Serine hydroxymethyltransferase from *Mycobacterium tuberculosis*
From the genome analysis of the *Mycobacterium tuberculosis* two putative genes namely *GlyA* and *GlyA2* have been proposed to encode for the enzyme serine hydroxymethyltransferase. We have cloned, overexpressed, and purified to homogeneity their respective protein products, serine hydroxymethyltransferase, SHM1 and SHM2. The recombinant SHM1 and SHM2 exist as homodimers of molecular mass about 90 kDa under physiological conditions, however, SHM2 has more compact conformation and higher thermal stability than SHM1. The most interesting structural observation was that the SHM1 contains 1 mol of pyridoxal 5'-phosphate (PLP)/mol of enzyme dimer. This is the first report of such a unique stoichiometry of PLP and enzyme dimer for SHMT. The SHM2 contains 2 mol of PLP/mol of enzyme dimer, which is the usual stoichiometry reported for SHMT. Functionally, both the recombinant enzymes showed catalysis of reversible interconversion of serine and glycine and aldol cleavage of a 3-hydroxyamino acid. However, unlike SHMT from other sources both SHM1 and SHM2 do not undergo half-transamination reaction with D-alanine resulting in formation of apoenzyme but L-cysteine removed the prosthetic group, PLP, from both the recombinant enzymes leaving the respective inactive apoenzyme. On detailed analysis of the enzyme kinetic for hydroxymethyltransferase reaction it was observed that both the SHMTs has $K_m$ value for L-Serine comparable to that reported for SHMTs from other sources. However, a significantly lower catalytic efficiency ($k_{cat}/K_m$) for the reaction was observed. Comparative structural studies on the two enzymes showed that the SHM1 is resistant to alkaline denaturation up to pH 10.5, whereas the native SHM2 dimer dissociates into monomer at pH 9. Urea- and guanidinium chloride-induced two-step unfolding of SHM1 and SHM2 with the first step being dissociation of dimer into apomonomer at low denaturant concentrations followed by unfolding of the stabilized monomer at higher denaturant concentrations.
INTRODUCTION

*Mycobacterium tuberculosis* is an opportunistic pathogen that claims more lives than any other single disease, infecting about 32% world's population. Its characteristic features include slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity. Several unique biosynthetic pathways generating cell wall components such as mycolic acids, mycoserosic acid, arabinogalactan etc. contribute to mycobacterial longevity, pathogenesis and trigger inflammatory reactions in host. This mode of pathogenesis is attributed to the array of proteins synthesized by the organism differently under different stress conditions, ranging from low profile dormant state to active state responsible for chronic conditions of the disease. There has been a wide spread emergence of the drug resistant strains against the most important front line drugs Isoniazid and Rifampicin, making the treatment difficult (Cole *et al.*, 1995). With the global spread of human immunodeficiency virus (HIV) in recent years, the deadly combination of TB in HIV patients has caused alarming rise in mortality. Hence there is an urgent need to develop new therapeutics, which offer more effective, improved and shorter treatment over the existing regimens. Conception of such novel prophylactic or chemotherapeutic interventions will have to be based on a deeper understanding of the molecular and genetic basis of biosynthetic pathways of the bacillus, the diversity of enzymes involved and metabolites synthesized. The availability of the complete genome sequence of *M. tuberculosis* has accelerated the pace of understanding biology of these slow growing bacteria greatly. To gain complete insight into the biochemistry of the bacteria, coordinated structural genomics projects have been initiated in order to determine the structure of a large number of proteins from *M. tuberculosis* (www.rcsb.org/pdp/strucgen.html). Detailed structural and functional study of these proteins will offer insight into its virulence mechanism, multiple drug resistance to available chemotherapy and may also suggest new drug targets.

Sequence analysis studies on the available H37Rv genome sequence have identified 3924 ORFs, which accounts for 91% potential coding capacity. By using detailed database comparisons, precise function has been attributed to ~40% of predicted proteins and some information is available for another 44%, remaining 16% proteins are
unique to the organism and are hypothesized to account for specific mycobacterial functions (Cole et al., 1998).

On the basis of function, genes in M. tuberculosis genome have been categorized in 11 functional classification codes. These include virulence, detoxification and adaptation (0), lipid metabolism (1), information pathways (2), cell wall and cell processes (3), stable RNAs (4), Insertion sequences and phages (5), PE/PPE (6), Intermediary metabolism and respiration (7), unknown (8), Regulatory proteins (9), Conserved hypotheticals (10). The class of conserved hypotheticals includes proteins that have not been characterized but are found to be widely conserved. These may be significant in some metabolic pathways and their precise function is yet to be determined.

Examination of amino acid composition of the M. tuberculosis by proteome by correspondence analysis (Greenacre, 1984), and comparison with other microorganisms whose genome sequences are available revealed statistically significant preference for the amino acids Ala, Gly, Pro, Arg and Trp, which are all encoded by G + C-rich codons (Cole et al., 1998). The fraction of the proteome that has arisen through gene duplication is similar to that seen in E. coli or B. subtilis (~51%), except that there may be extensive redundancy or differential production of the corresponding polypeptides. The apparent lack of divergence following gene duplication is consistent with the hypothesis that M. tuberculosis is of recent descent (Sreevatsan et al., 1997).

In recent years, SHMT has attracted considerable attention. To date (January, 2005), 60 sequences have been deposited in the data-base (http://www.ncbi.nlm.nih.gov/Entrez/). It was considered worthwhile to examine all the sequences of SHMT to identify residues that have been conserved in most, if not, all of the sequences. It was hoped that the conserved residues with functional group (s) could participate in catalysis or pair with an oppositely charged residue to stabilize the structure. The availability of the three dimensional structures of SHMT from 3 eukaryotic (Renwick et al., 1998; Scarsdale et al., 1999; Szebenyi et al., 2000) sources and 2 prokaryotic source (Scarsdale et al., 2000; Trivedi et al., 2002) could facilitate an understanding the role of some of these residues in the structure and function of SHMT. This chapter describes biochemical and structural characterization of two SHMTs from M. tuberculosis.
Searches of the *Mycobacterium tuberculosis* genome (http://www.sanger.ac.uk/Projects/M_tuberculosis/) for enzymes related to SHMT have lead to the identification of two genes namely *GlyA* and *GlyA2* which have been proposed as putative genes encoding for the same enzyme, serine hydroxymethyltransferase (SHMT). This is a very good example of gene duplication in pathogenic microorganism. We have cloned, over-expressed and purified to homogeneity the respective protein product of *GlyA* and *GlyA2* gene of *M. tuberculosis* and named it as SHM1 and SHM2, respectively. This chapter reports the cloning, overexpression, and purification of the recombinant SHM1 and SHM2, the protein products of the genes *GlyA* and *GlyA2*. This chapter also discusses the comparative structural, functional, and catalytic properties of both recombinant proteins along with the comparative stability studies against thermal, pH-, urea- and GdmCl–induced denaturation of both SHMTs.
RESULTS AND DISCUSSION

Expression and Purification

Both GlyA and GlyA2 genes of Mycobacterium tuberculosis were cloned and their respective protein products (SHM1 for GlyA and SHM2 for GlyA2) were overexpressed by the method described in “Experimental Procedures.” The expression of both the SHM1 and SHM2 was good and the expressed proteins were present predominantly (>90%) in the soluble fraction. The enzymes present in the soluble fraction were purified by the method described under “Experimental Procedures.” The yield of both the enzymes was in the range of 12 to 15 mg/liter. The purified proteins were homogenous as indicated by a single protein band on SDS-PAGE (figure 4.1) and a single peak in ESI-MS (figure 4.2).

The purified enzymes were assayed for SHMT activity (Taylor et al., 1965, Manohar et al., 1982). The 280/425 absorbance ratio was 4.78 and 3.43 for the purified recombinant SHM1 and SHM2, respectively, which is within the limits of the values reported for SHMT from various sources (Schirch et al., 1985).

Molecular Weight and Subunit Structure

The molecular masses of the purified recombinant SHM1 and SHM2 were determined under non-dissociating conditions as described by Andrews (1965) using the data of gel filtration experiments. Gel filtration of SHM1 and SHM2 on a Superdex S-200 column, calibrated with the various molecular weight standards, showed a single peak although with a slight difference in retention volume of 14.55 and 14.7 ml, respectively, for the two enzymes (figure 4.3). When the elution volumes of the marker proteins were plotted as a function of log of molecular masses, the molecular masses of 91.2 and 87.1
kDa were obtained for SHM1 and SHM2, respectively. From the primary amino acid sequence, the molecular masses of 45.03 and 45.52 kDa were obtained for SHM1 and SHM2, respectively. The subunit masses of purified SHM1 and SHM2 were determined by SDS-PAGE. A single homogeneous protein band corresponding to molecular mass of

Figure 4.2: ESI-MS of SHM1 (A) and SHM2 (B). Masses are taken after extensive dialysis of protein against deionised triple distilled water
about 45 kDa was observed for the two proteins (figure 4.1A and 4.1B). The precise molecular weights of the two recombinant enzymes were obtained by ESI-MS experiments (figure 4.2A and 4.2B). Molecular masses of 45.0 and 45.5 kDa were observed for SHM1 and SHM2, respectively, demonstrating that both the recombinant enzymes have very similar molecular masses.

The results of the studies on subunit masses along with the size exclusion chromatography as reported above demonstrate that both SHM1 and SHM2 exist as dimers under physiological conditions. Furthermore, as both the proteins have similar molecular masses but significant differences are observed in their retention volumes (on size exclusion chromatography), it suggests that significant differences exist in the molecular dimensions of the two recombinant enzymes with SHM2 having a slightly more compact conformation (higher retention volume) than the SHM1 under physiological conditions.

Content of PLP

The SHMT is a PLP-dependent enzyme in which the PLP is covalently attached to the enzyme, bound as an aldimine to Lys-229 that is conserved in SHMT from various sources (Renwick et al., 1998, Scarsdale et al., 1999, Venkatesha et al., 1998). Visible CD studies on SHMT have shown that the visible CD signal is different between the holo- and the apo- or unfolded enzyme. The holoenzyme has a unique peak at 425 nm because of the bound PLP, which disappears on unfolding of the enzyme or on removal of PLP from the enzyme, i.e. on stabilization of apoenzyme (Cai et al., 1995). The visible CD spectra of SHM1 and SHM2 were recorded (figure 4.5A). For both the recombinant proteins, a visible CD spectrum centered at about 430 nm, showing the presence of the enzyme-
bound PLP, was observed. However, at a similar enzyme concentration the visible CD signal for SHM2 was about 8 times greater than that observed for the SHM1 indicating that it probably has a higher content of PLP as compared with SHM1. To confirm this, the amount of PLP bound to the enzyme was determined for both recombinant enzymes by the method of Ulevitch et al., 1977 after extensive dialysis of the purified recombinant enzymes against 25 mM potassium phosphate buffer, pH 7.6, 50 mM NaCl, and 1 mM EDTA. The SHM1 and SHM2 were found to contain 1 ± 0.2 and 2.3 ± 0.5 mol of PLP/mol of enzyme, respectively.

The SHMT from mammalian sources are homotetramers and contain 4 mol of PLP/mol of enzyme (Renwick et al., 1998, Scarsdale et al., 1999), whereas the SHMT from E. coli as well as from other bacterial sources are dimeric and contain 2 mol of PLP/mol of enzyme (Venkatesha et al., 1998). All the SHMTs reported to date including SHM2 from M. tuberculosis (reported in this thesis) contain 2 mol of PLP/mol of enzyme dimer, however, SHM1 was found to contain only 1 mol of PLP/mol of enzyme dimer. This is the first report on such a unique stoichiometry of PLP and enzyme for SHMT.

**Catalytic Properties of SHM1 and SHM2**

SHMT is a homo-oligomer either being a homotetramer or a homodimer depending on the source (Schirch et al., 1963). The enzyme contains 2 mole of PLP per mole of enzyme dimer. The SHM1 reported in this thesis is the only SHMTs reported till date that have only one mole PLP per mole of enzyme dimer. Each monomeric subunit within the dimer or obligate dimer (in case of tetramer) contributes catalytically important amino acid residues to both the active sites. SHM1 have a unique cofactor to protein ratio we thought it worthwhile to study the catalytic property of this enzyme in comparison to SHM2, which is similar to the other SHMTs. The SHM1 and SHM2 catalyzed the H\(_4\)folate dependent retro-aldol cleavage of L-Serine to form Glycine and 5,10-CH\(_2\)-H\(_4\)-folate with a specific activity 1.35 U/mg and 1.5 U/mg respectively. These values were significantly lower compared to that reported for SHMT from other sources (table 4.1). The apparent \(K_m\) and \(V_{\text{max}}\) values were determined at saturating concentrations of the co-substrates and the optimal pH. Using L-serine as the variable substrate at saturating concentrations of THF, a hyperbolic curve with saturation at around 3 mM for SHM1 and
Table 4.1. Kinetic properties of SHM1 and SHM2 with respect to other SHMTs using L-Serine as substrate

<table>
<thead>
<tr>
<th>Protein</th>
<th>Specific Activity (units*/mg of protein)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$sec$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHM1</td>
<td>1.35</td>
<td>0.96</td>
<td>1.17</td>
<td>1.22</td>
<td>This Study</td>
</tr>
<tr>
<td>SHM2</td>
<td>1.5</td>
<td>0.89</td>
<td>1.67</td>
<td>1.88</td>
<td>This Study</td>
</tr>
<tr>
<td>ScSHMT</td>
<td>4.2</td>
<td>0.9</td>
<td>4.0</td>
<td>4.4</td>
<td>Jagath et al., 1995</td>
</tr>
<tr>
<td>BsSHMT</td>
<td>6.7</td>
<td>0.9</td>
<td>5.0</td>
<td>5.6</td>
<td>Venkatakrishna et al., 2002</td>
</tr>
<tr>
<td>Human mSHMT</td>
<td>N.R.</td>
<td>0.6</td>
<td>5.0</td>
<td>8.3</td>
<td>Girgis et al., 1998</td>
</tr>
<tr>
<td>eSHMT</td>
<td>1.0</td>
<td>0.3</td>
<td>10.7</td>
<td>35.67</td>
<td>Angelaccio et al., 1992</td>
</tr>
<tr>
<td>Methanococcus jannaschi SHMT</td>
<td>NR</td>
<td>0.8</td>
<td>3.5</td>
<td>4.4</td>
<td>Angellicco et al., 2003</td>
</tr>
</tbody>
</table>

*μmol HCHO formed per min per mg of protein at 37 °C and pH 7.6.
NR - Not reported

3.5 mM for SHM2 was obtained with maximum velocity of 1.3 for SHM1 and 1.5 for SHM2 (figure 4.4). From the double reciprocal plots of the same data, the straight lines were obtained by linear regression analysis using Sigma plot software. The $K_m$ values for L-Serine observed for the SHM1 and SHM2 were 0.96 mM and 0.89 mM respectively, which were similar to that observed for other SHMTs (table 4.1). The mycobacterium enzymes also showed significantly lower $k_{cat}$ values of 1.17 sec$^{-1}$ and 1.67 sec$^{-1}$ for SHM1 and SHM2 respectively. These observations demonstrate that mycobacterium SHMTs in general have lower catalytic efficiency for hydroxymethyltransferase reaction as compared to enzyme from other sources. Furthermore, within both SHMTs from M. tuberculosis, the...
enzymes having one mole of PLP per mole of enzyme dimer i.e. SHM1) have lower catalytic efficiency than those having two mole of PLP per mole of enzyme dimer (i.e. SHM2).

**Comparative Structural and Functional Properties of the Recombinant SHM1 and SHM2**

**Secondary Structure**—Far-UV CD studies were carried out on both SHM1 and SHM2 to analyze the differences in the secondary structure that exist between the two enzymes. For both SHM1 and SHM2, the far-UV CD spectra characteristic of a protein having both α-helix and β-sheet secondary structure was observed (figure 4.5B). However, for similar molar concentrations of enzyme, a significantly higher ellipticity was observed for SHM2 over the whole far-UV region (figure 4.5B). This observation suggests that SHM2 have a significantly higher secondary structure as compared with SHM1. Despite having similar subunit molecular mass as SHM1, the SHM2 was found to have a more compact conformation. One possible reason for this may be the presence of a significantly higher secondary structure in SHM2, which would result in stabilization of a compact conformation.

![CD profile](image)

**Figure 4.5**: CD profiles of SHM1 and SHM2 at pH 7.6 and 25 °C. A, visible CD spectra of native recombinant proteins. B, far-UV CD spectra of native recombinant proteins. In both the figures the **solid lines** represent data for SHM1 and the **dashed line** that for SHM2. The experimental details are given under "Experimental Procedures".

**Tryptophan Fluorescence**—According to the primary amino acid sequence, SHM1 has two tryptophan molecules at positions 172 and 421, whereas SHM2 has a single
tryptophan at position 175. The fluorescence spectra of SHM1 and SHM2 were recorded (figure 4.6). For both the enzymes the emission wavelength maxima for the tryptophan fluorescence was observed at about 339 nm, however, there were significant differences in fluorescence intensities of the two enzymes because of the difference in number of tryptophan moieties present in them. The buried tryptophan residues in folded protein show fluorescence emission maxima at 330–335 nm, whereas on unfolding of protein the tryptophan fluorescence emission maxima shifts to about 350 nm (Lakowicz, 1983). Hence, both in SHM1 and SHM2 the tryptophan molecule(s) is not completely buried but partially exposed to the solvent.

**Fluorescence Resonance Energy Transfer**—For eSHMT it has been demonstrated that reduction of the PLP aldimine bond results in absorbance maxima at 355 nm that overlaps the emission spectrum of tryptophan. Hence, the PyP can be used as an energy acceptor of tryptophan fluorescence. For native eSHMT, FRET is observed that abolishes on unfolding of enzyme (Cai et al., 1996b). For both the SHM1 and SHM2 in the native conformation two emission maxima, one around 339 nm (tryptophan fluorescence) and

![Figure 4.6: Tryptophan fluorescence emission spectra of native recombinant proteins at pH 7.6 and 25°C after excitation at 290 nm. In the figure, the solid lines represent data for SHM1 and the dashed line that for SHM2. The experimental details are given under "Experimental Procedures."](image)

![Figure 4.7: Fluorescence Resonance Energy Transfer of SHM1 (A) and SHM2 (B) at pH 7.6 and 25°C. Fluorescence emission spectra of native, pH 7.6, and unfolded (in presence of 8 M urea) PyP-SHM1 (A) and PyP-SHM2 (B) after excitation at 290 nm. In both figures, the solid lines represent data for native protein and dashed lines for unfolded protein. The experimental details are given under "Experimental Procedures."](image)
the other at 385 nm (PyP emission resulting from FRET) were observed when it is excited at 290 nm showing FRET (figure 4.7A and 4.7B). However, on unfolding by incubation in 8 M urea only single emission maximum at 352 nm was observed. These observations demonstrate that for SHM1 and SHM2 in the native conformation there is FRET between tryptophan residues and the bound PLP suggesting that the tryptophan residue(s) is located less than 5 Å from PyP in the native conformation of these two enzymes and on denaturation of the enzymes these two moieties move apart resulting in

**Figure 4.8:** Alignment of the amino acid sequence of SHMT from various sources. The alignment was done with the software CLUSTALW. The *arrow* denotes the site of the octapeptide having five threonine residues, which is conserved in all SHMT except SHM1 and SHM2. The *light shaded letters* indicate the amino acids involved in binding of enzyme to the PLP.
the loss of FRET.

**Functional Properties**—All the forms of SHMT for which a primary sequence is known contain the eight-residue conserved sequence, VTTTHK(Pyr)T, near the active-site lysyl residue (Lys-229 as in eSHMT) that forms the internal aldimine with PLP. The active site octa-peptide from SHMT is unusual in the sense that it has five threonine residues conserved in all the reported primary sequence of SHMTs (figure 4.8). Mutation studies have demonstrated that Thr-226 plays an important role in converting the gem-diamine complex to external aldimine complex. A T226A mutant of eSHMT has been shown to distinguish between substrates and substrate analogs in formation of the gem-diamine complex (Schirch et al., 1962). The primary sequence of SHM1 and SHM2 show significant changes in the conserved threonine sequence (figure 4.8). In SHM1, the Thr-225 (corresponding to Thr-227 of eSHMT) and Thr-222 (corresponding to Thr-224 of eSHMT) are replaced with valine and serine, respectively. In SHM2 the Thr-226 (corresponding to Thr-225 of eSHMT) is replaced by serine (figure 4.8). These alterations may lead to significant changes in substrate binding and complex formation in these enzymes.

It has been reported that L-alanine and D-alanine reacts with SHMT to undergo a slow half-transamination reaction resulting in the formation of apoenzyme, pyridoxamine-P, and pyruvate (Schirch et al., 1964). Experimentally this reaction is detected by a

![Figure 4.9](image-url)
decrease of the absorption at 420 nm (present in native SHMT) and appearance of an absorption peak at 327 nm on incubation of SHMT with L-alanine or D-alanine (Schirch et al., 1964). Incubation of approximately 100 mM L-alanine or D-alanine did not result in any change in absorption intensity at 410 nm for the native SHM1 or SHM2 (figure 4.9A and 4.9B), which demonstrates that both do not react with either SHM1 or SHM2.

L-cysteine has been shown to be an agent, which can be used to resolve the SHMT at neutral pH probably by combining with the enzyme-bound PLP and forming a more stable thiazolidine complex (Schirch et al., 1962). Experimentally, this reaction is detected by decrease of absorption at 410 nm (present in the native SHMT) and appearance of an absorption peak at 330 nm on incubation of SHMT with L-cysteine (figure 4.10A and 4.10B) Incubation of SHM1 and SHM2 with 100 mM L-cysteine resulted in absolute loss of absorption intensity at 410 nm for both the recombinant enzymes and appearance of an absorption peak at 330 nm demonstrating that the enzyme-bound PLP in SHM1 or SHM2 has been removed by L-cysteine.

Figure 4.10: Comparative analysis of functional properties of SHM1 (A) and SHM2 (B) in presence of L-cysteine at pH 7.6 and 25 °C. Effect of cysteine on the absorption spectra of SHM1 (A) and SHM2 (B). In both A and B the solid curves represent absorption spectrum of enzyme (15 μM) in 0.01 M potassium phosphate, pH 7.6, the dashed curve represents absorption spectra of enzyme after addition of 100 mM L-cysteine after incubation for 20 min at 25°C. The experimental details are given under “Experimental Procedures.”

Figure 4.11: pH-induced changes in enzyme activity of SHM1 (triangles) and SHM2 (circles). The results are expressed as percentage with the value observed at pH 7.8 taken as 100 percent. The experimental details are given under “Experimental Procedures.”
The changes in enzymatic activity of SHM1 and SHM2 with respect to change in pH were studied (figure 4.11). For both enzymes, bell-shaped curves centered at about pH 7.8 were observed. Furthermore, a complete loss of activity was observed at pH below 4.0 and above 10.0. These observations demonstrate that both SHM1 and SHM2 have similar pH optima for the enzymatic activity.

Comparative Denaturation Studies on SHM1 and SHM2

**Thermal Denaturation**—The thermal unfolding of SHM1 and SHM2 were characterized by monitoring the loss of secondary structure of enzymes with temperature. This was monitored the changes in CD ellipticity at 222 nm for SHM1 and SHM2 at increasing temperatures (figure 4.12). For both enzymes, a single irreversible sigmoidal transition was observed suggesting a cooperative thermal unfolding of native enzyme dimer to unfolded monomer. Furthermore, for both enzymes the transition was observed in the temperature region 40–80 °C, however, it was centered at about 60 and 69.4 °C for SHM1 and SHM2, respectively. As a difference of about 9 °C in the Tm was observed for the two enzymes it demonstrates that SHM2 has higher thermal stability than SHM1, which is probably because of the presence of a more compact native conformation having higher secondary structure than SHM1.

**pH-induced Denaturation**—Far-UV and visible CD studies on SHM1 and SHM2 at increasing pH were performed to study the pH-induced alterations in the secondary structure and the PLP-binding site of the two enzymes. In the pH range between 4 and 5,
aggregation was observed for both enzymes (data not shown).
However, at about pH 3.0, monomers of both enzymes were found to
be stabilized (figure 4.13A and 4.13B). In contrast, in the alkaline pH range significant
differences in the structural properties of the two enzymes were observed. Because of this
reason structural changes in the recombinant enzymes in the alkaline pH range are being
discussed in detail.

The effect of increasing pH, on secondary structure of SHM1 and SHM2 was
studied by monitoring the CD ellipticity at 222 nm between pH 6 and 11 (figure 4.14A).
For SHM2, a sigmoidal transition was observed between pH 8 and 10 with about 50 %
loss of the CD ellipticity at 222 nm associated with this transition. In contrast, for SHM1
no significant alteration in CD ellipticity at 222 nm was observed between pH 6 and 11.
These results demonstrate that secondary structure of native SHM1 is not significantly

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Figure 4.13: pH-induced alterations subunit configuration of SHM1 (A) and
SHM2 (B). SDS-PAGE profiles of glutaraldehyde cross-linked pH 3-10.5-treated
SHM1 (A) and SHM2 (B). In both the figures, lanes 1 and 2 represent molecular
weight markers, and uncross-linked native protein, respectively and lane 3-5
represent cross-linked samples of pH 3-, 7.5-, and 10- treated protein, respectively.
The experimental details are given under 'Experimental Procedures.'

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Figure 4.14: pH-induced changes in conformation of SHM1 and SHM2. A, pH-induced changes in
secondary structure of SHM1 (triangles) and SHM2 (circles) as monitored by following the changes in
ellipticity at 222 nm obtained from far-UV CD curves at increasing pH. B, pH-induced changes to
enzyme- bound PLP of SHM1 (triangles) and SHM2 (circles) as monitored by following the changes in
ellipticity at 425 nm obtained from visible CD spectra at increasing pH. In both, the results are expressed
as percentage with the maximum value observed taken as 100 percent. The experimental details are given
under ‘Experimental Procedures.’

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affected by enhancing the pH to 11.0, however, for SHM2 a significant denaturation occurs under similar conditions.

Visible CD signal at 425 nm was monitored between pH 6 and 11 to study the effect of increasing pH on enzyme bound PLP of SHM1 and SHM2 (figure 4.14B). For both enzymes, a sigmoidal transition for loss of CD signal in the visible region was observed in the alkaline pH region. However, for SHM2 the transition was observed between pH 6.5 and 11 centered at pH 8.0, whereas for SHM1 it was between pH 8 and 11 centered at about pH 9.25. These results demonstrate that compared with SHM2, the SHM1 shows higher stability against alkaline denaturation. Furthermore, for both SHM1 and SHM2 a complete loss of visible CD signal was observed at about pH 10 and above suggesting that at high pH the enzyme-bound PLP molecule gets dissociated from both recombinant enzymes resulting in stabilization of apoenzymes.

For studying the effect of alkaline pH on the molecular dimensions of the native SHM1 and SHM2, size exclusion chromatographic studies were carried out. The elution profile of SHM1 and SHM2 on the S-200 Superdex column between pH 7.5 and 10.0 were recorded (figure 4.15). For native SHM1 at pH 7.5, a
single peak with a retention volume of 14.06 ml corresponding to the dimeric species of enzyme was observed. On increase in pH from 7.5 to 10.5, no significant changes in the retention volumes of the native SHM1 were observed (figure 4.15A). This suggests that no significant alteration in the molecular dimension or subunit configuration of SHM1 occurs on enhancement of pH in alkaline region. This was confirmed by the glutaraldehyde cross-linking studies where a single protein band corresponding to that of the dimer of the enzyme was observed between pH 7.5 and 10.5 (figure 4.13A). In contrast for SHM2, on increase in pH from 7.5 to 9 an enhancement in the retention volume from 14.13 to 15.2 ml was observed (figure 4.13B). The enhancement in the retention volume for pH 9-treated SHM2 compared with the native enzyme is indicative of significantly reduced hydrodynamic radii for the stabilized enzyme intermediate under these conditions. On comparing the elution volume of pH 9.0-treated SHM2 with the molecular mass obtained for marker proteins in the size exclusion chromatography (with citrate/glycine/Hepes buffer), a molecular mass of about 45 kDa was obtained. As the SHM2 monomer has a molecular mass of about 45 kDa (figure 4.1), these observations indicate that alkaline treatment of SHM2 probably leads to dissociation of native dimer of enzyme resulting in stabilization of the enzyme monomer. This possibility was confirmed by the glutaraldehyde cross-linking studies where a single protein band corresponding to monomer of enzyme was observed on SDS-PAGE for pH 9-treated SHM2 (figure 4.13B).

Urea-Induced Denaturation—The urea-induced denaturation of SHM1 and SHM2 were studied by monitoring the changes in the secondary structure, the tertiary structure, and the enzyme-bound PLP of the recombinant proteins at increasing urea concentrations. The changes in the far-UV CD signal of SHM1 and SHM2 on treatment with increasing concentrations of urea were studied to see the effect on secondary structure (figure 4.16A). For both enzymes, an exponential decrease in CD ellipticity at 222 nm between 0 and 6 M urea and complete loss of CD signal above 6.5 M urea were observed. These observations suggest that both SHM1 and SHM2 undergo a continuous loss of secondary structure on treatment with increasing concentration of urea.

The urea-induced alterations in tertiary structure of recombinant proteins SHM1 and SHM2 was studied by monitoring the changes in the tryptophan fluorescence
emission wavelength maxima at increasing concentrations of urea (figure 4.16B). An exponential increase in tryptophans fluorescence emission wavelength maxima from 339 to 356 nm for both the SHM1 and SHM2 were observed with increasing urea concentrations between 0 and 5 M urea. These observations suggest that treatment of enzyme with increasing concentrations of urea lead to movement of partially buried tryptophans molecules present in the native enzyme toward the solvent (Lakowicz, 1983). Such a situation can arise only when the enzyme molecule undergoes unfolding on treatment with urea. The far-UV CD and tryptophan fluorescence results presented above collectively demonstrate that during the urea-induced denaturation a simultaneous unfolding of both the secondary and tertiary structures occurs in both SHM1 and SHM2.

The urea-induced changes in the enzyme-bound PLP of SHM1 and SHM2 were studied by monitoring the changes in the visible CD signal of the enzymes at increasing urea concentration (figure 4.16B). A sharp decrease in CD signal at 425 nm was observed (figure 4.16C).
observed between 0–1 and 0–1.2 M urea for SHM1 and SHM2, respectively. Above 1.25 M urea concentration, a complete loss of visible CD signal was observed for both recombinant enzymes suggesting that for both SHM1 and SHM2 the enzyme-bound PLP gets dissociated from the enzyme on treatment with about 1.25 M urea. However, at about 1.25 M urea only about 40% loss of CD ellipticity at 222 nm and partial exposure of tryptophans residues of enzyme to solvent was observed for the two enzymes. This indicates a possibility of stabilization of a partially unfolded apo-intermediate of SHM1 and SHM2 on treatment with low concentrations of urea.

![Figure 4.17: Urea-induced alterations in molecular dimension and subunit configuration of SHM1 and SHM2.](image)

**A**, size-exclusion chromatographic profiles for SHM1 on Superdex 200HR column at increasing urea concentration. The curves 1–4 represent profiles for 0, 0.8, 1.6, and 2 M urea-treated protein samples, respectively. **B**, size-exclusion chromatographic profiles for SHM2 on Superdex 200HR column at increasing urea concentrations. Curves 1–2 represent profiles for 0, 0.8, and 2 M urea-treated protein samples, respectively. For these studies buffer containing the required urea concentration is prepared and the columns were run at the same urea concentration at which the protein samples were incubated. **C** and **D** show SDS-PAGE profiles of glutaraldehyde cross-linked urea-treated SHM1 and SHM2, respectively. In both the figures, lanes 1 and 2 represent molecular weight markers and uncross-linked native protein, respectively, and lanes 3–6 represent cross-linked samples of 0, 0.8, 1.6, and 2 M urea-treated protein samples, respectively. The experimental details are given under "Experimental Procedures".
The effect of urea on the subunit configuration of SHM1 and SHM2 was studied by carrying out glutaraldehyde cross-linking studies at various urea concentrations (figure 4.17C and 4.17D). For 0.8 M urea-treated SHM1 single band is observed at dimer whereas SHM2 two protein bands corresponding to monomer and dimer, respectively, were observed. To check the molecular dimension at respective urea concentration, size exclusion chromatographic studies were carried out the S-200 Superdex column, which gives a single peak at 14.32 ml corresponding to the dimer in case of SHM1 whereas in case of SHM2, two peaks at 14.4 and 15.4 ml corresponding to slightly open dimer and monomer are observed. For 1.6 M urea-treated SHM2, a single protein band corresponding to monomer of enzyme was observed, whereas for SHM1 two protein bands corresponding to monomer and dimer, respectively, were observed, which also correlated the peaks obtained by size exclusion chromatographic studies. These observations in conjunction with the results of CD and tryptophans fluorescence studies demonstrate that treatment of SHM1 and SHM2 with low urea concentrations leads to dissociation of native dimer of these enzymes resulting in stabilization of an apomonomer.

The enzymatic activity of SHM1 and SHM2 was also studied with respect to the increasing urea concentration (figure 4.18). For both enzymes, a sharp decrease in activity is observed between signal at 425 nm was observed between 0–0.8 and 0–1 M urea for SHM1 and SHM2, respectively. Above 1.2 M urea concentration, a complete loss of activity was observed for both SHM1 and SHM2, which demonstrate that with loss in enzymes bound PLP the activity also decreases.

Guanidinium Chloride-Induced Denaturation—The GdmCl-induced denaturation of SHM1 and SHM2 was studied by monitoring the alterations in far-UV and visible CD and tryptophan fluorescence profiles of the two enzymes at increasing GdmCl
concentrations (figure 4.19). For both enzymes, a sharp exponential decrease in CD ellipticity at 425 nm with increasing GdmCl concentrations were observed between 0 and 1.25 M GdmCl. Above 1.5 M GdmCl a complete loss of visible CD signal for SHM1 and SHM2 were observed suggesting dissociation of the enzyme-bound PLP under these conditions (figure 4.19B). However, for CD ellipticity at 222 nm an initial sharp loss of about 60% of signal was observed between 0 and 0.5 M GdmCl followed by a gradual loss of ellipticity between 0.75 and 4 M GdmCl (figure 4.19A). As the GdmCl concentration at which the dissociation of enzyme-bound PLP was found to occur only about 60% loss of secondary structure of enzyme was observed, these observations indicate a possibility of stabilization of a partially unfolded intermediate during GdmCl denaturation of both enzymes (Prakash et al., 2002).

Figure 4.19: GdmCl-induced structural changes. A, GdmCl-induced changes in secondary structure of SHM1 (triangles) and SHM2 (circles) as monitored by following the changes in ellipticity at 222 nm obtained from far-UV CD curves at increasing concentrations of GdmCl. B, GdmCl-induced changes of enzyme-bound PLP of SHM1 (triangles) and SHM2 (circles) as monitored by following the changes in ellipticity at 425 nm obtained from visible CD spectra at increasing concentrations of GdmCl. C, changes in the wavelength of maximum fluorescence emission of tryptophans of SHM1 (triangles) and SHM2 (circles) versus GdmCl concentration. The inset shows the changes between 0 and 2 M GdmCl. In all these studies the results are expressed as percentage with the value observed for recombinant mSHMT at no urea taken as 100 percent. The experimental details are given under “Experimental Procedures”.
The stabilization of a partially unfolded intermediate of SHM1 and SHM2 during GdmCl-induced unfolding of enzymes was further supported by the tryptophan fluorescence studies (figure 4.19C). For both SHM1 and SHM2, two distinct transitions were observed between GdmCl concentrations of 0 and 6 M. For SHM1, the two transitions were in the GdmCl concentration ranges 0–1.5 and 1.5–6 M, whereas for SHM2 they were in the GdmCl concentration ranges 0–1 and 1.1–7 M. At GdmCl concentration about 5 M and above the tryptophan fluorescence emission wavelength

![Figure 4.20: GdmCl induced alterations in molecular dimension and subunit configuration of SHM1 and SHM2. A, size-exclusion chromatographic profiles for SHM1 on Superdex 200HR column at increasing GdmCl concentration. The curves 1–3 represent profiles for 0, 0.2 and 2 M GdmCl-treated protein samples, respectively. B, size-exclusion chromatographic profiles for SHM2 on Superdex 200HR column at increasing GdmCl concentrations. Curves 1–3 represent profiles for 0, 0.2 and 2 M urea-treated protein samples, respectively. For these studies Buffer containing the required GdmCl concentration is prepared and the columns were run at the same GdmCl concentration at which the protein samples were incubated. C and D show SDS-PAGE profiles of glutaraldehyde cross-linked urea-treated SHM1 and SHM2, respectively. In both figures, lanes 1 and 2 represent molecular weight markers and uncross-linked native protein, respectively, and lanes 3–5 represent cross-linked samples of 0, 0.2, and 2 M GdmCl-treated protein samples, respectively. The experimental details are given under "Experimental Procedures".](image-url)
maxima of about 354 nm was observed suggesting complete unfolding of enzymes under these conditions (Lakowicz, 1983).

The subunit configuration of the GdmCI-stabilized intermediate of SHM1 and SHM2 was characterized by glutaraldehyde cross-linking experiments and the results were confirmed by carrying out size exclusion size exclusion chromatographic studies on S-200 Superdex column (figure 4.20). For 0.2 M GdmCI-treated SHM1 and SHM2, two protein bands corresponding to dimer and monomer, respectively, of the recombinant enzymes were observed. However, for SHM1 the population corresponding to the monomer of the enzyme was significantly higher compared with that for the dimer (figure 4.20C). In contrast for SHM2 an almost equal population of the monomer and dimer was observed (figure 4.20D). With respect to size exclusion size exclusion chromatographic studies, for SHM1 two peaks at 14.4 and 15.8 are observed which differ in intensity corresponding to open dimer and monomer showing monomer population is more (figure 4.20A) whereas for SHM2, two peaks of equal intensity are observed at 14 and 15.2 ml, which correspond to partially open dimer and monomer of approximately equal population (figure 4.19B). The above reported observations collectively demonstrate that treatment of SHM1 and SHM2 with low concentrations of GdmCI lead to dissociation of the native dimer of enzymes along with dissociation of enzyme bound PLP, resulting in stabilization of apomonomer of enzymes.

The enzymatic activity of SHM1 and SHM2 was also studied with respect to the increasing GdmCI concentration (figure 4.21). For both enzymes, a sharp decrease in activity is observed between signal at 425 nm was observed between 0–0.5 and 0–0.75 M GdmCI for SHM1 and SHM2, respectively. Above 1.0 M GdmCI concentration, a complete loss of activity was observed for both SHM1 and SHM2, which demonstrate that with loss in enzymes bound PLP the activity also decreases.

Figure 4.21: GdmCI induced changes in enzyme activity of SHM1 (triangles) and SHM2 (circles). The activity is expressed as percentage with the value observed for recombinant mSHMT at no urea taken as 100 percent. The experimental details are given under "Experimental Procedures".
CONCLUSIONS

The results presented in this chapter demonstrate that *M. tuberculosis* contains two genes (gene duplication), which code for the same enzyme serine hydroxymethyltransferase. These enzymes had about 66% sequence identity and 74% sequence homology. Despite all this, they have significantly different structural, functional, and stability properties. The most significant structural difference between SHM1 and SHMTs from various other sources including SHM2 is the presence of 1 mol of PLP/enzyme dimer in SHM1 as compared with 2 mol of PLP/enzyme dimer for all other reported SHMTs. Such a change in the stoichiometry of PLP and enzyme in SHMTs would have significant consequences in the structure and stability of SHM1 as PLP has been shown to play a significant role in functional activity and stability of SHMTs (Venkatesha et al., 1998). Besides this, the SHM1 was found to have a lesser secondary structure and less compact conformation than the SHM2. Regarding the stability differences between the SHM1 and SHM2, it was observed that the SHM2 was more stable than SHM1 against thermal denaturation. However, SHM1 showed higher stability than SHM2 against alkaline denaturation. Comparative analysis of mammalian SHMTs (Renwick et al., 1998, Scarsdale et al., 1999, Venkatesha et al., 1998) as reported in literature and the *M. tuberculosis* SHMTs (both the SHM1 and SHM2) reported in this chapter suggest that significant differences exist in their structural and functional properties between these enzymes. For example, the mammalian SHMTs are tetramers, whereas the *M. tuberculosis* SHMTs are dimers. The mammalian SHMT contains the eight-residue conserved sequence VTTTTHK(Pyr)T near the active site lysyl residue (Lys-229 as in eSHMT) that forms the internal aldimine with pyridoxal phosphate. However, the *M. tuberculosis* SHMTs show significant changes in the conserved threonine sequence in the octapeptide (Figure 3). These unique features of *Mycobacterium* SHMTs compared with the mammalian SHMTs can be exploited for designing drugs against *M. tuberculosis*. 