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A functionally active dimer of *Mycobacterium tuberculosis* Malate synthase G

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Abstract Malate synthase G is an important housekeeping enzyme of glyoxylate shunt in mycobacterium. The pleotropic function of this protein by virtue of its intracellular/extracellular localization and its behavior as an adhesin and virulence factor is quite enigmatic. Despite its importance in mycobacterium persistence, we do not know much about its biophysical and biochemical properties. Earlier reports suggest that the enzyme exists only as a monomer in prokaryotes; however, we observed the existence of both active monomer and dimer forms of the enzyme under physiological conditions. The dimeric form of the enzymes is more stable as compared to the monomeric form as evident from various biophysical parameters. In addition, the dimeric enzyme also shows enhanced stability against proteolysis than the monomers. Based on these studies, it seems that dimerization is an important factor in regulating stability. The differential localization and diverse functions of malate synthase other than its enzymatic role might be triggering the stabilization of the enzyme dimer and modulation of activity and stability in vivo.

Keywords Dimer · Inter-chain beta sheet · Structural cooperativity · Stability · Enzymatic activity

Abbreviations

MtbMS	Mycobacterium tuberculosis malate synthase G
SEC	Size exclusion chromatography
GdmHCl	Guanidine hydrochloride
CD	Circular dichroism
ICBS	Inter-chain beta sheet

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Introduction

Tuberculosis is the second leading infectious cause of mortality across the globe (Sacchettini et al. 2008). It is responsible for approximately 2.5 million deaths annually and about one-third of the population being latently infected (Pieters and Gatfield 2002). The persistence of multidrug-resistant strains demands more effective drugs. Enzymes of the mycobacterial glyoxylate shunt have received global focus of contemporary drug research because of their roles in latent infection (Zhang 2004). This cycle has two key enzymes: isocitrate lyase (ICL; EC4.1.3.1) and malate synthase (MS; EC 4.1.3.2). The pathway is present in most prokaryotes, lower eukaryotes and plants, but has not been observed in vertebrates (Sharma et al. 2000). Since humans do not have a functional glyoxylate pathway, this enzyme is a very effective target for combating tuberculosis (Kumar and Bhakuni 2008).

Malate synthase (MS) falls broadly into two major families, isoforms A and G. The 80-kDa monomeric malate synthase G (MSG) is found exclusively in bacteria, whereas oligomeric malate synthase A (MSA, 65 kDa per subunit) is present in plants and several other organisms. Members of the G family share ~56% sequence identity (Anstrom and Remington 2006). In mycobacterium, MSG is a multifunctional protein that besides its enzymatic role, has also evolved to promote the adherence of the bacterium to host cells by its ability to bind lamimin and thus act as a

virulence factor (Kinhikar et al. 2006). The attempt to knockout the gene for MSG has been unsuccessful, indicating its pivotal role in survival (Sassetti and Rubin 2003).

The present studies focus on addressing the *Mycobacterium tuberculosis* malate synthase, oligomeric status, stability and function.

Materials and methods

Materials

Chemicals were purchased from Sigma-Aldrich. Chromatographic columns were purchased from GE Healthcare and Ni-NTA agarose from Qiagen. The clones of malate synthase were gifts from Prof. S. James Remington of the University of Oregon.

Methods

Purification of recombinant MtbMS

The MtbMS clone in PET 23b was transformed into C41 *E. coli* host strain. A single colony was inoculated into 5 ml of LB broth (Hi-media) having 100 μg/ml of ampicillin and allowed to grow overnight at 37°C. It was then sub-cultured in 400 ml of LB broth overnight after induction with 0.1 M IPTG at A_{600} of 0.6. The histidine-tagged protein was finally purified using Ni-NTA affinity chromatography.

Size-exclusion chromatography

SEC was carried out on a Superdex 200 column on AKTA FPLC (GE Healthcare, UK) pre-equilibrated with 50 mM potassium phosphate buffer; 200 μl of the sample was loaded on the column and run at a flow rate of 0.3 ml/min at 25°C with detection at OD 280.

Circular dichroism measurements

CD measurements were made using a Jasco J810 spectropolarimeter. The spectrum was recorded at an enzyme concentration of 2.0 μM in a 1-mm path length at 25°C.

Thermal denaturation

Thermal denaturation was studied by monitoring changes in molar ellipticity at 222 nm as a function of temperature on a Jasco J810 spectropolarimeter.

Enzyme assay

MS activity was determined as follows. To 980 μl of buffer (50 mM potassium phosphate pH 8.0, 5 mM $MgCl_2$, 0.15 mM acetyl-CoA), 0.5 μM of enzymes was added and incubated until a constant absorbance was achieved. Reaction was started by addition of 20 μl, 35 mM glyoxylate. The cleavage of the thioester bond of acetyl-CoA was monitored at 232 nm.

pH denaturation

Protein (2 μM) in 10 mM CGH (citrate-glycine-HEPES) buffer of different ranges was incubated for 6 h at 4°C before the measurements were made.

Fluorescence measurements

Fluorescence was recorded on a Perkin-Elmer spectrophotometer. The samples were excited at 285 nm, and emission was recorded in the wavelength range of 300–400 nm in triplicate.

Results and discussions

MtbMS forms a monomer and dimer

Figure 1a shows the purification of recombinant MtbMS. The purified enzyme on SDS-PAGE showed a single band of about 80 kDa; however, on a Superdex^{TM} 200 column two peaks having retention volumes of 13.9 ml (~80 kDa) and 11.8 ml (~160 kDa) were observed (Fig. 1b). The first smaller peak corresponded to a dimer and the second abundant peak to a monomer of the enzyme. This was an interesting observation as according to the reports the enzyme is known to exist only as a monomer (Anstrom and Remington 2006; Smith et al. 2003). The presence of both a monomer and dimer of MtbMS was further supported by the results of glutaraldehyde crosslinking (Fig. 1c) and native PAGE analysis of the sample obtained from both the peaks (Fig. 1d). The equilibrium between a monomer and dimer was studied by reloading the samples of both the peaks separately after 48 h. The reloaded sample shows little conversion of either of the populations (Fig. 1b). These observations demonstrate that a dimer and monomer of MtbMS exist in equilibrium; however, their conversion rate is very slow.

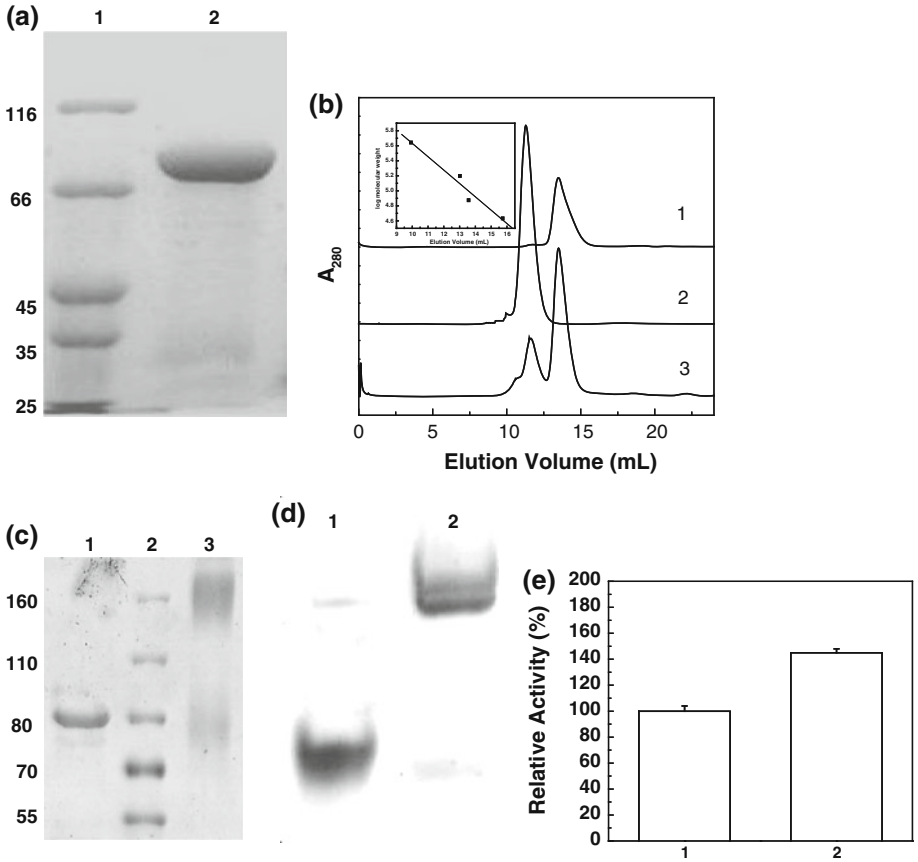


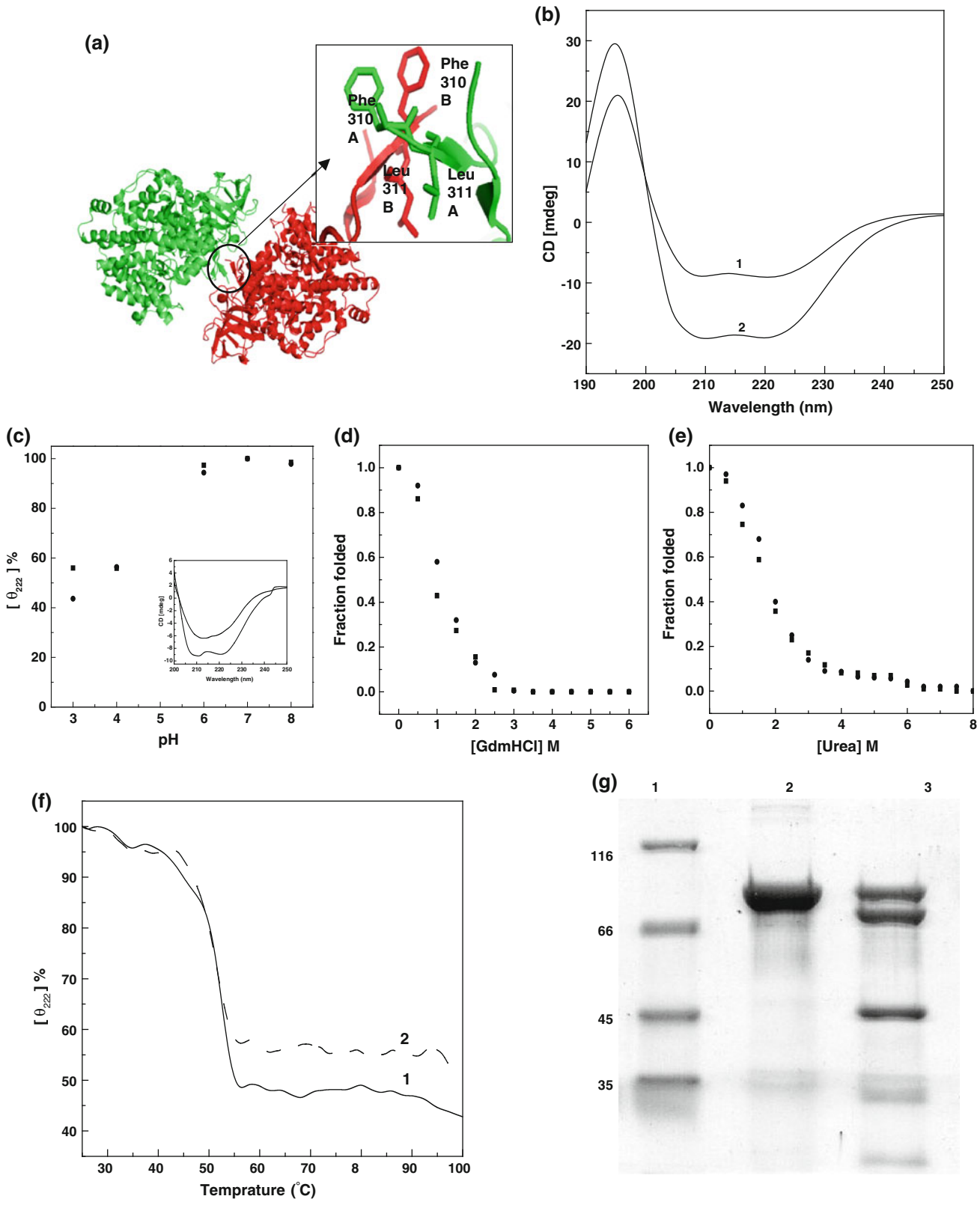
Fig. 1 Purification of MtbMS. **a** SDS-PAGE showing over-expression of MtbMS and purified protein. *Lanes 1–2* represent molecular weight markers and purified MtbMS from the Ni-NTA column, respectively. **b** SEC profile of MtbMS on Superdex 200 h column at pH 8.0 and 25°C (3). (1) Shows reloaded samples of a monomer, whereas (2) shows a dimer population pooled and loaded after 48 h. The inset shows the curve of the elution volume plotted against the log of molecular mass for standard protein markers. The proteins are (1) 440 kDa (ferritin), (2) 158 kDa (aldolase), (3) 75 kDa (conalbumin) and (4) 43 kDa (ovalbumin). **c** SDS-PAGE analysis of glutaraldehyde crosslinked MtbMS. *Lanes 1–3* represent the protein monomer, molecular weight markers and protein dimer, respectively. **d** Native gel profile for the MtbMS monomer and dimer, respectively. **e** Relative enzyme activity of the monomer (*bar 1*) and dimer (*bar 2*) form of malate synthase. The data are presented as percent activity with the activity of the MtbMS monomer taken as 100%. The data represent the mean + SD of three separate measurements

Modulation of the functional activity

At equimolar concentration the dimeric form showed about 45% enhanced activity as compared to the monomer (Fig. 1e). A possible explanation for the slight enhancement but not the doubling of activity between monomer and dimer can be that there is an allosteric interaction between the 300 and 312 loop and the coenzyme A binding pocket. Hence, the crystal packing involving this loop could control the substrate-binding affinity of individual subunits within the asymmetric unit and thus show lower activity (Anstrom and Remington 2006).

Evidences of dimerization

The molecular packing at the interface deduced from the crystal structure of MtbMS (1N8 W) (Smith et al. 2003) indicates formation of the interchain β-sheet (Fig. 2a). This is firmly corroborated by detailed analysis from interface servers ICBS (http://contact14.ics.uci.edu) (Dou et al. 2004) and HotSprint (http://prism.ccbb.ku.edu.tr/hotsprint) (Guney et al. 2008) for non-polar and polar interactions. The hydrophobic as well as electrostatic interactions together make a stable active dimer. The highly conserved residues involved in hydrophobic interaction are phenylalanine (310) and leucine (311) from chain A to phenylalanine (310) and leucine (311) from chain B. Conserved hotspot (Ali and Imperiali 2005) residues are serine (247), arginine (312) from chain A and serine (247), arginine (312) and aspergine (315) from chain B are involved in making H-bonds and van der Waals contacts. There is a strong possibility of phenylalanine (310)-phenylalanine (310) stacking interaction at the interface, which would induce obligate homodimer formation. These analyses indicate a possibility of stabilization of the MtbMS dimer.


(a)
Phe 310 A
Phe 310 B
Leu 311 B
Leu 311 A
(b)
CD [mdeg]
Wavelength (nm)
1
2
(c)
[θ222] %
pH
(d)
Fraction folded
[GdmHCl] M
(e)
Fraction folded
[Urea] M
(f)
[θ222] %
Temprature (°C)
1
2
(g)
1
2
3
116
66
45
35

◀ **Fig. 2** **a** Interacting interfacial region of dimer MtbMS. Pymol (DeLano 2002) representation of dimer interface of MtbMS (PDB ID 1N8 W) of *Mycobacterium tuberculosis*. **b** Circular dichorism profile of monomer (1) and dimer (2). **c** pH-induced structural changes in the MtbMS monomer (*square*) and dimer (*circle*). Inset represents CD profile of the monomer at pH 3 and 8, respectively. **d** Guanidium chloride-induced changes in CD ellipticity at 222 nm in MtbMS monomer (*square*) and dimer (*circle*). **e** Urea-induced changes in CD ellipticity at the 222-nm MtbMS monomer (*square*) and dimer (circle). **f** Thermal stability of MtbMS CD ellipticity at the 222-nm monomer (1) and dimer (2). **g** Enhanced stability of dimer against proteolysis. *Lane 1* Marker, *lane 2* dimer MtbMS, *lane 3* monomer MtbMS

Stability of MtbMS dimer and monomer against pH, chaotropic agents and thermal denaturations

Since the G isoform of MS is predominantly monomer, we studied if the dimerization has any effect on the stability of the enzyme. Comparative stability of the monomer and dimer was evaluated by studying the loss of the secondary structure of the enzyme under different denaturing conditions. For both forms of the enzyme, far-UV CD spectra having both α/β were observed with the signal for the dimer being exactly the double of the monomer at equimolar concentration (Fig. 2b).

pH-induced changes in the secondary structure of the enzyme in the monomeric and dimeric form were monitored (Fig. 2c). The far-UV CD spectra at 222 nm were found to be stable between pH 6.0 and 8.0; however, a decrease at pH below 4.0 resulted in partial denaturation as indicated by about 45% loss in the secondary structure (2C and inset). A similar pattern was observed for enzymatic activity with a maximum at pH 8 and loss below pH 6.0. These findings are inline with recently reported observations on *Pseudomonas aeruginosa* MSG (Roucourt et al. 2009).

To study the GdnHCl and urea-induced changes in the secondary structure, far-UV CD studies were carried out. Figure 2d and e summarizes the effect of increasing concentrations on CD ellipticity at 222 nm. For both conformations of the enzyme, a sigmoidal loss of the CD signal at 222 nm was observed between 1.0 and 3.0 M GdnHCl, with $C_{1/2}$ for the transition being about 1.0 M. For urea-induced denaturation, a similar sigmoidal loss of signal at 222 nm with $C_{1/2}$ of about 2.0 M urea was observed for both the conformers.

Figure 2f summarizes the effect of increasing temperature on the CD ellipticity at 222 nm. For both configurations, a similar sigmoidal loss of the CD signal at 222 nm was observed. However, only about 50% loss of CD signal was found to be associated with the thermal transition suggesting that MtbMS consists of two major domains, and one of these domains is resistant to thermal denaturation while the other is sensitive. A Tm of about 49°C was observed for both conformers.

Stability studies demonstrate that dimers and monomers have very similar stability against pH, chemical chaotrops and heat-induced denaturation of enzymes.

Enhanced stability of the dimer against proteolysis

Malate synthase has an inherent tendency to undergo autoproteolysis on storage giving a protein fragment of 45 kDa (Beeckmans et al. 1997). Furthermore, the co-purification of this proteolytic contaminating species has been reported for several MSs from different sources (Beeckmans et al. 1997). The monomeric population of MtbMS showed slow degradation, and finally the 45-kDa band started accumulating, as observed on SDS-PAGE (Fig. 2f). In contrast, the dimer of MtbMS did not show any significant proteolysis even after 7 days. These differences in proteolytic behavior of both forms of MtbMS suggested that the dimer is more stable than the monomer because of its inaccessible interface.

The discrimination of true oligomeric protein-protein contacts from nonspecific crystal contacts remains problematic, thus apart from crystal a detailed analysis of the molecule in solution is essential (Elcock and McCammon 2001). The PDB (1n8w) structure reflects clearly that the molecule has a tendency to oligomerize as a dimer. Noncovalent contacts between residue side chains are the basis for protein folding, assembly and interaction. Interactions between the edges of protein beta-sheets occur widely in the formation of quaternary structures, protein-protein interactions, and aggregation. Hydrophobic interactions play an important role in defining homo-oligomeric interfaces (Ali and Imperiali 2005). This is well supported by non-polar residues at interfacial proximity forming antiparallel β-sheets in MS.

Our studies reflect for the first time the presence of dimeric MtbMS, and this may not be an experimental artifact, but may be a biological necessity whereby a highly flexible beta sheet of one molecule comes in close proximity to another, providing stable interaction in a dynamically fluctuating interfacial region.

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