6}$ cm^2 s^{-1}) for	
membrane	7.9[b]
NADH	1.4[c]
OAA	4.0[c]
external transport coefficient (NADH or OAA) ($\times 10^{-2}$ cm s^{-1})	1.6[d]
radius(NADH) (Å)	14[e]
radius(OAA) (Å)	6
crystal pore radius (Å)	23[f]
crystal size (μm)	10 × 10 × 0.5

[a] Calculated from crystal unit-cell dimensions reported by Banaszak et al. (1971). [b] For NADH diffusion in a glutaraldehyde-cross-linked bovine serum albumin membrane (Millis & Wingard, 1981). [c] Estimated as described by Renkin (1954). [d] Paul et al. (1978). [e] Estimated from the data of Banaszak & Webb (1975). [f] Estimated from the schematic packing diagram of the s-MDH unit cell (Tsernoglou et al., 1972).

Engasser (1978) has presented graphical correlations between external or internal effectiveness factors and readily calculated quantities derived from crystalline enzyme activity and crystal properties. Using these correlations, both external and internal diffusion effects were evaluated for the conditions actually used to measure crystalline s-MDH activity. K_M's were required in these correlations. Values for solution-state s-MDH K_M's (Table IV) were used since these parameters have not been determined for crystalline-phase s-MDH, and a number of reports indicate crystallization has a relatively modest effect on K_M (Spilburg et al., 1977; Kavinsky & Madsen, 1976).

The external effectiveness factor was correlated with the quantity:

$$\frac{V}{Ah[S]} \qquad (5)$$

where A is the surface area of the crystals, determined from average crystal dimensions (Table IV), h is the external transport coefficient, V is the activity of the crystalline enzyme on a volume basis, and [S] is the bulk substrate or cofactor concentration. Since the external transport coefficient for crystalline enzyme preparations is not reported, it was estimated by approximating the crystals as spheres of similar size (about 10 μm in diameter) and using the relationships of Brian & Hales (1969). The resulting value (Table IV) was assumed to be identical for NADH and oxaloacetate. External effec-

tiveness factors greater than 0.98 were found for both NADH and oxaloacetate, suggesting external diffusion does not significantly contribute to the crystalline s-MDH activity under the conditions used here.

Assuming that crystals can be treated as small membranes, the quantity correlated with the internal effectiveness factor was

$$\frac{(l/2)^2 V}{D_0'[S]_s \nu} \tag{6}$$

where l and ν are the crystal thickness and volume, respectively, V is the measured solid-state enzyme activity expressed on a volume basis, D_0' is the internal diffusion coefficient (estimated as already described) (Table IV), and $[S]_s$ is the substrate concentration at the crystal's surface. $[S]_s$ was estimated by using the relationship:

$$[S]_s = [S] - \frac{\nu}{Ah} \tag{7}$$

where [S] is the bulk substrate concentration while A and h are the crystal surface area and the external transport coefficient, respectively. Although the D_0' values in eq 6 are subject to the uncertainties already described, internal effectiveness factors greater than 0.95 were still obtained by using these estimates. This suggests that internal diffusion does not contribute to the crystalline s-MDH activity reported here.

Taken together, the external and internal effectiveness factors indicate that diffusion cannot account for the low activities of crystalline-state s-MDH observed here.

Registry No. MDH, 9001-64-3; NAD, 53-84-9; NADH, 58-68-4; OAA, 328-42-7; IAA, 64-69-7.

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Elementary Steps in the Reaction Mechanism of Chicken Liver Fatty Acid Synthase: Reduced Nicotinamide Adenine Dinucleotide Phosphate Binding and Formation and Reduction of Acetoacetyl-Enzyme†

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ABSTRACT: The kinetics of reduced nicotinamide adenine dinucleotide phosphate (NADPH) binding to fatty acid synthase from chicken liver and of the reduction of enzyme-bound acetoacetyl by NADPH (β-ketoacyl reductase) and the steps leading to formation of the acetoacetyl-enzyme have been studied in 0.1 M potassium phosphate–1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0, at 25 °C by monitoring changes in NADPH fluorescence with a stopped-flow apparatus. Improved fluorescence detection has permitted the use of NADPH concentrations as low as 20 nM. The kinetics of the binding of NADPH to the enzyme is consistent with a simple bimolecular binding mechanism and four equivalent sites on the enzyme (presumably two β-ketoacyl reductase sites and two enoyl reductase sites). The bimolecular rate constant is 12.7×10^6 M^{-1} s^{-1}, and the dissociation rate constant is 76.7 s^{-1}, which gives an equilibrium dissociation constant of 6.0 μM. The formation of the acetoacetyl-enzyme and its subsequent reduction by NADPH could be analyzed as two consecutive pseudo-first-order reactions by mixing enzyme–NADPH with acetyl-CoA and malonyl-CoA under conditions where [acetyl-CoA], [malonyl-CoA] >> [enzyme] >> [NADPH]. From the dependence of the rate of reduction of acetoacetyl-enzyme by NADPH on enzyme concentration, an independent estimate of the equilibrium dissociation constant for NADPH binding to the enzyme of 5.9 μM is obtained, and the rate constant for the reduction is 17.5 s^{-1}. The kinetic studies and analysis of the products obtained with [^{3}H]-NADPH suggest that the dehydration and enoyl reductase reactions occur much faster than the β-ketoacyl reductase reaction. The mechanism of formation of the acetoacetyl-enzyme is complex, but the inhibition of the rate of this process by high concentrations of both acetyl- and malonyl-CoA can be explained semiquantitatively in terms of a relatively rapid equilibration of these substrates with the enzyme followed by a rate-determining condensation reaction with a specific rate constant $\geq$30 s^{-1}. If CoA is scavenged from the reaction mixture, the rate of formation of the acetoacetyl-enzyme is greatly decreased; this indicates CoA is important for rapid formation of the acetoacetyl-enzyme. These results are consistent with those from steady-state kinetic studies.

The fatty acid synthase from chicken liver is a multienzyme complex containing two identical polypeptide chains of molecular weight 250000 (Yun & Hsu, 1972; Kumar et al., 1972; Arslanian et al., 1976; Stoops & Wakil, 1981). The enzyme catalyzes the synthesis of palmitic acid from AcCoA,[1] MalCoA, and NADPH through a series of reactions: after covalent transfer of malonyl and acetyl from MalCoA and AcCoA to the enzyme, malonyl and acetyl are condensed to form an acetoacetyl-enzyme intermediate; the enzyme-bound acetoacetyl is then reduced to hydroxybutyryl by NADPH; this is followed by dehydration to form crotonyl and a second reduction by NADPH to give enzyme-bound butyryl. This cycle is repeated 7 times by successive condensation of malonyl on to the growing hydrocarbon chain until palmitic acid is released by a thioesterase [cf. Bloch & Vance (1977)]. This mechanism is consistent with steady-state kinetic studies (Katiyar et al., 1975; Cox & Hammes, 1983) that have permitted an estimate of substrate binding constants and rate constants for the overall process. A systematic study of the individual steps in the reaction mechanism is being undertaken in our laboratory. A study of the acetylation and deacetylation of the enzyme by AcCoA and CoA, respectively, already has been reported (Cognet & Hammes, 1983). The rate constants for acetylation and deacetylation by enzyme-bound AcCoA and CoA are approximately 40 and 100 s^{-1}, respectively, at pH 7.0, 25 °C. In this work, the binding of NADPH to the enzyme, the reduction of enzyme-bound acetoacetyl by NADPH, and the formation of the acetoacetyl-enzyme from enzyme, AcCoA, and MalCoA have been studied with the stopped-flow method. Substrate dissociation constants and rate constants for individual steps in the mechanism have been obtained.

Materials and Methods

Materials. *N*-Acetyl-*S*-(acetoacetyl)cysteamine, 2,6-dichlorophenolindophenol, L-ascorbic acid, dithiothreitol, MalCoA, AcCoA, Mops, phosphotransacetylase, acetyl phosphate, and NADPH (type X) were from Sigma; ethyl butyrate, (*R*)-(−)-methyl 3-hydroxybutyrate, ethyl DL-2-

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[1] Abbreviations: AcCoA, acetyl coenzyme A; MalCoA, malonyl coenzyme A; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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