Chapter-5

Serine hydroxymethyltransferase from *Mycobacterium laprae*
ABSTRACT

From the genome analysis of the *Mycobacterium laprae* a putative gene *GlyA* (ML1953) has been proposed to encode for the enzyme serine hydroxymethyltransferase. We have cloned, over-expressed and purified to homogeneity the protein product of the *GlyA* gene of *M. laprae*. The recombinant mISHMT exists as homo-dimer of molecular mass about 90 kDa under physiological conditions and contains one mole of PLP per mole of enzyme dimer. Studies on catalytic properties of mISHMT shows that the enzyme catalyzes the H₄ folate dependent retro-aldol cleavage of L-Serine however, like SHMTs reported from same genus *Mycobacterium tuberculosis*, D-alanine dependent transaminase activity was found to be absent in the enzyme but L-cysteine removed the prosthetic group, PLP, from the recombinant enzymes leaving its respective inactive apoenzyme. The detailed analysis of the enzyme kinetic for hydroxymethyltransferase reaction it was observed that the mISHMT has *Kₘ* value for L-Serine comparable to that reported for SHMTs from other sources. However, a significantly lower catalytic efficiency (*kₐₑₜ/Kₘ*) for the reaction was observed with mISHMT which is slightly less than that reported for *M. tuberculosis* SHMTs which suggests that mycobacterium SHMTs (mISHMT, SHM1 and SHM2) in general have lower *kₐₑₜ/Kₘ* values. The mISHMT was found to be less stable than SHM1 both against thermal and pH denaturation. Structural studies suggested that the lesser stability of mISHMT against pH denaturation was probably due to loose binding of cofactor PLP to the enzyme. The urea- and guanidinium chloride- induced dissociation of mISHMT dimer into monomer at low denaturant concentrations resulting in loss of enzymatic activity.
INTRODUCTION

Leprosy, one of the oldest recorded diseases, remains a major public health problem especially in Asian and African countries. It is a chronic human neurological disease, results from the infection with the obligate intracellular pathogen *Mycobacterium leprae*, which is a close relative of the tubercle bacillus, is encrypted in its genome. *M. leprae* is an exceptional bacterium because of its longest doubling time of all known bacteria and no growth in artificial media (Shepherd, 1960). Entire sequencing of the bacillus genome showed that it possesses a single circular chromosome that differs from other known mycobacterium chromosomes with regard to size (3.2 Mb) genes encoding proteins (1,605) and for stable RNA species (50) and G + C content (57.8%) (Cole et al., 2001).

Comparison with the genome sequence of *Mycobacterium tuberculosis* (table 5.1) revealed an extreme case of reductive evolution, since less than half of the genome contains functional genes while inactivated or pseudogenes (inactive reading frames with functional counterparts in *M. tuberculosis*) are highly abundant. Disappearance of numerous enzymatic pathways in comparison with *M. tuberculosis*, an intracellular pathogen comparable to *M. leprae*, could explain the differences observed between the two organisms (Cole et al., 1999, 2001).

Table 5.1-Comparison of genome of *M. leprae* and *M. tuberculosis* (Cole et al., 2001)

<table>
<thead>
<tr>
<th>Feature</th>
<th>M. leprae</th>
<th>M. tuberculosis</th>
</tr>
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<tbody>
<tr>
<td>Genome size (bp)</td>
<td>3,268,203</td>
<td>4,411,532</td>
</tr>
<tr>
<td>G+C (%)</td>
<td>57.79</td>
<td>65.61</td>
</tr>
<tr>
<td>Protein coding (%)</td>
<td>49.5</td>
<td>90.8</td>
</tr>
<tr>
<td>Protein-coding genes (no.)</td>
<td>1,604</td>
<td>3,959</td>
</tr>
<tr>
<td>Pseudogenes (no.)</td>
<td>1,116</td>
<td>6*</td>
</tr>
<tr>
<td>Gene density (bp per gene)</td>
<td>2,037</td>
<td>1,114</td>
</tr>
<tr>
<td>Average gene length (bp)</td>
<td>1,011</td>
<td>1,012</td>
</tr>
<tr>
<td>Average unknown gene length (bp)</td>
<td>338</td>
<td>653</td>
</tr>
</tbody>
</table>

The level of gene duplication was approximately 34% and, on classification of the proteins into families, the largest functional groups were found to be involved in the metabolism and modification of fatty acids and polyketides, transport of metabolites, cell envelope synthesis and gene regulation. Reductive evolution, gene decay and genome downsizing have eliminated entire metabolic pathways, together with their regulatory...
circuits and accessory functions, particularly those involved in catabolism. This may explain the unusually long generation time and account for our inability to culture the leprosy bacillus (Eiglmeier et al., 2001).

The information deduced from the genome sequence of Mycobacterium leprae is of immense value for the chemotherapy of leprosy. Knowing the complete set of genes, enzymes and proteins allows us to understand why some drugs are without effect whereas others are fully active. It may also enable better use to be made of existing drugs, such as β-lactams, and opens new avenues for the development of novel compounds (Grosset et al., 2001). M. leprae is relatively susceptible to a wide range of drugs, unlike the highly related tubercle bacillus, and several new multidrug regimens are in clinical trials. Genomic analysis of the leprosy bacillus also provided insight into the molecular basis for resistance to various antibiotics and allowed identification of several potential targets for new drug treatments. For most of M. leprae proteins, the primary amino acid sequence (obtained on translation from the gene sequence) shows about 90 percent sequence homology with the homologous M. tuberculosis proteins (Poulet et al., 1995). So the development of new tuberculosis drugs and vaccines should also benefit directly to the leprosy treatment from our comparative genomic analysis through definition of the core mycobacterial genes.

However, in spite of such high sequence homology significant differences have been observed in their structural and stability properties (Cole et al., 2001). Hence, in order to have a better understanding of the biochemistry of M. leprae, it is essential to have detailed structural and functional characterization of the proteins from this organism.

From the genome search of mycobacterium species namely the M. tuberculosis, M. leprae and M. bovis putative genes encoding for SHMT have been proposed. From M. tuberculosis genome two genes namely the GlyA and GlyA2 have been proposed as encoding for SHMTs in the organism (Chaturvedi et al., 2003). In contrast, for M. leprae and Mycobacterium bovis, a single putative gene namely GlyA and GlyA2, respectively, have been proposed to encode for the enzyme SHMT. The primary amino acid sequence of the GlyA gene of M. leprae and GlyA2 gene of M. bovis shows a very high sequence identity (about 89.9 percent) (figure 1) to the protein product of GlyA and GlyA2 gene respectively, of M. tuberculosis. The structural and functional properties of SHMT1 and
SHMT from *M. laprae*

SHMT1 and SHMT2 are the protein products of *GlyA* and *GlyA2* genes respectively, of *M. tuberculosis* have been discussed in detail in chapter 4 (Chaturvedi et al., 2003). The SHMT1 protein is a unique SHMT as it contains one mole of PLP per mole of dimer unlike other SHMTs from euukaryotes or prokaryotes or SHMT2 from *M. tuberculosis*, which contains two mole of PLP per mole of dimer or four mole of PLP per mole of tetramer.

M. *laprae*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>SHMT1</td>
<td>MSLAPEVDPDIAEELGKELGRORUTELEIASENFVPVRGQLAQGSGVLTSKYNAYEGFGRRYGDCEHVENVIL</td>
</tr>
<tr>
<td>SHMT2</td>
<td>MSLAPEVDPDIAEELGKELGRORUTELEIASENFVPVRGQLAQGSGVLTSKYNAYEGFGRRYGDCEHVENVIL</td>
</tr>
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</table>

Figure 5.1: Alignment of the amino acid sequence of SHMTs from *M. laprae* (mISHMT) and SHMT1 of *M. tuberculosis*. The alignment was done with the software CLUSTALW. The light shaded letters indicate homologous amino acids and dark shaded indicate non-homologous amino acids. The non-shaded letters indicate identical amino acids.

Searches of the *Mycobacterium laprae* genome (http://www.sanger.ac.uk/Projects/M_laprae/) for enzymes related to SHMT have lead to the identification of a single gene namely *GlyA* which have been proposed as putative genes encoding for the enzyme, serine hydroxymethyltransferase (SHMT). We have cloned, over-expressed and purified to homogeneity the respective protein product of *GlyA* gene of *M. laprae* and named it mISHMT. This chapter discusses the structural and catalytic properties of recombinant mISHMT along with the stability studies against thermal, pH, urea and GdmCl denaturation have also been reported.
RESULTS AND DISCUSSION

Expression and Purification

GlyA gene of Mycobacterium laprae was cloned and its respective protein product (mISHMT) was overexpressed by the method described in “Experimental Procedures.” A good expression of mISHMT was obtained and the expressed protein was present predominantly (>90%) in the soluble fraction. The enzyme was purified from the soluble fraction by the method described under “Experimental Procedures.” A yield of about 14-16 mg/liter of purified enzyme was obtained. The purified protein on SDS-PAGE when stained with Coomassie blue showed a single band (figure 5.1) and a single symmetrical peak was observed on ESI-MS (figure 5.2) showing that the purified protein was greater than 95 percent pure.

The purified enzymes were assayed for SHMT activity (Taylor et al., 1965, Manohar et al., 1982). The purified recombinant mISHMT shows a ratio was 4.64 for 280/425 absorbance, 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 mass of the purified recombinant mISHMT was determined under non-dissociating conditions using the data of gel filtration experiment (Andrews, 1965). mISHMT on a Superdex S-200 column calibrated with the various molecular weight standards, showed a single peak with a retention volume of 14.05 ml (figure 5.4), which corresponds to a molecular mass of 89 kDa for mISHMT. The predicted subunit size of mISHMT based on the amino acid sequence is 45.23 kDa. For purified mISHMT on SDS-PAGE, a single homogeneous protein band corresponding to molecular weight of about 45 kDa was observed (figure 5.3). The ESI-MS showed a molecular mass of 45.2 kDa for the protein.
The results of the studies on subunit mass along with the size exclusion chromatography (SEC) as reported above demonstrate that mlSHMT exists as dimer under physiological conditions.

Content of PLP

SHMT is a PLP-dependent enzyme. 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). Due to the presence of protein-bound...
PLP in SHMT, a unique peak at 425 nm is observed in the visible CD spectra of enzyme, which disappears on removal of PLP from the enzyme or on unfolding of the enzyme (Cai et al., 1995). A visible CD spectra centered at about 430 nm, showing the presence of the enzyme bound PLP, was observed (figure 5.5). The amount of PLP bound to the mlSHMT dimer was determined 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 mlSHMT was found to contain 1±0.2 mole of PLP per mole of enzyme dimer.

The SHMT from E. coli as well as from other bacterial sources are dimeric and contain 2 mole of PLP per mole of enzyme dimer (Venkatesha et al., 1998). However, SHM1 of M. tuberculosis (Chaturvedi et al., 2003) and now mlSHMT of M. laprae are the only two SHMTs that have been found to contain one mole of PLP per mole of enzyme dimer.

Catalytic Properties of mlSHMT

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 of M. tuberculosis (Chaturvedi et al., 2003) and this mlSHMT are 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. As mlSHMT like SHM1 has a unique cofactor to protein ratio we thought it worthwhile to study the catalytic properties of these enzymes.
**Hydroxymethyltransferase Reaction**- The mlSHMT catalyzed the H$_2$-folate dependent retro-aldol cleavage of L-Serine to form Glycine and 5,10-CH$_2$-H$_2$-folate with a specific activity of 0.96 U/mg. This value was significantly lower compared to that reported for SHMT from other sources except SHMTs from M. tuberculosis (Table 4.1). This mlSHMT like SHMTs from Mycobacterium tuberculosis also showed significantly lower k$_{cat}$ values of 0.98 sec$^{-1}$. The K$_m$ values for L-Serine observed for the mlSHMT was 1.03 mM (figure 5.6), which were similar to that observed for other SHMTs (Table 4.1). The catalytic efficiency of mlSHMT is 0.95 mM$^{-1}$sec$^{-1}$ in comparison to 1.22 mM$^{-1}$sec$^{-1}$ and 1.88 mM$^{-1}$sec$^{-1}$ for SHM1 and SHM2 of M. tuberculosis, 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 mycobacterium SHMTs, the enzymes having one mole of PLP per mole of enzyme dimer (i.e. mlSHMT and SHM1) have lower catalytic efficiency than those having two mole of PLP per mole of enzyme dimer (i.e. SHM2) and SHMT from M. laprae like other proteins reported have lower catalytic efficiency in comparison to M. tuberculosis proteins.

**Transaminase Activity**- In addition to the physiological reaction of reversible interconversion of serine to glycine, SHMT also catalyses the transamination of D- and L-alanine resulting in the formation of apo-enzyme, pyridoxamine-P and pyruvate (Schirch et al., 1964). This reaction is detected by a decrease of the absorption at 425 nm (present in native SHMT) and appearance of an absorption peak at 327 nm on incubation of SHMT with D-alanine (Schirch et al., 1964). Incubation of up to 100 mM of L-alanine or D-
alanine for even 20 minutes did not result in any change in absorption intensity at 425 nm for mlSHMT (figure 5.7A) demonstrating that both D-alanine does not react with the enzyme. This property of mlSHMT is similar to that of *Mycobacterium tuberculosis* enzymes SHM1 and SHM2 reported earlier (Chaturvedi *et al*., 2003).

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). Incubation of mlSHMT with 100 mM of L-cysteine for about 20 minutes resulted in absolute loss of absorption intensity at 425 nm for the native mlSHMT and an appearance of an absorption peak at 335 nm demonstrating that the enzyme-bound PLP in mlSHMT has been removed by L-cysteine (figure 5.7B).

![Figure 5.7: Functional properties of mlSHMT. A, Effect of L-alanine and D-alanine on the absorption spectra of mlSHMT, 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-alanine and the dotted curve represents absorption spectra of enzyme after addition of 100 mM D-alanine. B, Effect of cysteine on the absorption spectra of mlSHMT, 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. All samples were incubated for 20 min at 25°C. The experimental details are given under “Experimental Procedures”.](image)

**Structural Properties of the Recombinant mlSHMT**

Studies on the model polypeptide and proteins have demonstrated that the α-helical and β-sheet proteins show characteristic far-UV CD spectra, with α-helical proteins having two minima at 222 and 208 nm and β-sheet proteins having a single minima at 216 nm (Chen *et al*., 1972). Hence, for analyzing the secondary structure present in mlSHMT, far-UV CD studies were carried. A far UV-CD spectra characteristic of a protein with both α-helix and β-sheet secondary structure was observed for mlSHMT (Figure 5.8A).
According to the primary amino acid sequence, mlSHMT has tryptophan molecules at position 172, 195 and 421. A good fluorescence intensity with emission wavelength maximum at about 339 nm was observed for tryptophan fluorescence of mlSHMT (figure 5.8B). The buried tryptophan residues in folded protein show fluorescence emission maxima at 330 to 335 nm whereas, on unfolding of protein the tryptophan fluorescence emission maxima shifts to 350 to 355 nm (Lakowicz, 1983). Based on these facts it seems that the tryptophan residues in mlSHMT are not significantly buried in the protein core but partially exposed to the solvent.

The reduction of the PLP aldimine bond leading to formation of PyP results in an absorbance maxima at 355 nm that overlaps the emission spectrum of tryptophan (Manohar et al., 1982). Hence, the PyP can be used as an energy acceptor of tryptophan fluorescence and FRET studies can be carried out. For mlSHMT in the native conformation two emission maxima, one around 339 nm (tryptophan fluorescence) and the other at 380 nm (PyP emission resulting from FRET) were observed (figure 5.8C). However, on unfolding of enzymes by incubation in 8 M urea only single emission

Figure 5.8: Structural properties of mlSHMT at pH 7.6 and 25°C. A, Far-UV CD spectra of recombinant protein. B, Tryptophan fluorescence emission spectra of recombinant mlSHMT after excitation at 290 nm at pH 7.6 (solid line) and unfolded enzyme, in presence of 8 M urea (dotted line). C, Fluorescence emission spectra of recombinant PyP-mlSHMT after excitation at 290 nm, pH 7.6 (solid line) and unfolded, in presence of 8 M urea, PyP-mlSHMT (dotted line). The experimental details are given under "Experimental Procedures".
maxima at 352 nm was observed. These observations demonstrate that for mlSHMT in the native conformation there is FRET between tryptophan residues and the bound PLP suggesting that the tryptophan residues in native conformation of enzyme are located less than 5 Å from the cofactor PLP.

Stability Studies on mlSHMT

**Thermal Stability**—The thermal stability of mlSHMT was characterized by monitoring the loss of secondary structure of enzyme with increasing temperature. The changes in CD ellipticity at 222 nm were monitored at increasing temperature at the rate of 1°C per minute to study the unfolding pathway for mlSHMT. A single irreversible sigmoidal transition centered at about 54°C was observed for mlSHMT suggesting that the protein undergoes cooperative thermal unfolding (figure 5.9). However, the T_m is about 6°C lesser than that observed for SHM1 (60°C) thus demonstrating that mlSHMT is thermally less stable than SHM1.

**pH Stability**—The pH dependent changes in enzymatic activity, PLP microenvironment and secondary structure of mlSHMT were studied by incubating the enzyme samples at varying pH and then measuring the enzymatic activity and monitoring the changes in the visible and far-UV CD spectra, respectively. For changes in enzyme activity (Figure 5.10) and visible CD signal (Figure 5.11A), a bell shaped curve centered at about pH 7.8 and 8.3 respectively, were observed. The interesting observation was that a slight shift in pH from the optima resulted in significant loss of both the enzymatic activity and visible CD signal. An almost complete loss of enzyme activity and visible CD signal were observed at

![Figure 5.9: Thermal denaturation of native recombinant mlSHMT measured by loss of CD ellipticity at 222 nm. A linear extrapolation of baselines in pre- and post-transitional regions was used to determine the fraction-unfolded protein within the transition region by assuming a two-state mechanism of unfolding. The thermal transition was found to be irreversible and the precipitation of the recombinant enzymes was observed at about 90 °C irrespective of the buffer used. The experimental details are given under “Experimental Procedures”.](image-url)
pH below 6.0 and 10 and above. These observations suggest that in mLISHMT the loss of protein bound PLP and the enzyme activity occurs simultaneously. For SHM1, a significantly higher stability of enzyme to acidic pH is observed as even at pH 6 about 80 percent residual enzymatic activity has been reported (Chaturvedi et al., 2003). The above reported observations suggest that in mLISHMT, the interaction between the PLP cofactor and the protein amino acid side chain residues are much weaker as compared to those present in SHM1 due to which protein bound PLP gets detached easily from mLISHMT at slightly acidic pH. However, only a slight variation, maximum loss of about 30%, in CD ellipticity at 222 nm was observed for mLISHMT in pH range 6 to 11 (Figure S.11B) suggesting that the enzyme secondary structure is significantly resistant to alkaline pH induced modifications.

This was further supported by studying the effect of pH on the molecular dimensions and subunit configuration on the native protein using SEC and glutaraldehyde crosslinking. The elution profile of mLISHMT on the S-200 Superdex column between pH 7.5 and 10.0 were recorded (figure S.11C). For native mLISHMT at pH 7.5, a single peak with a retention volume of 14.6 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 mLISHMT were observed (figure S.11C). 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 crosslinking studies where a single protein band corresponding to that of the dimer of the enzyme was observed between pH 7.5 and 10.5 However, at about pH 3.0, monomers was found to be stabilized. These studies demonstrate that the dimeric configuration of mLISHMT is maintained in the pH range 7 to 11 (Figure S.11D). The above-presented results suggest that the pH dependent loss of activity of mLISHMT is due to detachment of
protein bound PLP under these conditions and not due to significant structural change in the protein.

![Figure 5.11](image)

**Figure 5.11**: pH-induced alterations in structure and subunit configuration of mlSHMT at 25°C. A, pH-induced changes in microenvironment of enzyme bound PLP of mlSHMT as monitored by following changes in ellipticity at 425 nm obtained from near-UV CD curves at increasing pH. B, pH-induced changes in secondary structure of mlSHMT as monitored by following changes in ellipticity at 222 nm obtained from far-UV CD curves at increasing pH. In both, the results are expressed as percentage with the value observed at pH 8.5 taken as 100 percent. C, Size-exclusion chromatographic profiles for mlSHMT on superdex 200HR column at increasing pH. The curves 1 and 2 represent profiles for mlSHMT incubated in buffer of pH 7.6 and 10.5, respectively. The columns were run at the pH at which the protein samples were incubated. D, SDS-PAGE profiles of glutaraldehyde cross-linked pH 7.6 and 10.5 incubated protein samples. The lanes 1 and 2 represent molecular weight markers, pH 7.6 uncrosslinked protein respectively, and lanes 3 to 5 represent crosslinked samples of pH 3, 7.6 and 10 incubated protein samples, respectively. For these studies citrate, glycine, Hepes buffer (10 mM each) was used and the desired pH was maintained with 1 N NaOH or 1 N HCl. The experimental details are given under “Experimental Procedures”.

**Urea–induced Denaturation**—The stability of mlSHMT against urea denaturation was studied by monitoring the loss of enzymatic activity, secondary structure, tertiary structure and enzyme bound PLP of protein with increasing denaturant concentration (figure 5.12).
Figure 5.12: Urea-induced alterations in activity, structure, molecular dimension and subunit configuration of mISHMT. A, Urea-induced changes in enzyme activity of mISHMT. B, Urea-induced changes in secondary structure of mISHMT as monitored by following changes in ellipticity at 222 nm obtained from far-UV CD curves at increasing urea concentration. C, urea-induced changes of enzyme-bound PLP of mISHMT as monitored by following the changes in ellipticity at 425 nm obtained from visible CD spectra at increasing the concentrations of urea. D, changes in the wavelength of maximum fluorescence emission of tryptophans of mISHMT versus increasing urea concentration. In all these studies the results are expressed as percentage with the value observed for recombinant mISHMT at no urea taken as 100 percent. E, size-exclusion chromatographic profiles for mISHMT 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. 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. F, SDS-PAGE profiles of glutaraldehyde cross-linked urea-treated mISHMT. 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".
For urea-induced changes in the enzyme activity of mISHMT, a sharp exponential decrease from 100 to almost complete loss of activity was observed between 0 to 2 M urea (figure 5.12A). Similarly, a sharp decrease in CD signal at 425 nm was observed between 0–1.8 M urea and above 2.0 M urea concentration, a complete loss of visible CD signal was observed the recombinant enzyme suggesting the enzyme-bound PLP gets dissociated from the enzyme on treatment with about 2.0 M urea (figure 5.12B). However, under these conditions only about 60 percent loss of secondary structure (figure 5.12C) and partial exposure of tryptophans residues of enzyme to solvent was observed for the enzyme (figure 5.12D), which suggests that there is a significant structure left in the enzyme.

The molecular dimension and subunit configuration of the mISHMT under conditions where the enzyme activity is completely lost during denaturant treatment was studied by size exclusion chromatographic and glutaraldehyde cross-linking experiments, respectively. For 0.8 M urea-treated glutaraldehyde crosslinked mISHMT, single band is observed (figure 5.12F), which gives a single peak at 14.32 ml corresponding to the dimer (figure 5.12E). For 1.6 M urea-treated crosslinked enzyme a significant population of monomeric species along with the dimeric species of enzyme is observed which also correlated the peaks obtained by size exclusion chromatographic studies. These studies suggest that treatment of mISHMT with low concentrations of the denaturants results in dissociation of dimeric enzyme into monomers.

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.

**Guanidinium Chloride-induced Denaturation**—The GdmCl-induced denaturation of SHM1 and SHM2 was studied by monitoring the loss in enzymatic activity, alterations in far-UV and visible CD and tryptophan fluorescence profiles of the two enzymes at increasing GdmCl concentrations (figure 5.13). A sharp exponential decrease in CD ellipticity at 425 nm with increasing GdmCl concentrations was observed between 0 and 1.0 M GdmCl. Above 1.25 M GdmCl a complete loss of visible CD signal for mISHMT
was observed suggesting dissociation of the enzyme-bound PLP under these conditions (figure 5.13B). Similarly, a sharp exponential decrease from 100 to almost complete loss of activity was observed between 0 to 0.75 M GdmCl suggesting that loss of PLP leads to loss in activity of protein (figure 5.13A). 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 5.13C). 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 were supported by tryptophan fluorescence studies, which show at that till 1 M GdmCl there is partial exposure while at GdmCl concentration of about 4 M and above the tryptophan fluorescence emission wavelength maxima of about 354 nm was observed suggesting complete unfolding of enzymes under these conditions (figure 5.13D). These studies indicate a possibility of stabilization of a partially unfolded intermediate during GdmCl denaturation of both enzymes (Prakash et al., 2002).

The subunit configuration of the GdmCl-stabilized intermediate mlSHMT 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 5.13E). For 0.4 M GdmCl-treated crosslinked SHMT, two protein bands corresponding to dimer and monomer, respectively, of the recombinant enzyme were observed. With respect to size exclusion size exclusion chromatographic studies, for SHM1 two peaks at 14.4 and 15.6 are observed which differ in intensity corresponding to open dimer and monomer showing monomer population is more (figure 5.13F).

The above reported observations collectively demonstrate that treatment of mlSHMT with low concentrations of GdmCl lead to dissociation of the native dimer of enzymes along with dissociation of enzyme bound PLP, resulting in stabilization of apomonomer of enzymes.
Figure 5.13: GdmCl-induced alterations in activity, structure, molecular dimension and subunit configuration of mISHMT. A, GdmCl-induced changes in enzyme activity of mISHMT. B, GdmCl-induced changes in secondary structure of mISHMT as monitored by following changes in ellipticity at 222 nm obtained from far-UV CD curves at increasing urea concentration. C, GdmCl-induced changes of enzyme-bound PLP of mISHMT as monitored by following the changes in ellipticity at 425 nm obtained from visible CD spectra at increasing the concentrations of urea. D, changes in the wavelength of maximum fluorescence emission of tryptophans of mISHMT versus increasing GdmCl concentration. In all these studies the results are expressed as percentage with the value observed for recombinant mISHMT at no urea taken as 100 percent. E, size-exclusion chromatographic profiles for mISHMT on Superdex 200HR column at increasing GdmCl concentration. The curves 1–3 represent profiles for 0, 0.4 and 2 M GdmCl-treated protein samples, respectively. For these studies buffer containing the required GdmCl concentration is prepared and the columns were run at the same urea concentration at which the protein samples were incubated. F, SDS-PAGE profiles of glutaraldehyde cross-linked GdmCl-treated mISHMT. Lanes 1 and 2 represent molecular weight markers and uncross-linked native protein, respectively, and lanes 3–5 represent cross-linked samples of 0, 0.4, and 2 M GdmCl-treated protein samples, respectively. The experimental details are given under "Experimental Procedures".
CONCLUSIONS

The studies presented in this chapter demonstrate that the mlSHMT is very similar to its 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 mSHMT. The light shaded letters indicate the amino acids involved in binding of enzyme to the PLP.
to the SHM1 of the *Mycobacterium tuberculosis* both in terms of cofactor to protein ratio i.e. one mole of PLP per mole of enzyme dimer and also the catalytic activity and the kinetic parameters associated with the hydroxymethyltransferase activity. This is because these enzymes had about 89% sequence identity and 95% sequence homology. Despite of all this they have significantly different structural, functional and stability properties. It had secondary structure comparable to SHM1 but was less stable than SHM1 against thermal denaturation. mlSHMT behaved similarly to SHM1 against alkaline denaturation. MI SHMT shows higher stability as compared to SHM1 at low concentrations of urea but behave similarly at higher concentration. The GdmCl denaturation curves are approximately same. In the sequence comparison of the SHMTs from various sources (Figure 9) it can be seen that all the SHMTs other than the SHM1 and mlSHMT contain the eight residue conserved sequence V-T-T-T-T-H-K(Pyr)-T near the active-site lysyl residue (K229 as in eSHMT) that forms the internal aldimine with pyridoxal phosphate. The *Mycobacterium tuberculosis* SHM1 and the mlSHMT are the only two enzymes that show significant changes in the conserved threonine sequence in the octapeptide. This might be a possible reason for these two SHMTs to have a unique cofactor to protein stoichiometry of one mole of PLP per mole enzyme dimer rather than the conventional stoichiometry of two mole of PLP per mole of enzyme dimer as observed for other SHMTs.