Chapter 5

Structural and Stability characteristics of *Mycobacterium tuberculosis* Malat synthase G and its Comparison with *E.coli* homolog
Introduction

Tuberculosis is the second leading infectious cause of mortality across the globe claiming one life every ten seconds. It is responsible for approximately 2.5 million deaths annually with one third of the people on this planet being latently infected\cite{102}. The problem in compliance and emergence of multidrug resistance, even after existence of current cocktail of drugs due to prolonged treatment regimen has paved the path for more effective drug that can shorten the therapy and particularly target the persistence pathway. The inherent notion being that the drugs that are effective against persistent bacteria will clear an infection more quickly, reducing chemotherapy time there by decreasing potential risk of developing of multi-drug-resistant TB.

Enzymes of glyoxylate shunt have received global focus of contemporary drug research because of its proven role as virulence factor \cite{61, 103, 104}. This cycle has two key enzymes; isocitrate lyase (ICL; EC4.1.3.1) and malate synthase (MS; EC 2.3.3.9). In the first step, ICL catalyzes the cleavage of D-isocitrate (six carbons) to glyoxylate (two carbons) & succinate (four carbons). In the second step, glyoxylate formed by ICL reaction is condensed with acetyl-CoA to produce L-malate (four carbons) by malate synthase\cite{68}. This pathway is present in most prokaryotes, lower eukaryotes and plants, but has not been observed in vertebrates. As humans do not have functional glyoxylate pathway the enzymes of the pathway are promising drug target \cite{67, 105, 106}. MS is a multifunctional protein which, besides its traditional enzymatic role, has evolved to promote the adherence of the bacterium to host cells by its ability to bind lamamin thus acting as virulence factor \cite{70, 103}

All malate synthases described till date fall broadly in to two major families, isoforms A and G. The 80-kDa monomeric malate synthase isoform G (MSG) has been found exclusively in bacteria whereas, the oligomeric malate synthase isoform A (MSA, 65 kDa per subunit) occurs in plants and several other organisms. MSG is a magnesium-dependent enzyme \cite{107}. The members of this family of enzymes are structurally based on TIM barrel fold. They also contain an insertion forming a separate α/β domain and an additional C-terminal helical plug. The cleft between the TIM barrel and C-terminal plug forms the active site \cite{74}. Members of the MSG family share about 50% amino acid sequence identity \cite{68}. The E. coli and Mtb MSGs have essentially identical backbone conformation \cite{59} and active site. However, the overall amino acid sequence identity is about 56%. Thus the possibility remains that the two enzymes are functionally distinct and might not be strictly comparable.
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Figure 1. Sequence alignment of MtbMS and ecMS Multiple sequence alignment of MtbMS and ecMS using ESPript[108]

The present study was carried out in pursuit of comparatively delineating the structure, activity, and stability properties of recombinant MtbMS and E. coli MSG (ecMS). As significant differences in the structural properties of the two enzymes were observed so attempts were made to unveil the differential role of ionic interactions in modulation of stability of native conformation, and functional activity as well as induction of structural cooperativity in these molecules using various biophysical techniques including fluorescence...
spectroscopy, circular dichroism in combination with size exclusion chromatography, limited proteolysis and ANS binding studies.

**Results and Discussion**

**MtbMS and ecMS are stabilized predominantly as a monomer**

The MtbMS and ecMS were over-expressed and purified. Figure 2A shows the overexpression and purification of MtbMS and ecMS. On SDS PAGE both the enzymes show the expected molecular mass of about 80 kDa and a purity of about 95%. The quaternary structures of the enzymes were analyzed by SEC. On the Superdex™ 200 column the proteins showed two peaks with high abundance of one compared to other. However, the retention volumes of the peaks were significantly different for the two enzymes indicating difference in the hydrodynamic radii of their stabilized conformations. For MtbMS, the prominent peak was observed at a retention volume of 13.9 mL corresponding to the molecular mass of about 80 kDa as calculated from the values obtained for the protein standards (Figure 2B and Inset). For ecMS the retention volume of major peak was 14.36 ml which corresponds to the molecular mass of 70 kDa (Figure 2B). The smaller peak observed for MtbMS corresponded to the dimeric configuration of the enzymes as estimated by the mobility of protein standard. The monomer does not get converted to dimer on storage even after one week and it takes long time for the equilibrium to attain. An interesting observation is that according to primary amino acid sequence both the enzymes should have similar molecular mass of about 80kDa however, on SEC ecMS shows a molecular mass of about 70kDa. This demonstrates that in solution the ecMS is stabilized in a significantly compact conformation compared to MtbMS.

**Effect of limited proteolysis on the functional activity and cooperativity of MtbMS and ecMS.**

The factors determining the vulnerability of a protein for proteolysis by protease depends on the conformational parameters such as accessibility, segmental motion, and protrusions. For this reason limited proteolysis has been effectively used to monitor structural domains in proteins, ligand-induced conformational changes, and protein folding/unfolding[109]. Limited proteolysis of MtbMS and ecMS with trypsin resulted in cleavage of the both the enzymes. For MtbMS a major protein fragment of molecular mass about 48 and 30kDa was observed whereas, for ecMS three fragments corresponding to
molecular masses of about 50kDa and 35kDa respectively were observed (Figure 3B). The difference in proteolysis pattern of ecMS and MtbMS by trypsin demonstrates that the two enzymes have different three-dimensional structure.

Figure 2 Purification of MtbMS and EcMS (A) SDS-PAGE showing purified recombinant MtbMS and ecMS Mtblcl and purified protein. Lanes 1-3 represent molecular weight markers and, purified MtbMS and ecMS respectively. (B) SEC profile of MtbMS (1) and EcMS (2) on Superdex 200HR column at pH 8.0 and 25°C. The inset shows the curve of elution volume plotted against log of molecular mass of standard protein markers. The proteins are (1) 440KDa (ferritin), (2)158KDa (aldolase), (3) 75KDa (conalbumin) and (4)43KDa (ovalbumin).

Limited trypsinolysis of MS from maize, a 62kDa A-isoform of enzyme, results in cleavage of enzyme into two prominent fragments of molecular mass 45kDa and 19kDa[107]. Sequence alignment and structural characterization of malate synthase enzymes belonging to the family of A and G isoform show that they have similar overall fold with conserved C-terminal cap and TIM barrel domain[59]. The difference in size between the two classes of enzymes is due to the insertion of an additional α/β thumb domain in the G-form [110]. Furthermore, this additional α/β domain in the G-form of enzyme protrudes out from the main fold of the structure and does not make significant contact/interaction with the rest of the molecule (Figure 3A). This demonstrated that the G-isoform of MS undergoes a similar proteolytic cleavage as the A-form of enzyme thus;
supporting the fact the TIM barrel domain and the C-terminal cap portion in the A- and G-isoforms of MS have similar conformation. The result also suggests that, the additional α/β domain in the G-form of enzyme does not have any significant interaction with the other part (domains) of the molecule and hence does not influence the fragmentation pattern.

One very interesting observation on the A-form of enzyme had been that limited proteolysis had been that does not result in complete inactivation of the enzyme and even when no more intact subunit was observed on the SDS PAGE, enzymatic activity of about 30% of the original activity is seen[107]. Based on the overall structural similarity between the A and G-isoforms of enzyme we wanted to see whether MtbMS and ecMS (G-isoform of enzyme) also show a similar behavior. Furthermore, we intrigued to comprehend the basis of retention of residual activity in the proteolysed enzyme when no significant protein band is observed in SDS PAGE.

The trypsin digested enzyme samples of MtbMS and ecMS retained about 35 % activity of the native enzyme (Figure 3C). However a significantly different proteolytic pattern was observed for the two enzymes for MtbMS two prominent protein bands of 48kDa and 30kDa whereas for ecMS three distinct bands of 50kDa and 35kDa were observed respectively. These observations indicate that MtbMS and ecMS are stabilized in significantly different conformations due to which different proteolytic pattern are observed for the two observations.

We wanted to see whether a fragment is responsible for residual activity observed for the proteolysed protein sample, we carried out SEC studies in which we obtained a very interesting The trypsin treated enzyme samples on SEC eluted as a single peak at the same position as the native enzyme (Figure 3D). This demonstrates that during the proteolysis of MS by trypsin only nicking of the enzyme occurs as the fragments obtained have tendency to remain associated. Due to this unique property of the structural domains, the proteolysed enzyme retains significant enzymatic activity.

**Effect of enzyme nicking of structural cooperativity of MtbMS and ecMS**

We were interested in studying the effect of enzyme nicking on the structural cooperativity of the MtbMS and ecMS. Studying the thermal denaturation of the protein by monitoring the loss of CD ellipticity at 222 nm provides significant information on the
cooperativity existing in the protein molecule under different experimental conditions[111](Figure 4Aand B). For the native enzymes a sigmoidal loss of the CD signal

Figure 3. Limited proteolysis of MtbMS and ecMS using trypsin. (A) Pymol representation of superimposed coordinates of MSA of e.coli (PDB ID 3CUZ) and MtbMS (PDB ID 1D8C) of Mycobacterium tuberculosis.(B) SDS-PAGE profile of trypsin treated samples of MtbMS and ecMS. The lanes 1-5 in the figure represents molecular weight marker, native MtbMS, trypsinolised MtbMS, native ecMS and trypsinolised ecMS, respectively.(C) Relative enzyme activity of Native and trypsinolysed Malate synthase bars 1-4 represents native malate Synthase , trypsinolysed MtbMS,native ecMS and trypsinolysed ecMS respectively the data is presented as percent activity with the activity of MtbMS taken as 100 percent. The data is represented as mean ± SD of three separate measurements.(D) SEC profile of native and trypsinolysed MtbMS (profile 1 and 2) and native ecMS and trypsin treated ecMS (profile 3 and 4)
at 222 nm was observed with increasing temperature. However, only a partial loss (about 50% and 35% for MtbMS and ecMS, respectively) of CD signal was associated with their thermal denaturation. This indicates that the native enzyme undergo non-cooperative thermal denaturation reflecting the fact that structurally one of the domain is sensitive to temperature while the other part being recalcitrant similar results have been seen in case of mycobacterium tuberculosis isocitrate lyase[60]. However, for trypsin digested protein samples a sigmoidal curve with complete loss of secondary structure was observed for both the enzymes. As even after the enzyme nicking the protein fragments obtained remain associated (discussed earlier), these results suggest that limited proteolysis removes the inherent strain in the protein molecule and induces enhanced interaction between the respective structural domains by virtue of which structural cooperativity is brought about in the inherently non-cooperative MSG molecule on proteolysis.

Chemical denaturant-induced unfolding of MtbMS and ecMS

The spectral changes associated with the unfolding of MtbMs and ecMS were studied by CD at 222nm. To reach unfolding equilibrium at each denaturant concentration, measurements were performed after incubation of protein solution for 24 hours at 27°C.

![Figure 4. Thermal denaturation of Malate Synthase](image)

**Figure 4. Thermal denaturation of Malate Synthase** Thermal unfolding of Malate synthase studied by monitoring the loss of CD signal at 222 nm with increasing temperature. Panel A and B: Solid lines represent the profile of native MtbMS and ecMS whereas dotted line represent the trypsinolyzed samples of the respective proteins. The data has been represented as percentage with the value observed for protein at 30°C taken as 100 percent.
Urea-induced unfolding

The comparative unfolding and stability characteristics of the MtbMS and ecMS were studied by monitoring the urea-induced changes in the proteins. Figure 5 shows the equilibrium unfolding of MtbMS and ecMS monitored by CD at 222nm. For both the enzymes the reduction in secondary structure monitored by CD appeared to be sigmoidal. However, the denaturation curve observed for MtbMS was significantly steeper as compared to that observed for ecMS suggesting that MtbMS was more susceptible to urea denaturation than ecMS. This is clearer from the $C_{1/2}$ value associated with the urea denaturation of the two enzymes. The MtbMs and ecMs had $C_{1/2}$ value of about 2.0M and 4.0M, respectively. This demonstrates that ecMS has significantly higher stability against urea denaturation as compared to MtbMS. The difference in susceptibility between the two enzymes towards urea-induced denaturation is probably due to stronger hydrophobic interactions present in protein core in case of ecMS due to compact conformation of the enzyme as compared to MtbMS (discussed earlier).

![Figure 5. Urea induced perturbations in secondary structure MtbMS and ecMS](image)

Figure 5. Urea induced perturbations in secondary structure MtbMS and ecMS
Changes in CD ellipticity at 222nm for MtbMS (circle) and EcMS (square) on incubation with increasing concentrations of urea at pH 8.0 and 25° C. Represented as fraction folded where one represent the native and zero represent the unfolded form in the two state model.
GdnHCl-induced unfolding

To study the changes in the secondary structure of enzyme induced by GdnHCl, far-UV CD studies were carried out. Figure 6 summarizes the GdnHCl-induced unfolding of MtbMS and ecMS monitored by CD measurements at 222nm. For MtbMS, 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 GdnHCl. This demonstrates that MtbMS undergoes GdnHCl-induced two-state unfolding. However, significant difference in the GdnHCl-induced denaturation of ecMS was observed. For ecMS, the reduction of secondary structure monitored by CD appeared to be biphasic (Figure 6A). The first phase of unfolding of ecMS was a sharp decrease in CD signal from 100 to 40% between GdnHCl concentration of 0 and 0.5M. The second phase was a plateau between 0.5 and 2.5M GdnHCl and finally a sigmoidal loss of CD signal between 2.5 and 4M GdnHCl. These observations indicate the possibility of stabilization of a partially unfolded intermediate of ecMS at low GdnHCl concentration which was further probed by carrying out ANS binding studies under these conditions[95].

Figure 6. GdnHCl induced unfolding of MS (A) Guanidium chloride induced structural alteration in MtbMS (square) and ecMS (circle) represented as fraction folded.(B) ANS binding profiles of GdnHCl treated ecMS (Square) and MtbMS (circle) as function of increasing GdnHCl concentration.

The hydrophobic fluorescent dye ANS was used to probe the exposure of the hydrophobic region upon GdnHCl-induced unfolding of ecMS (Figure 6B). ANS
fluorescence quantum yield increases upon noncovalent binding to hydrophobic regions of proteins. In presence of native ecMS the fluorescence emission spectra of ANS was similar to that of ANS in buffer. A steep increase in ANS fluorescence emission accompanied by a blue shift in emission wavelength maxima (Data not shown) was observed during the first transition (as seen from Figure 6A) of GdnHCl-induced unfolding of ecMS. After a sharp maximum, the ANS fluorescence intensity decreased sharply, preceding the second transition observed for ecMS in the CD experiment. This observation confirms the stabilization of a partially unfolded intermediate of enzyme with exposed hydrophobic clusters at low GdnHCl concentration.

Effect of modulation of ionic interactions in ecMS and MtbMS on their unfolding characteristics

GdnHCl is an electrolyte and in aqueous solution is ionized into Gdn$^+$ and Cl$^-$ ions. Structurally urea and Gdn$^+$ are very similar however; urea is a neutral molecule whereas the guanidinium ion has a positive charge delocalized over the planar structure due to which at low concentration it can preferentially interact with negatively charged amino acid residues present in the protein molecule. This may lead to perturbations and/or weakening of the electrostatic interactions present in the native conformation of the enzyme as a result of which stabilization of intermediates can be observed under these conditions[112]. As a significant difference in the GdnHCl-and urea-induced unfolding of ecMS and MtbMS was observed we studied the effect of NaCl, an electrolyte, on these unfolding profiles. Figure 7A summarizes the effect of increasing concentrations of GdnHCl on the CD ellipticity at 222 nm for MtbMS and ecMS. For MtbMS, no significant difference in the GdnHCl-induced unfolding of enzyme both in presence and absence of 150mM NaCl was observed. However, for ecMS presence of 150mM NaCl influenced the unfolding profile of enzyme significantly. For ecMS treated with 150 mM NaCl, a sigmoidal loss of CD signal with increasing concentration of GdnHCl was observed. The C1/2 of GdnHCl associated with the transition was about which is close to that observed for the MtbMS. This suggest that GdnHCl-induces a two-state unfolding of NaCl treated ecMS that is the partially unfolded intermediate stabilized during GdnHCl unfolding of ecMS is abolished by presence of NaCl. This inference is also supported by the observation that no binding of ANS was observed for the GdnHCl-induced unfolding of 150mM NaCl-treated ecMS (Figure 7B). These observations suggest that NaCl brings about structural cooperativity in otherwise non-
cooperative ecMS molecule and abolishes the stabilization of partially unfolded intermediate during GdnHCl unfolding of enzyme.

The results of the studies presented in this paper demonstrate that although the backbone structure or the secondary structure of ecMS and MtbMS is very similar but significant differences exist in their molecular packing which are very visible in the differences in the unfolding characteristics of the two enzymes. The ecMS seems to have predominance electrostatic interactions as compared to MtbMS. Analysis of surface charge distribution by GRASP, reflects subtle differences with *E. coli* having a more electronegative surface as compared to MtbMS. The presence of predominant electrostatic interactions in the ecMS might be the possible reason for the stabilization of a compact conformation of ecMS in the native state and its higher stability. The other important observation is that the interactions prevailing between the structural domains in both the A and G isoform of Malate synthase are very similar although they have pronounced differences in sequence and the molecular mass.

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**Figure 7. Modulation of ionic interactions in MtbMS and ecMS** (A) Guanidium chloride induced structural alteration in MtbMS (square) and MtbMS incubated with 150mM (circle) represented as fraction folded. (B) Guanidium chloride induced structural alteration in ecMS (square) and ecMS incubated with 150mM (circle) represented as fraction folded.
Conclusion

Mycobacterium metabolic plasticity renders a high degree of adaptive advantage in hostile environment and persistence. This characteristic metabolic enigma is characterized by upregulation of glyoxylate shunt. The studies demonstrate that Mycobacterium tuberculosis Malate Synthase (MtbMS) which belongs to the G isoform is expressed predominantly as monomer and interestingly has a similar overall catalytic domain fold as A isoform present in E.Coli (a smaller variant not present in mycobacterium). It further unveils the comparative analysis of the two known MSG's from E.Coli (ecMS) and Mycobacterium; it showed that in spite of possessing a high sequence identity, they show differential behavior as far as stability and unfolding, and functional attributes of the two enzymes are concerned. The study further demonstrates that the differences in the stability and unfolding of the two enzymes are by virtue of electrostatic modulation.