Atomic resolution structures of *Escherichia coli* and *Bacillus anthracis* malate synthase A: Comparison with isoform G and implications for structure-based drug discovery

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Abstract

Enzymes of the glyoxylate shunt are important for the virulence of pathogenic organisms such as *Mycobacterium tuberculosis* and *Candida albicans*. Two isoforms have been identified for malate synthase, the second enzyme in the pathway. Isoform A, found in fungi and plants, comprises ~530 residues, whereas isoform G, found only in bacteria, is larger by ~200 residues. Crystal structures of malate synthase isoform G from *Escherichia coli* and *Mycobacterium tuberculosis* were previously determined at moderate resolution. Here we describe crystal structures of *E. coli* malate synthase A (MSA) in the apo form (1.04 Å resolution) and in complex with acetyl-coenzyme A and a competitive inhibitor, possibly pyruvate or oxalate (1.40 Å resolution). In addition, a crystal structure for *Bacillus anthracis* MSA at 1.70 Å resolution is reported. The increase in size between isoforms A and G can be attributed primarily to an inserted α/β domain that may have regulatory function. Upon binding of inhibitor or substrate, several active site loops in MSA undergo large conformational changes. However, in the substrate bound form, the active sites of isoforms A and G from *E. coli* are nearly identical. Considering that inhibitors bind with very similar affinities to both isoforms, MSA is as an excellent platform for high-resolution structural studies and drug discovery efforts.

Keywords: drug discovery; atomic resolution; isoform; conformational change

Malate synthase (MS) and isocitrate lyase make up the glyoxylate shunt (Kornberg and Krebs 1957), which bypasses a portion of the citric acid cycle to convert isocitrate to malate. The actions of these enzymes circumvent the steps in the Krebs cycle that evolve CO₂, permitting bacteria, fungi, and plants to utilize two-carbon sources such as acetate and fatty acids for net

biosynthesis. Enzymes of the glyoxylate shunt have been implicated for the virulence and persistence of many different pathogens, especially *Mycobacterium tuberculosis* (mTB) (Honer Zu Bentrup et al. 1999; McKinney et al. 2000; Munoz-Elias and McKinney 2005) and *Candida albicans* (Lorenz and Fink 2001; Lorenz et al. 2004; Ramirez and Lorenz 2007). As mammals do not have genes encoding either glyoxylate shunt enzyme, the enzymes have become attractive targets for drug discovery (Smith et al. 2004).

Two distinct isoforms of MS, A (abbreviated MSA) and G (abbreviated MSG) (Falmagne et al. 1965), have been identified. Members of the isoform G family share >50% identity and are found only in bacteria (Smith et al. 2003).

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Likewise, MSA isoforms share high identity but are found in fungi and plants as well as bacteria. The eukaryotic MSAs form homomultimers, which distinguishes them from prokaryotic MSAs (Durchschlag et al. 1981). Both isoforms are represented in pathogenic organisms; for example, mTB utilizes a G isoform, whereas *C. albicans* utilizes isoform A.

Escherichia coli is thus far unique in that it differentially expresses both MSA and MSG, encoded by the genes *aceB* and *glcB*, respectively (Molina et al. 1994). Isoform A expression is regulated by fatty acids and acetate while isoform G expression is regulated by the presence of glycolate (Vanderwinkel and De Vlieghere 1968). *E. coli* MSA (ecMSA) comprises 533 amino acids, whereas *E. coli* MSG (ecMSG) comprises 723 amino acids. The two isoforms are very distantly related. Sequence comparisons suggest that the larger molecular size of MSG isoforms can be attributed to the presence of one or more insertions (Molina et al. 1994; Howard et al. 2000; Smith et al. 2003), while the conserved segments of the amino acid sequence show only ~18% identity. Crystal structures of MSG from *E. coli* (Howard et al. 2000; Anstrom et al. 2003) and from mTB (Smith et al. 2003; Anstrom and Remington 2006) have been determined, but unfortunately the resolution of the diffraction data is modest, which limits structure-based drug discovery efforts (Anstrom et al. 2005).

Structural studies of mTB MSG and ecMSG (which share about 56% amino acid sequence identity) reveal four structural domains (Howard et al. 2000; Tugarinov et al. 2002, 2005; Anstrom et al. 2003; Smith et al. 2003; Anstrom and Remington 2006). An $\alpha 8/\beta 8$ (TIM) barrel is centrally located and contains the active site. It is buttressed on one side by an N-terminal α -helical clasp and on the other by an α/β domain consisting of two insertions into the barrel. The C-terminal segment, which comprises several α -helices, caps the active site. As of yet, no function has been attributed to the α/β domain. Sequence alignments proposed by various groups disagree with respect to which domains are missing from the structure of MSA. Alignments by Smith et al. (2003) and Howard et al. (2000) suggest the N-terminal clasp domain is missing, whereas the alignment of Molina et al. (1994) predicts the absence of the α/β -domain; however, both alignments agree that the C-terminal cap and the TIM barrel are well conserved.

In the second step of the glyoxylate shunt pathway, the condensation and subsequent hydrolysis of glyoxylate and acetyl-CoA is performed by MS to produce malate and CoA. The modes of substrate binding are known in some detail from crystallographic and NMR structural studies (Howard et al. 2000; Tugarinov et al. 2002, 2005; Anstrom et al. 2003; Smith et al. 2003; Tugarinov and Kay 2005; Anstrom and Remington 2006), and a catalytic

mechanism has been proposed. Briefly, bound glyoxylate is activated toward nucleophilic attack by salt bridges to an essential Mg^{2+} ion and by hydrogen bonds to the protein backbone and a conserved arginine. An essential aspartic acid residue is believed to accept a proton from the acetyl-CoA terminal methyl group. The proposed enolate intermediate is stabilized by interaction with the essential arginine.

The binding site for the substrate glyoxylate is deep within the protein, connected by a channel to the solvent-exposed acetyl-CoA binding site. Prior to the structural studies, the results of small angle X-ray scattering experiments suggested “opening” or separation of domains so that the active site could sequester substrates from the solvent (Zipper and Durchschlag 1977). Substrate-induced conformational changes were also consistent with circular dichroism data on MS isolated from maize and yeast (Schmid et al. 1974; Beeckmans et al. 1994). In contrast, data from subsequent NMR studies of apo ecMSG were inconsistent with domain motions but did suggest disorder in a loop (Thr451–Asp455) that could control access to the active site (Tugarinov et al. 2002).

We report here crystal structures for ecMSA and *Bacillus anthracis* MSA (baMSA) at atomic resolution. These structures reveal that the conserved portions of MSA and MSG are very similar and that the difference in size is due to the presence of an additional α/β domain in the G isoform. Comparisons of *E. coli* MSA apo and ternary complex structures reveal significant loop movements, which we suggest are necessary for substrate binding and product release and, in addition, serve to order the reaction sequence. Active site comparisons of the two substrate-bound isoforms, combined with kinetic studies, suggest that either isoform will be useful as a platform for drug discovery.

Results

The gene for ecMSA was cloned from genomic DNA. Since genomic DNA was not readily available for baMSA, the gene was synthesized commercially. The primary structures share 68% similarity and 54% identity (BLOSUM62 matrix). In both cases, overexpression in *E. coli* yielded large quantities of protein. Kinetic and inhibition constants were obtained for both ecMSA and baMSA, allowing comparison with ecMSG. Both ecMSA and baMSA crystallized readily, but ecMSA produced crystals of much higher diffraction quality.

Kinetics and inhibition of malate synthase

The kinetic properties of ecMSA and baMSA were evaluated for the substrates acetyl CoA, glyoxylate, and several inhibitors. They are very similar for the two A isoforms and, in addition, are similar to those found for

ecMSG as shown in Table 1. Both A and G isoforms have a highly conserved cysteine residue in the active site, which potentially has a regulatory role (Anstrom et al. 2003). ecMSA Cys438, corresponding to Cys617 in ecMSG, was mutated to serine as performed by Anstrom et al. (2003). The ecMSA C438S mutation resulted in a modest reduction of activity and acetyl-CoA K_m , as observed for the ecMSG C617S mutation. The active site cysteine mutations were also necessary for determination of inhibition constants for compounds absorbing near 232 nm (see Materials and Methods). Inhibition by the competitive inhibitors pyruvate, parabanic acid, and 4-amido-2,3,4-pyrrolidenetrione was comparable for the different isoforms, indicating that either isoform should be useful for drug discovery. The binding of pyruvate and parabanic acid are relatively weak with low to sub millimolar inhibition constants, whereas oxalate and 4-amido-2,3,4-pyrrolidenetrione bind more tightly, with mid-micromolar inhibition constants. Parabanic acid evidently undergoes base and enzyme catalyzed hydrolysis under the conditions used in the assay (as determined by loss of parabanic acid absorbance at 280 nm); however, the reaction is slow enough (uncatalyzed ~ 80 nM/sec, ecMSG catalyzed ~ 800 nM/sec) that approximate inhibition constants could be obtained. While the affinity of parabanic acid for the enzyme is modest, in theory it is readily functionalized; therefore, parabanic acid could be an excellent lead compound for drug discovery. MS has long been known to employ Mg^{2+} for catalysis and, accordingly, ecMSA exhibits negligible activity in buffer containing Ca^{2+} as the primary divalent cation, as determined for mTBMSG (Smith et al. 2003).

ecMSA apo form structure

E. coli MSA crystallized readily from calcium acetate/PEG 8K in space group $P2_1$ with a single monomer per asymmetric unit and unit cell dimensions of $a = 52.0$ Å, $b = 74.1$ Å, $c = 71.9$ Å with $\beta = 97.8^\circ$. These crystals diffracted well, and data were collected using the home source to 1.68 Å resolution with excellent statistics (Table

2). The structure was solved using the SIRAS method using a $HgCl_2$ heavy atom derivative data set that diffracted to 1.80 Å. The final model has a crystallographic R -factor of 0.177 and $R_{free} = 0.208$. It consists of residues 5–533 with two Ca^{2+} ions and one molecule each of glycerol and acetate. Only one amino acid, Glu119, is found in the generously allowed region of the Ramachandran diagram, with none in disallowed regions. The conformation of Glu119 is supported by clear electron density.

Bacillus anthracis MSA apo structure

Crystals of *Bacillus anthracis* malate synthase A (baMSA) grew from ammonium sulfate/PEG 4K in space group $C2$ with unit cell dimensions of $a = 97.8$ Å, $b = 52.0$ Å, $c = 89.1$ Å; $\beta = 98.9^\circ$. Data reduction statistics were reasonable for diffraction data collected to 1.70 Å resolution. The structure was solved using molecular replacement with the ecMSA model after truncation of nonconserved residues. The model was constructed using ARP/wARP. The final model has a crystallographic R -factor of 0.194 ($R_{free} = 0.240$). There is one protomer per asymmetric unit containing residues 7–14, 19–325, 345–495, 497–529, two Mg^{2+} (one at a crystal contact), and 180 water molecules. The metal ion at the crystal contact may be a weakly bound Ni^{2+} ion, as addition of $NiCl_2$ to the crystallization medium promotes crystal growth. Structural comparison with apo ecMSA using DALI (Holm and Park 2000) results with a rmsd of 1.1 Å for all observed α -carbons. Residues 326–344 could not be located because of weak electron density, and in the crystal cannot adopt the conformation of the corresponding region of apo ecMSA (residues 329–346) as they would clash with a symmetry mate.

ecMSA ternary complex structure

Solutions containing ecMSA, acetyl-CoA, and pyruvate in ammonium sulfate/PEG 8K yielded ternary complex crystals in space group $P2_1$ with two protomers per asymmetric unit. The unit cell dimensions are $a = 75.2$ Å, $b = 71.5$ Å, $c = 103.3$ Å with $\beta = 90.1^\circ$. These

Table 1. Kinetic and inhibition constants for malate synthases

Enzyme	ecMSA WT	ecMSA C438S	ecMSG WT ^b	ecMSG C167S ^b	baMSA
Specific activity ^a	2500 $\pm$ 100	1850 $\pm$ 50	3400 $\pm$ 40	3000 $\pm$ 60	2300 $\pm$ 20
Percent of wild-type activity	100	74	100	88	100
K_m glyoxylate (μ M)	55 $\pm$ 7	58 $\pm$ 7	21 $\pm$ 2	50 $\pm$ 4	85 $\pm$ 2
K_m acetyl-CoA (μ M)	22 $\pm$ 3	56 $\pm$ 2	9.0 $\pm$ 1.1	46 $\pm$ 3	23 $\pm$ 3
K_i pyruvate (μ M)	650 $\pm$ 90	600 $\pm$ 8	1000 $\pm$ 20	1000 $\pm$ 90	750 $\pm$ 30
K_i parabanic acid (μ M)		370 $\pm$ 50		550 $\pm$ 60	
K_i 4-amido-2,3,4-pyrrolidenetrione (μ M)		40 $\pm$ 7		113 $\pm$ 12	
K_i oxalate (μ M)	37 $\pm$ 4		23 $\pm$ 4		

^aUnits of mol malate/min/mol protein.

^bValues taken from Anstrom et al. (2003), except data regarding parabanic acid, 4-amido-2,3,4-pyrrolidenetrione, and oxalate.

Table 2. Malate synthase A data collection and refinement statistics

Crystal	ecMSA native	ecMSA HgCl ₂	ecMSA Mg ²⁺	ecMSA inhibitor	baMSA
Data collection					
Total observations	214,408	186,790	1,018,867	785,650	138,489
Unique reflections	59,002	48,087	228,574	229,489	43,852
Cell dimensions (a, b, c) (Å)	52.0, 74.1, 71.9	51.9, 73.7, 71.9	52.1, 73.9, 71.7	75.2, 71.5, 103.3	97.8, 52.0, 89.1
(α, β, γ)	α = γ = 90°, β = 97.8°	α = γ = 90°, β = 97.7°	α = γ = 90°, β = 98.0°	α = γ = 90°, β = 90.1°	α = γ = 90°, β = 98.9°
Resolution (Å)	50–1.68	50–1.80	30–1.04	30–1.40	50–1.70
Highest resolution shell (Å)	1.74–1.68	1.86–1.80	1.08–1.04	1.45–1.40	1.76–1.70
Completeness ^a (%)	96.9 (97.8)	97.6 (94.6)	89.5 (50.9)	97.1 (94.7)	89.9 (62.3)
Average <i>I</i> /σ ^a	26.1 (2.6)	19.8 (2.3)	12.2 (2.15)	25.1 (1.8)	19.1 (2.4)
<i>R</i> _{merge} ^{a,b}	0.055 (0.427)	0.081 (0.454)	0.078 (0.399)	0.057 (0.551)	0.057 (0.280)
Refinement					
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>C</i> 2
No. of molecules ^c	1	1	1	2	1
No. of protein atoms ^c	4236		4341	8504	3991
No. of solvent atoms ^c	504		625	1156	168
Resolution range (Å)	30–1.68		25–1.04	25–1.40	30–1.70
Crystallographic <i>R</i> -factor ^d	0.177		0.152	0.170	0.201
<i>R</i> -free	0.208		0.166	0.200	0.235
Average <i>B</i> -factors (Å ²)					
Protein atoms (main chain)	14.3		10.2	18.7	25.3
Solvent	21.5		19.2	29.6	31.6
Root mean square deviations					
Bond lengths (Å)	0.013		0.006	0.010	0.017
Bond angles (°)	1.22		1.07	1.21	1.63

^a Values in parentheses indicate statistics for the highest resolution shell.

^b $R_{\text{merge}} = \sum I < I > / \sum < I >$, where *I* is observed intensity, *<I>* is average intensities from multiple observations of symmetry-related reflections.

^c Per asymmetric unit.

^d $R\text{-factor} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where *F*_o and *F*_c are the observed and calculated structure amplitudes, respectively.

cocrystals diffracted well, and data with good statistics were collected to 1.40 Å (Table 2). The final model was solved by molecular replacement using the apo-form model and has a final crystallographic *R*-factor of 0.17 (*R*_{free} = 0.20). The final ternary complex model consists of residues 9–533, one Mg²⁺ ion, either pyruvate or oxalate, and one molecule of acetyl-CoA for each protein chain, and 1156 water molecules were placed. Alignment of the A and B molecules in the asymmetric unit shows them to be essentially identical with a rmsd of 0.05 Å. A single residue, Glu119 in both monomers, was in a generously allowed region of the Ramachandran diagram, and no residues are in the disallowed regions. However, the unusual conformation of Glu119 is supported by clear electron density.

Overview of ecMSA structure

E. coli malate synthase A (ecMSA) is fashioned from an N-terminal α-helical clasp (Domain I, residues 5–60), which interfaces with an α8/β8 TIM barrel (Domain II, residues 92–383) and a C-terminal α-helical plug (Domain III, residues 414–533), as shown in Figure 1A. Domains I and II are linked by a loop region (residues 61–91) of approximately the diameter of the protein, covering the “bottom” of the TIM barrel. Residues 384–413

form a similar strand that extends the diameter of the protein to connect Domains II and III.

Active site of apo ecMSA

The active site of apo ecMSA has two channels leading to the solvent, in an approximately perpendicular orientation. Residues 439–446 in the C-terminal plug form a strand-β-turn-strand, within which residues 443–445 interface with residues 332–334 in the TIM barrel to separate the channel openings. The active site contains a large peak in the electron density map that was initially modeled as an octahedrally coordinated magnesium atom ligated by four waters and the carboxylates of residues Glu250 and Asp278. However, significant positive peaks in the (*F*_o – *F*_c) difference electron density map and low *B*-factors (6.28 Å² for Mg²⁺ vs. 14.3 Å² main chain average) at the metal site suggested that instead a calcium ion, which is present in the mother liquor, is bound. After redefinition of the atom type, the *B*-factor for Ca²⁺ refined to the more reasonable value of 13.4 Å². In addition, in initial SIRAS phased maps the metal site had an electron density of 14.5 σ, similar to nearby sulfurs (12.3 σ), whereas the metal ligands had densities

Figure 1. Ternary complex of ecMSA, acetyl-CoA, and inhibitor. (A) Stereo cartoon image of overall structure. TIM barrel (green); C-terminal plug (red); N-terminal clasp (blue). CoA fragment (lavender) and inhibitor (black) shown in ball-and-stick with magnesium as orange sphere. (B) Stereo image of active site inhibitor with omit $F_o - F_c$ density contoured at 3σ for the inhibitor, magnesium, and adjacent residues. Black dashes represent hydrogen bonds and red dashes show the presumptive steric clash with pyruvate or an acceptable $2.7 \text{ \AA}$ hydrogen bond to modeled oxalate. Based on structural comparisons with MSG, the blue dashes represent a hypothetical interaction of Asp447 and the terminal methyl group of acetyl-CoA. (C) Stereo image of the CoA binding site with omit $F_o - F_c$ density shown at 2σ for CoA and Cys438. The electron density corresponding to the panthothenic acid moiety is too weak to permit a satisfactory model thereof to be constructed. The presumed water molecules at the top, shown interacting with D447, represent the hypothetical location of the terminal acetyl group.

ranging from 3.4 to 6.8σ . Finally, the average of the metal-ligand distances is $2.33 \pm 0.16 \text{ \AA}$, which is more consistent with calcium (2.36 – $2.39 \text{ \AA}$) than magnesium ($2.07 \text{ \AA}$) (Harding 2006).

To test this hypothesis, crystals were transferred to artificial mother liquor in which Ca^{2+} was replaced by Mg^{2+} , and synchrotron diffraction data were collected to $1.04 \text{ \AA}$ resolution (Table 2). The final atomic model consists of residues 5 – 533 , 4 Mg^{2+} ions, and 641 water molecules. An anomalous scattering difference map exhibited no significant peaks at the presumed metal site, indicating that calcium is not present in the magnesium-substituted crystals (data not shown). The divalent metal B -factor refined to $7.3 \text{ \AA}^2$, compared with $10.2 \text{ \AA}^2$ for the main chain. The final $F_o - F_c$ difference electron density exhibits no significant features near the site. The average metal-ligand coordination distance is $2.08 \pm 0.03 \text{ \AA}$, appropriate for Mg^{2+} (Harding 2006). The remainder of the model is essentially identical to the model obtained for crystals grown from solutions containing Ca^{2+} (rmsd $0.09 \text{ \AA}$), except for residues 335 – 343 . These form a flexible loop with B -factors that are much higher than the average for both structures. In addition, many side chains showed lowered occupancy for carboxylate groups and sulfur atoms, presumably due to radiation damage.

Active site of ecMSA ternary complex

The active site of ecMSA is buried between the TIM barrel and the C-terminal plug. The electron density map was initially interpreted as containing a molecule of pyruvate (Fig. 1B, see also Discussion). Although pyruvate was included in the crystallization mixture, the electron density map clearly shows that the bound species is not planar as expected. The dihedral angle defined by the oxygen atoms coordinated to Mg^{2+} and the intervening carbon atoms refines to a value of 27° . The bound species makes hydrogen bonds to the backbone amides of Trp277, Asp278, and Mg^{2+} , the guanidinium group of Arg166, and three water molecules (Figs. 1B and 2). Of particular interest are the two interactions from bound water molecules to the group that was interpreted to be the methyl carbon of pyruvate, which is out of the molecular plane. One interaction is typical of a hydrogen bond ($2.74 \text{ \AA}$) while the other ($3.10 \text{ \AA}$) is typical of a van der Waals contact. Considering the deviation from planarity and that either interaction would be favorable for an oxygen atom, we interpret the bound species to be oxalate rather than pyruvate. Furthermore, considering the hydrogen bond pattern, oxalate would most likely be bound as the monoanion rather than the dianion (see Discussion).

Acetyl-CoA binding pocket

Acetyl-coenzyme A (acetyl-CoA) is clearly bound to the enzyme, as there is strong, well-resolved electron density for the adenine ring and ribose. However, density is weaker for the phosphate groups (see Fig. 1C). The

Met445 and Gly443 through the contacts with residues 330–338, thus widening the entrance to the acetyl binding site. Asp447, the proposed catalytic base, is located at the end of the strand- β -turn-strand and moves about 1 Å away from the electrophilic substrate, possibly to make room for the incoming acetyl group.

Comparison of ecMSG and ecMSA structures

Overall fold

As expected, the overall fold of MSA is similar to that described previously for MSG. Structural alignment of the ecMSG (PDB entry 1D8C) and apo ecMSA structures using DALI results in 486 α -carbon positions aligned with an rmsd of 2.4 Å. The difference in size between MSG and MSA can be attributed largely to the appended α/β domain, comprising residues 135–262 and 296–333 in ecMSG. Residues 135–252 in MSG can be thought of as an insertion between residues 109–110 in MSA and, likewise, residues 296–333 form an insertion between residues 158 and 159 in MSA (Fig. 4A). No function has yet been ascribed to the α/β -domain of MSG, yet there are highly conserved positions within the family that suggest a conserved binding pocket (Fig. 4A, see also Discussion). Finally, the N-terminal helical clasp in MSA is shorter by ~25 residues, which in MSG forms two short helices.

Catalytic site comparison

The active sites of the ternary structures of ecMSA and ecMSG are very similar, with identical catalytic groups, in identical conformations. The catalytic bases are Asp447 and Asp631 for ecMSA and ecMSG, respectively, while the corresponding acid groups are Arg166 and Arg338 for ecMSG and ecMSG, respectively (Fig. 4B). Not surprisingly, the catalytic Mg^{++} is bound by identical residues in both isoforms.

Alignment of 16 active site α -carbons yields an rmsd of 0.32 Å. Residues Cys274, Gly275, Arg276, and Trp277 in ecMSA (corresponding to Thr451, Gly452, Phe453, and Leu454 in ecMSG) are proposed to form a gate into the active site in either case (Fig. 3). These residues are highly conserved within each isoform family even though they are different between the isoforms. Interestingly, the active sites of the two isoforms also contain many bound water molecules with conserved positions, as shown in Figure 4B.

Acetyl-CoA binding pockets

A common theme in crystal structures of the G and A isoforms, regardless of biological origin, is that CoA derivatives appear to be partially disordered and/or only fractionally bound (Anstrom et al. 2003; Smith et al.

Figure 4. (A) Stereo ribbon diagram showing potential allosteric regulatory binding site on MSG. Sequence homology scores were threaded on to the ecMSG (1P7T, chain A) α/β domain (residues 134–263 and 314–331), with homology scores from high to low represented by red, orange, yellow, green, teal, and blue segments. Bound acetyl-CoA is shown as a ball-and-stick model. The proximity of AcCoA with highly conserved residues 134–146 suggests a regulatory binding site. Homology scores computed with the Scoresons program (Valdar 2002) based on alignment of 57 annotated MSG sequences from the Swiss-Prot database (Boeckmann et al. 2003). ArchPRED (Fernandez-Fuentes et al. 2006) was used to construct the model residues 151–159 as they were missing from the 1P7T model. (B) Stereo overlay showing active site alignment of ecMSA (black) and ecMSG (magenta) with bound inhibitors. Black dashes represent hydrogen bonds between atoms in ecMSA. The isolated spheres represent presumed water molecules (ecMSA, blue; ecMSG, red), or magnesium (ecMSG, green; ecMSA, orange).

2003; Anstrom and Remington 2006). Acetyl-CoA as determined in the ecMSG model from Anstrom et al. (2003) exhibits *B*-factors higher than the surrounding residues for the panthothenic acid moiety. Similarly, the panthothenic acid moiety of CoA bound in mTB MSG had high *B*-factors in the models of Smith et al. (2003) whereas in the mTB MSG model of Anstrom et al. (2003) the CoA occupancy is low. In our model of the ecMSA ternary complex, density could not be observed for the panthothenic acid moiety (Fig. 1C). Thus, comparison of the CoA binding modes to the two isoforms can only be made for the adenine, ribose, and phosphate moieties.

The defined portion of acetyl-CoA, as modeled in the ecMSA structure, is bound in a very similar manner as seen in ecMSG. An alignment between ecMSA and ecMSG of nine α -carbons in the acetyl-CoA binding site

has an rmsd of 0.57 Å. The adenine ring sits on top of a proline in both MS, which caps a TIM barrel α -helix in both isoforms, and is Pro369 and Pro538 for ecMSA and ecMSG, respectively. For isoform A, a conserved methionine at position 102 lies above the ring, whereas in ecMSG the residue is Tyr126. The N6 position of the adenine ring is bound by backbone carbonyls in both isoforms. In both isoforms a conserved asparagine, Asn105 in ecMSA, makes a hydrogen bond to the ribose hydroxyl. For MSG the 3'-phosphate is ligated by two arginines, whereas in MSA these residues are a lysine and tyrosine. The 5'-phosphate is bound by a histidine in MSA, whereas in MSG the diphosphate interacts with the tyrosine capping the adenine ring.

An interesting feature of the CoA binding pocket concerns conserved Cys438 of ecMSA (Cys617 in ecMSG), which lines the pocket and presumably interacts with the panthothenic acid moiety. This Cys has been implicated to have catalytic and/or regulatory functions, and in both cases, the sulfur atom is found to be oxidized to the sulfenic acid form (Anstrom et al. 2003).

Discussion

Given that well-diffracting crystals of ecMSG and mTB MSG are difficult to obtain and tend to diffract to moderate resolution, we investigated the possibility that other isoforms of malate synthase might be more amenable to structural and/or drug discovery studies. *E. coli* MSA was found to crystallize readily and the crystals to diffract to extremely high resolution. Furthermore, these crystal forms facilitated crystal structure determinations in both "open" and "closed" enzyme conformations. The "open" conformation revealed two openings to the active site and suggests how glyoxylate, inhibitors, and malate might enter and exit. Inhibitor binding results in a "closed" conformation, in which one remaining opening allows entry of acetyl-CoA. The closed conformation for ecMSA, particularly in the active site region, is extremely similar to that observed for MSG. In the following, we examine in greater depth several details of interest.

Proposed oxalate binding

In the active site of the ecMSA ternary structure, the electron density map has a clear feature with the shape expected for a C_2 compound. We suggest that this molecule is oxalate bound as the monoanion, rather than pyruvate as included in the mother liquor, based on the deviation from planarity and the details of the hydrogen bonding pattern. There is evidence that oxalate is produced as solutions of polyethylene glycol age (Uekawa et al. 2000; Kryuk et al. 2002; Ellis 2006), consistent with the inadvertent use of a PEG solution that had been made

up two years previously for crystal setups. ecMSA binds oxalate (K_i 37 ± 4 μ M) much more tightly than pyruvate (K_i 648 ± 89 μ M), which is similar to the results of previous inhibition studies conducted with *Saccharomyces cerevisiae* MS [K_i for oxalate and pyruvate is 19 μ M and 540 μ M, respectively (Durchschlag et al. 1981)]. It has previously been shown that the rate of the enolization reaction (proton abstraction) catalyzed by MS is strongly dependent on the nature and presence of 2-keto acids such as pyruvate (Eggerer and Klette 1967; Durchschlag et al. 1981). In particular, oxalate does not stimulate the enolization reaction, giving rise to the idea that oxalate prevents the acetyl group from binding. In another study, it was shown for *Zea mays* malate synthase (an A isoform) that the affinity for acetyl-CoA is increased almost 50-fold when pyruvate is bound in the active site (Beeckmans et al. 1994). These data were interpreted to mean that binding of an appropriate 2-keto acid compound is essential for productive binding of acetyl-CoA.

The results of the present structural study are consistent with these observations and provide a molecular basis to explain this behavior. In the ternary complex, the electron density map indicates that the acetyl group of bound acetyl-CoA is disordered. If oxalate, instead of pyruvate, is bound as we have assumed, the water molecules associated with the oxalate OH would interfere with productive binding of the acetyl group of acetyl-CoA. Furthermore, the conformation of bound oxalate is subtly different from planar compounds such as pyruvate, which would presumably influence the surrounding active site configuration.

Weak acetyl-CoA binding

A theme common to all MS structures analyzed to date is the observed low occupancy and disorder in bound CoA. Two ternary product complex structures of mTB MSG have been described. In the first report (Smith et al. 2003), the *B*-factors for CoA are high, and CoA is observed to be bound to only one of the two protomers in the asymmetric unit. The second report (Anstrom and Remington 2006), conducted with crystals similar but not identical to those of the first study, revealed similar binding behavior. Density was apparent for CoA in only one of the two protomers, and the ligand was modeled at 70% occupancy. The nucleotide portion of the CoA binding site is surface exposed; hence, it has been suggested that crystal contacts can inhibit binding of CoA. In only one instance has acetyl-CoA binding with full occupancy been observed, and this was for the ecMSG abortive ternary complex containing pyruvate and acetyl-CoA (Anstrom et al. 2003). However, the *B*-factors reported for acetyl-CoA are higher than those observed for nearby protein atoms.

In this study, electron density for the panthothenic acid moiety of acetyl-CoA weak or absent, which we suggest is due to the presence of oxalate rather than a substrate (or pyruvate) in the active site. Taken together, these observations suggest that acetyl-CoA is bound productively only during a small portion of the catalytic cycle and that binding is critically dependent on subtle changes in the enzyme conformation. Presumably, maximal acetyl-CoA binding takes place only when a productive substrate (glyoxylate) is present in the active site, consistent with induced fit models for enzyme catalysis.

Conformational transitions and reaction order

As described earlier, much evidence suggests that the reaction sequence of MS is ordered and that binding of glyoxylate creates the binding site for acetyl-CoA. The transition between open and closed conformations of ecMSA is characterized by large loop movements that are undoubtedly instrumental to this process. Somewhat counterintuitively, in the “open” conformation the binding site for the panthothenic acid moiety is partially blocked, preventing entry of the acetyl group. However, the binding pocket for the phospho-adenosine moiety remains accessible, allowing acetyl-CoA to bind non-productively to the enzyme. Upon binding of glyoxylate, active site loops close down on the substrate, and this motion presumably coordinates the rearrangements necessary to open the active site cavity for entry of the reactive acetyl group.

In indirect support of this proposal, it was long ago observed that malyl-CoA, the presumed intermediate in the reaction, is a poor substrate for the enzyme (Eggerer and Klette 1967). Those authors interpreted the low reactivity to be due to the difficulty of deprotonating the 2-hydroxyl of malyl-CoA prior to binding (Lill et al. 1991; Howard et al. 2000). However, another explanation, consistent with the structural results, is that in the absence of a bound C₂ species, the entry passageway for the malyl portion is simply blocked.

Possible regulatory function of the α/β domain in MSG

The most obvious feature distinguishing the A and G isoforms of MS is the presence of an α/β domain, comprising residues 135–262 and 296–333 in ecMSG (see Fig. 4A), of unknown function. Although some side chains emanating from this domain are close in space to the (acetyl-) CoA binding site, none participate directly in binding of substrate or product. DALI searches using only the α/β domain do not return positive structural matches, indicating that the domain has a novel fold. The first 13 amino acids (ecMSG 134–146) of the domain are highly conserved among G isoforms and lie at the bottom of a cleft close to the CoA binding site. This conserved

structure suggests the presence of an extended regulatory binding site that may accommodate a feedback inhibitor. It is worth noting that the identification of a regulatory agent would greatly expand the range of possible lead compounds for the effort to discover new antimicrobial reagents.

In conclusion, we suggest that bacterial malate synthases, especially the A isoforms, form an outstanding platform for drug discovery studies.

Materials and Methods

Cloning, mutagenesis, overexpression, and purification

The gene for ecMSA was amplified from genomic DNA using primers containing restriction endonuclease cut sites. After digestion, the fragments were cloned into a modified pBAD expression vector containing a TEV protease site in place of the enterokinase cleavage site. The ecMSA C438S mutant was prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), and the presence of the mutation was verified by DNA sequencing. A synthetic gene coding for the baMSA gene was produced by Blue Heron Biotechnology and subcloned similarly to the ecMSA gene. The ecMSG C617S mutant was prepared as described previously (Anstrom et al. 2003).

The pBAD vector containing ecMSA or baMSA was transformed into *E. coli* TOP10 cells. These cells were cultured overnight in 100 mL of Luria-Bertani media supplemented with ampicillin (100 μ g/mL). This culture was used to inoculate 4 L of media in a fermenter at 37°C. Once the culture reached an A₆₀₀ of 0.6–0.8, 2 g of arabinose was added to start overexpression. After 4 h, the culture was harvested by centrifugation and disrupted by sonication with the addition of lysosyme and DNase, followed by centrifugation to remove insoluble material.

Purification was performed using a Ni-NTA column. The His-tag was removed using 0.15 mg of TEV protease per 20 mg of purified protein and checked for cleavage using MALDI/TOF MS. After complete cleavage, the protein was further purified using size exclusion or anion exchange chromatography. The protein was dialyzed into 50 mM Tris pH 8.0, 20 mM KCl, 5 mM MgCl₂, 1 mM TCEP, 0.1 mM EDTA, then quantified using the calculated ϵ_{280} 81.8 mM⁻¹ cm⁻¹ based on the method of Gill and Vonhippel (1989), and concentrated to ~20 mg/mL prior to crystallization.

Crystallization, derivatization, and diffraction data collection

Large, diffraction-quality crystals of apo ecMSA were readily obtained from many conditions by using the hanging-drop vapor diffusion technique. The optimized condition for apo ecMSA was 0.1 M Tris pH 7.5, 0.2 M calcium acetate, freshly prepared 22% PEG 8K (1:1, protein: reservoir solution, 2 μ L drops). A heavy atom derivative was prepared by soaking a crystal for one hour in HgCl₂ saturated mother liquor (Sun et al. 2002). Crystals were flash-frozen, and data were collected in-house on a Rigaku rotating anode generator with a Raxis 4 detector. To produce the Mg²⁺ bound ecMSA structure, crystals were placed in artificial mother liquor where Ca²⁺ was replaced by Mg²⁺ and stored for

7 d before data collection at the Advanced Light Source (Berkeley, CA), beamline 5.0.2 using the ADSC-Q315 detector at a wavelength of 0.90 Å and temperature of 100K. To obtain the ecMSA acetyl-CoA/inhibitor ternary complex, protein was mixed with 0.1 M acetyl-CoA and 0.2 M pyruvate, which was then again subjected to crystallization trials. The ternary complex crystals appeared only in mother liquor consisting of 0.1 M PIPES pH 6.5, 0.1 M ammonium sulfate, 27% PEG 8K (aqueous stock solution prepared two years earlier), and data were collected 3 mo after crystallization setup. Crystals of baMSA grew from 0.2 M sodium citrate, 0.1 M Tris pH 8.0, 30% PEG 1550 from which two crystal forms are found, small needle clusters, and large plates. Large plates of baMSA were used for data collection. It was later found that inclusion of 30–70 mM NiCl_2 in the crystallization buffer encouraged formation of the plates over the needles. Synchrotron radiation at ALS beamline 5.0.2 (wavelength 1.00 Å) was used for data collection on the ecMSA inhibitor/acetyl-CoA complex and for the baMSA crystal.

Data reduction and structure determination

The home source diffraction data for apo ecMSA were processed and scaled with the HKL suite (Otwinowski and Minor 1997). Initial phases were obtained and modified with SHELXC/D/E using the SIRAS method (Schneider and Sheldrick 2002; Sheldrick 2002). Five heavy atom sites were found. ARP/wARP was then used to build a partial model into the experimentally phased electron density map (Morris et al. 2003). Automatic model building resulted in a partial model consisting of 500 out of the 533 amino acids. The phases from ARP/wARP were extended to 1.68 Å using REFMAC 5 (Murshudov et al. 1997). The final model was completed manually by using COOT (Emsley and Cowtan 2004). Water molecules were placed using COOT with the combined criteria of a peak of greater than 2.5 σ in the ($F_o - F_c$) difference map, or 1.0 σ in the ($2F_o - F_c$) map and reasonable intermolecular interactions. Synchrotron data were processed using the HKL2000 suite (Otwinowski and Minor 1997). Molecular replacement, using MOLREP (Vagin and Teplyakov 1997), was performed to solve the structures of the acetyl-CoA/inhibitor ecMSA and baMSA structures using the apo ecMSA structure as a starting point. ARP/wARP was also used to rebuild the models of the acetyl-CoA/inhibitor-bound ecMSA and baMSA (Morris et al. 2003). The magnesium-bound ecMSA, acetyl-CoA/inhibitor-bound ecMSA, and baMSA structures were all refined using REFMAC 5 (Murshudov et al. 1997). The atomic coordinates and structure factors have been deposited in the Protein Data Bank and may be retrieved using ID codes 3CV1 (ecMSA Ca^{2+}), 3CUZ (ecMSA Mg^{2+}), 3CV2 (ecMSA inhibited), and 3CUX (baMSA).

Enzyme activity assay

The reaction of acetyl-CoA and glyoxylate catalyzed by ecMSA and baMSA was quantified by measuring the decrease in absorbance at 232 nm because of cleavage of the thioester bond of acetyl-CoA as described previously (Durchschlag, et al. 1981; Anstrom et al. 2003). The reaction conditions were 0.2 mM acetyl-CoA, 0.5 mM glyoxylate, 50 mM Tris pH 8.0, and 10 mM MgCl_2 . The reaction was initiated by the addition of enzyme solution. Kinetic parameters for substrates were determined using the above conditions with varying substrate concentrations. Least squares fits for kinetic parameters were obtained using the program Enzfitter (Biosoft). Inhibition constants were

obtained for parabanic acid ($\epsilon_{280} = 1.77 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, Aldrich) and 4-amido-2,3,4-pyrrolidenetrione, synthesized as described (Merchant et al. 1972). These compounds absorb in the vicinity of 232 nm, thus enzymatic assays monitored the appearance of free CoA via its reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and the concomitant rise in absorbance at 412 nm (Nakata and Selitrennikoff 2002). Cys438, a conserved residue in the active site of MS, could potentially react with DTNB and block catalysis, so this residue was mutated to serine prior to kinetic analyses. The assay conditions were similar to above with a 1 mM concentration of DTNB.

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