The product complex of *M. tuberculosis* malate synthase revisited

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Abstract

Enzymes of the glyoxylate shunt have been implicated as virulence factors in several pathogenic organisms, notably *Mycobacterium tuberculosis* and *Candida albicans*. Malate synthase has thus emerged as a promising target for design of anti-microbial agents. For this effort, it is essential to have reliable models for enzyme:substrate complexes. A 2.7 Å resolution crystal structure for *M. tuberculosis* malate synthase in the ternary complex with magnesium, malate, and coenzyme A has been previously described. However, some unusual aspects of malate and Mg^{++} binding prompted an independent determination of the structure at 2.3 Å resolution, in the presence of saturating concentrations of malate. The electron density map of the complex reveals the position and conformation of coenzyme A to be unchanged from that found in the previous study. However, the coordination of Mg^{++} and orientation of bound malate within the active site are different. The revised position of bound malate is consistent with a reaction mechanism that does not require reorientation of the electrophilic substrate during the catalytic cycle, while the revised Mg^{++} coordination is octahedral, as expected. The results should be useful in the design of malate synthase inhibitors.

Keywords: malate synthase; protein crystallography; mycobacterium tuberculosis; drug design; ternary complex

The glyoxylate shunt enzymes isocitrate lyase and malate synthase have been implicated as virulence or persistence factors in several different pathogens. In particular for *Mycobacterium tuberculosis* (mTB) (Graham and Clark-Curtiss 1999; Höner zu Bentrup et al. 1999; Dubnau et al. 2002), it has been reported that genes encoding one or both of the enzymes are up-regulated in response to phagocytosis. An isocitrate lyase knockout of mTB shows severely attenuated virulence (McKinney et al. 2000), suggesting that the activity may be important for survival in the environment within the phagolysosome. Also sug-

gestive of the importance of this pathway is a recent study reporting that antibodies to malate synthase were found in 90% of patients during incipient subclinical tuberculosis (Singh et al. 2005). As the genes encoding glyoxylate cycle enzymes are not found in mammals, both malate synthase and isocitrate lyase have become attractive targets for the design of anti-microbial agents (Smith et al. 2004). Reliable structures of enzyme:substrate and enzyme:product complexes would clearly be helpful for such design efforts.

A good deal of structural information exists for malate synthase isoform G (MSG), which is a single-chain 80-kDa enzyme. Crystal structures have been reported for substrate, inhibitor, and product complexes with enzymes from *Escherichia coli* (Howard et al. 2000; Anstrom et al. 2003) and mTB (Smith et al. 2003), and an NMR solution structure of *E. coli* MSG is also available (Tugarinov et al. 2005). The overall fold is comprised of a central TIM barrel

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Abbreviations: RMSD, root-mean-square deviation; CoA, coenzyme A. Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.062300206>.

with an N-terminal α -helical clasp along one side, an α/β domain of unknown function formed from two insertions into the TIM barrel along the other, and a C-terminal five-helix domain that caps the active site.

The structures provided a preliminary basis for rationalization of the reaction mechanism, which is briefly as follows: Conserved Asp 633 acts as the catalytic base, abstracting a proton from the terminal methyl group of acetyl-CoA. The resulting enolate is stabilized by the positive charge of conserved Arg 339. Glyoxylate is polarized for nucleophilic attack by the catalytically required magnesium ion, and the resulting malyl-CoA intermediate is stabilized by magnesium and Arg 339. This intermediate is then hydrolyzed on the enzyme, presumably by an activated water molecule, and the products released. (For details, see Howard et al. 2000; Anstrom et al. 2003; Smith et al. 2003; and references therein.)

Smith et al. (2003) described the product complex of Mg^{++} , malate, and coenzyme A, determined at 2.7 Å resolution. In this structure, malate is modeled such that its two carboxylic acid groups coordinate the magnesium ion, which in turn has only five ligands, including one water molecule. However, glyoxylate-bound malate synthase structures reveal a second bound water molecule adjacent to the magnesium ion, resulting in the more usual octahedral coordination (Markham et al. 2002). Perhaps to reconcile these observations, Smith et al. proposed that this second water molecule is activated toward hydrolysis of the malyl-CoA intermediate by Glu 273 and/or Asp 274. Hydrolysis might then result in a pentacoordinate magnesium ion as modeled in their products-bound structure. However, a comparison of the proposed orientation of bound malate and those observed in the *E. coli* enzyme for glyoxylate (determined at 2.0 Å resolution) or for the competitive inhibitor pyruvate (determined at 1.95 Å resolution), suggest that the enzymatic activity would require reorientation of the conserved portions of the substrate during the catalytic cycle (Anstrom et al. 2003).

A product complex has not been reported for *E. coli* MSG. Although the active sites of MSG from *E. coli* and mTB are essentially identical, the overall amino acid sequence identity is ~56%. Thus the possibility remains that the enzymes are functionally distinct and, therefore, might not be strictly comparable. In order to reinvigorate discussion on these points, we redetermined the structure of mTB MSG in complex with magnesium and the products malate and coenzyme A at 2.3 Å resolution. In the revised structure, the coordination of Mg^{++} has the expected octahedral configuration, and the position of bound malate differs from that previously reported by Smith et al. (2003). However, the new orientation of bound malate is consistent with observations based on the

structures of substrate and inhibitor complexes of either enzyme and is consistent with a mechanism that does not require motion of conserved portions of the substrate/product during the catalytic cycle.

Results and Discussion

Overall structure

MSG crystallized from a solution containing 0.25 M malate in space group $P4_12_12$ with unit cell dimensions of $a = b = 120.4$ Å, $c = 238.9$ Å, with two protomers in the asymmetric unit. The final model includes two molecules of malate, one molecule of coenzyme A, four magnesium ions, two molecules of HEPES, and 617 water molecules. The crystallographic R-factor is 17.4% for all data, and the model has acceptable stereochemistry. Data collection and final refinement statistics are presented in Tables 1 and 2, respectively.

This crystal is very similar to that reported by Smith et al. (2003), which had space group $P4_12_12$ and unit cell dimensions $a = 120.8$ and $c = 232.8$ Å. The contents of the asymmetric unit are similar, and in each case the two protomers form a dimer related by an approximate non-crystallographic twofold axis. The choice of asymmetric unit in our case is related to that chosen by Smith et al. (2003) by a crystallographic twofold rotation along the Z-axis. Nevertheless, because of differences in unit cell dimensions (particularly the difference of ~6 Å in the length of the crystallographic C axis) and possibly also differences in the crystallization medium, the crystal packing is different in many details.

The overall fold of malate synthase is as described previously (Howard et al. 2000; Anstrom et al. 2003; Smith et al. 2003; Tugarinov et al. 2005). For the A molecule, electron density permitted residues 2–71, 75–585, and 589–727 to be modeled but was weak for residues 72–74 and 586–588; four side chains were similarly truncated due to weak electron density. In the B molecule residues 2–150, 154–302, and 311–726 were

Table 1. Data collection statistics

Total reflections	399,684
Unique reflections	78,420
Space group	$P4_12_12$
Cell dimensions (Å)	120.4, 120.4, 238.9
Resolution (Å)	49.09–2.30
Highest resolution shell (Å)	2.38–2.30
Completeness (%)	99.6 (98.6)
Multiplicity	5.3 (3.1)
Average I/ σ	9.7 (2.9)
R _{merge}	0.090 (0.350)

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

Table 2. *Refinement statistics*

Resolution (Å)	49.09–2.30
No. of protein molecules ^a	2
No. of protein atoms ^a	11,016
No. of water molecules ^a	617
No. of heteroatoms ^{a,b}	100
Crystallographic $R_{\text{factor}}/R_{\text{free}}^c$	17.8/24.4
RMSD bond length (Å)	0.023
RMSD bond angle (°)	1.986
Average B-factors (Å ²)	40.3
Protein	40.1
Malate	38.7
Coenzyme A	41.7

^aPer asymmetric unit.^bFour magnesium atoms, two molecules malate, two molecules HEPES, one molecule coenzyme A.^c $R = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$. Ten percent (10%) of the data was reserved for calculation of R_{free} . In the deposited coordinates, all reflections were used in the final cycles of crystallographic refinement, with $R_{\text{factor}} = 17.4\%$.

modeled with residues 151–153 and 303–310 omitted as well as eight side chains due to poor electron density. As defined by PROCHECK (Laskowski et al. 1993), there are no residues with disallowed backbone conformational angles and only six (Lys 207 A, Glu 273 A, Asp 29 B, Asp 206 B, Ser 264 B, and Glu 586 B) in “generously allowed” regions of the ϕ/ψ diagram. RMS deviations of α -carbon coordinates are ~ 0.51 Å for protomers A and B within the asymmetric unit and 0.44 Å for each protomer, when compared with the model deposited by Smith et al. (2003; PDB ID 1N8W).

Binding of coenzyme A

Electron density for bound coenzyme A (CoA) is clear in molecule A, although the electron density adjacent to the terminal sulfur is weak. When CoA was modeled at 100% occupancy, significant negative peaks appeared in the $F_o - F_c$ difference electron density map at the phosphate positions. These peaks were eliminated when the occupancy was set at 70%, so the occupancy was fixed at this value. There was no electron density for CoA in molecule B, so it was presumed absent. The CoA binding site, as described previously (Anstrom et al. 2003; Smith et al. 2003), is a pocket that is ~ 15 Å deep but quite narrow, located between the TIM barrel and the C-terminal plug domains. The adenine ring binds to a hydrophobic patch, wedged between TIM barrel residues Phe 126 and Pro 545. The phosphate groups are partially exposed to solvent, and there is a bend in the molecule such that an intramolecular hydrogen bond forms between the N7 of the adenine ring and a hydroxyl group on the pantethenic acid portion.

Compared to previous reports (Anstrom et al. 2003; Smith et al. 2003), there is some disorder in the config-

uration of the final two carbon atoms and the sulfur of bound CoA. The thiol portion is in contact with Met 631; however, negative features in a difference electron density map also suggested positional variability of the Met 631 side chain. Consequently, the three terminal atoms of Met 631 were modeled at 50% occupancy. This segment of the atomic model should be regarded as tentative.

It is not entirely clear why Coenzyme A appears to be bound to only one molecule of malate synthase in the asymmetric unit of the crystal. However, Smith et al. (2003) also reported only one molecule of bound coenzyme A. A possible explanation for this behavior is related to flexibility observed for a loop comprised of residues 300–312, which in turn forms part of the CoA binding site. In the malate synthase structures, the 300–312 loop has some variability in conformation and the number of residues that can be modeled, depending on crystal packing and the presence or absence of substrates and products. In the present crystal, the noncrystallographic twofold axis relating the two protomers in the asymmetric unit places the coenzyme A binding pockets near each other at a crystal packing interface. In the A subunit containing bound CoA, the 300–312 loop is well ordered. In molecule B, this loop is partly disordered; however, those residues that are visible appear to form an intermolecular β -sheet with the corresponding residues in molecule A. In other crystal structures of malate synthase (see, for example, Anstrom et al. 2003), as well as NMR studies (Tugarinov and Kay 2003; Tugarinov et al. 2005), the ordering of the 300–312 loop seems to be correlated with the presence of bound coenzyme A or acetyl-CoA, possibly due to formation of a salt bridge between the 3' phosphate group and Lys 305 and/or Arg 312. Thus, it is reasonable to assume that there is an allosteric interaction between the 300–312 loop and the coenzyme A binding pocket and, hence, that crystal packing interactions involving this loop could control the substrate binding affinity of individual subunits within the asymmetric unit.

Malate binding and magnesium coordination

Both subunits of the asymmetric unit show strong, well-resolved electron density for a feature that we have modeled as one magnesium ion, two water molecules, and one molecule of malate (Fig. 1A). The conformation of bound malate does not appear to differ between the subunits. It forms hydrogen bonds to the backbone of Asp 462 and Leu 461, as well as two hydrogen bonds with the side chain of Arg 339. Depending on the charge states, there may be an electrostatic clash between the side chain of Asp 633 and the 4-carboxylic acid of malate. These interactions are very similar to those observed in glyoxylate- and acetyl-CoA and pyruvate-bound structures of

Figure 1. (A) Stereo view of a ball-and-stick model of the active site of *tbMSG* superimposed on a portion of a σ_A -weighted $F_o - F_c$ omit electron density map contoured at 3.5σ . Malate, magnesium, and the two water molecules coordinating magnesium were omitted for the purpose of phase calculation. (B) Stereo view showing magnesium coordination and position of pyruvate and the acetyl terminus of acetyl-CoA as reported by Anstrom et al. (2003; PDB ID 1P7T). (C) Stereo image of magnesium coordination and malate position as reported by Smith et al. (2003; PDB ID 1N8W). In all figures relevant hydrogen bonds and salt bridges are shown as black dashed lines, while potential electrostatic clashes are shown as red dashed lines. In A and C, numbering is for mTB malate synthase, whereas in B, numbering is that of *E. coli* malate synthase G.

malate synthase (Howard et al. 2000; Anstrom et al. 2003; Smith et al. 2003) as shown (Fig. 1B).

Malate provides two coordinating groups to the catalytic magnesium ion, one of which is an oxygen atom from the 1-carboxylic acid group, while the 2-hydroxyl group provides a second, presumably deprotonated oxygen ligand. Malate thus likely exists in the active site as a trianion, stabilized by the +2 charge on magnesium as well as the positive charge on Arg 339. Arg 339 is in turn positioned to make charged hydrogen bonds with both the 2-hydroxyl oxygen and a 4-carboxylic acid oxygen (Fig. 1A). It was earlier predicted that malate would coordinate the active site magnesium using the 2-hydroxyl group (Howard et al. 2000). The requirement to deprotonate the 2-hydroxyl prior to binding to Mg^{++} may explain the fact that malyl-CoA, the presumed reaction intermediate, is a poor substrate of the enzyme (Eggerer and Klette 1967). Finally, the catalytic Mg^{++} is octahedrally coordinated by Glu 434, Asp 462, and two water molecules in addition to two malate ligands, as observed in most other complexes.

The coordination of Mg^{++} and the binding of malate reported here differ significantly from the results reported by Smith et al. (cf. Fig. 1C [this paper] and Fig. 3 in Smith et al. 2003). In that report the ion is coordinated by five oxygen atoms in a distorted square pyramid. One oxygen atom is provided by each of Glu 434 and Asp 462, one from a water molecule and two from malate. This proposal is inconsistent with the octahedral coordination observed for Mg^{++} in all other structures of malate synthase, but is also inconsistent with the overwhelmingly preferred coordination for Mg^{++} as seen in the Cambridge Structural Database (Markham et al. 2002). As previously pointed out (Anstrom et al. 2003), the reported conformation of bound malate would require reorientation of the substrate with respect to the catalytic Mg^{++} at some point during the catalytic cycle, which seems unlikely. Finally, in the previous model, bound malate exhibits highly unfavorable contacts with Asp 633 and Asp 274.

In an early report, malate was characterized as a weak, competitive inhibitor of malate synthase (Dixon et al. 1960). Later, quantitative inhibition constants were reported for 2-carbon acids such as fluoroacetic acid, glycolic acid, and L-malic acid against malate synthases from yeast and *E. coli*. The K_i s are similar and were reported to be in the vicinity of 300 μ M (Eggerer and Klette 1967; Zollner 1989). The crystal used by Smith et al. (2003) for data collection was prepared from a solution originally containing 2 mM acetyl-CoA and 2 mM glyoxylate. In the presence of enzyme, this reaction mixture will produce malate; however, the final concentration may not have been sufficient to guarantee saturation of the active site with malate. This is particularly a concern when one considers that malate binding must compete with residual glyoxylate, the K_m for which is

20–100 μ M depending on the enzyme source. It deserves mention that neither reported glyoxylate-bound structure of malate synthase had included glyoxylate in the crystallization conditions (Howard et al. 2000; Smith et al. 2003). In both cases, it was assumed that glyoxylate was scavenged from the growth medium, which underscores the high affinity of the enzyme for this substrate.

Thus, it seems reasonable to assume that the density observed by Smith et al. (2003) in the active site may have corresponded to a mixture of partially occupied species, leading to an unreliable interpretation. In our study, the final concentration of malate in the crystallization setup was 0.25 M, $\sim$ 1000-fold in excess of the K_i , and thus, the active site should be fully occupied. Taking into consideration the increased resolution of the diffraction data and the excellent refinement statistics, we suggest that our revised model for the binding of malate and the coordination of the catalytic Mg^{++} in the active site of *M. tuberculosis* malate synthase are sufficiently reliable to be of use in inhibitor design studies.

Materials and methods

Malate synthase cloning, overexpression, and purification

A pET23b plasmid encoding the gene for *Mycobacterium tuberculosis* malate synthase GlcB with a noncleavable C-terminal 6x His tag was obtained from John Belisle (Colorado State University). This construct crystallizes poorly (data not shown), and as residues C-terminal to Glu 727 were disordered in previously determined crystal structures (Smith et al. 2003), the gene segment coding for residues 1–727 was inserted into a pBH4 plasmid, together with an N-terminal TEV protease-cleavable 6x His tag. This construct was verified by DNA sequencing and used to transform a JM-109(DE3) cell line. Cells were grown in 4 L of SLBH media containing 0.1 mg/mL ampicillin at 37°C. Malate synthase expression was induced at OD₆₀₀ 1.2 with 50 μ g/mL IPTG, and the temperature was decreased to 20°C. Cultures were harvested by centrifugation 15 h post-induction and resuspended in lysis buffer (10% glycerol; 0.3 M NaCl; 50 mM HEPES at pH 7.9; 10 mM $MgCl_2$; 2 mM β -mercaptoethanol), with EDTA-Free Complete Mini protease inhibitor tablets (Roche) as per the manufacturer's instructions. The cells were disrupted by a single pass through a French press. Cellular debris was pelleted, and the supernatant was loaded onto a 8 mL Ni-NTA column (Qiagen) pre-equilibrated with lysis buffer, washed with 20 mL lysis buffer and then 7 mL 20 mM imidazole, 0.3 M NaCl, 50 mM HEPES (pH 7.9), 10 mM $MgCl_2$, and 2 mM β -mercaptoethanol, and eluted with 20 mL 100 mM imidazole, 0.3 M NaCl, 50 mM HEPES (pH 7.9), 10 mM $MgCl_2$, and 2 mM β -mercaptoethanol. Protein was treated with TEV protease for 3 h at room temperature with cleavage verified by MALDI-TOF mass spectrometry. Treated enzyme was then run over an 8 mL Ni-NTA column pre-equilibrated with 0.1 M NaCl, 0.1 M HEPES (pH 7.9), 10 mM $MgCl_2$, and 2 mM β -mercaptoethanol. Flow-through was dialyzed against 1 L of 0.1 M NaCl, 0.1 M HEPES (pH 7.9), 10 mM $MgCl_2$, and 2 mM DTT at 4°C with two changes. Malate synthase with the His tag removed was

concentrated to 30 mg/mL and then further purified by FPLC using a HiLoad 16/60 Superdex 200 gel filtration column (Amersham Biosciences). Activity was assayed as described previously (Anstrom et al. 2003).

Crystallization and data collection

Crystals were grown at room temperature in hanging drops containing 1 μ L of protein solution (22.1 mg/mL malate synthase, 0.5 M L-malate, 40 mM coenzyme A, 44 mM NaCl, 44 mM HEPES at pH 7.9, 4.4 mM MgCl₂, and 0.9 mM DTT) and 1 μ L well solution (23% PEG-8000, 14% MPD, 0.2 M Mg acetate, 0.1 M HEPES at pH 7.5). Crystals grew to a final size of $\sim 0.07 \times 0.15 \times 0.6$ mm after 5 d, which were then swept through cryoprotectant containing 20% PEG-8000, 18% MPD, 0.2 M Mg acetate and 0.1 M HEPES (pH 7.5) and then flash-frozen in a cryostream. A single crystal was used to collect data at beamline 8.2.1 at the Advanced Light Source in Berkeley, California.

Data reduction and structure refinement

Images were integrated using the HKL2000 suite (Otwinowski and Minor 1997). Molecular replacement solutions were found by using EPMR (Kissinger et al. 1999), using the glyoxylate-bound structure of *M. tuberculosis* malate synthase (PDB ID 1N8I; Smith et al. 2003) as a search model. Model building was done using O (Jones et al. 1991) and Xtalview (McRee 1999), while refinement was initially performed using TNT (Tronrud et al. 1987), but later refinement was done using Refmac5 (Murshudov et al. 1997) to utilize TLS parameterization (Winn et al. 2001). Simulated annealing was performed using CNS (Brünger et al. 1998). Solvent molecules were added where evidenced by positive difference density peaks with appropriate distances to hydrogen bonding partners.

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