Chapter 4

Methionine aminopeptidases from M. tuberculosis: Biochemical characterization
Methionine aminopeptidases from *M. tuberculosis:*
Biochemical characterization

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DISCUSSION 67-69
MetAPs catalyze the irreversible and co-translational removal of the initiator methionine from the polypeptide chain, if the penultimate residue is non-bulky and uncharged (Sherman et al., 1985). In prokaryotes and eukaryotic organelles the removal of methionine is preceded by the catalysis of N-formyl group attached to it by another enzyme, PDF (Solbiati et al., 1999, Saxena and Chakraborti, 2005). The activity of PDF is indispensable for MetAP to perform its regular function.

MetAPs are ubiquitous in distribution and are highly conserved throughout the phylogeny. They were initially grouped in two classes, MetAP1 and MetAP2 on the basis of sequence comparison. The type 2 enzymes, in contrast to type 1 have an approximately 60 amino acid long α helical domain inserted within the catalytic region of the enzyme (Arfin et al., 1995; Bradshaw et al., 1998). This helical subdomain shares neither sequence nor structural homology with any other known protein. MetAPs are further divided in two subclasses on the basis of absence (subclass a) or presence (subclass b) of N-terminal extension (Bradshaw et al., 1998). The N-terminal extension present in type 1b contains two zinc finger motifs while that of type 2b has alternate stretches of polyacidic and polybasic residues. Recently, two new subclasses, type 1c and 2c, have been introduced which contain a ~40 amino acid long N-terminal extension with neither zinc finger motif, nor polyacidic/polybasic residues (Addlagatta et al., 2005; Alvarado et al., 2009).

As mentioned in the earlier chapter, M. tuberculosis has two genes mapA (Rv0734) and mapB (Rv2861c) encoding MtMetAP1a and MtMetAP1c respectively. Although, crystal structure of MtMetAP1c (Addlagatta et al., 2005) was reported as an apoenzyme and in complex form, biochemical characterization of the two MtMetAPs was not performed until very recently (Zhang et al., 2009; Chai et al., 2009; Lu et al., 2010; Olaleye et al., 2010; Lu and Ye, 2010). However, detailed structure-function analysis has not yet been carried out. The estimation of the promoter activity of the two putative promoters for the genes encoding these two metalloproteases revealed that the mapA promoter is more active than that of mapB (chapter 3). Therefore, it is fascinating to see the biochemical features of both the proteins. This chapter deals with the cloning, expression and purification of the two mycobacterial MtMetAP1s. Further, the biochemical characteristics of the two mycobacterial MetAPs has been compared to find out if there are any differences in
the functionality of the two proteins. An attempt has been made to find the critical residues for the activity of these enzymes.

4.1 Isolation of mapA and mapB from M. tuberculosis

The genomic DNA from M. tuberculosis strain H37Ra was isolated as described in chapter 2 (Section 2.2.4.1) and was utilized to amplify both mapA and mapB genes through PCR. For this purpose based on published genome sequence (Cole et al. 1998), gene specific primers incorporating restriction sites were designed (primers CK74 with HindIII and CK140 with Nhel sites for mapA; primers CK64 with Ndel and CK65 with HindIII sites for mapB; Table 2.3 for primer sequences). PCR amplification was carried out as mentioned in Chapter 2 (Section 2.2.1.7) using Herculase II fusion DNA polymerase (Stratagene). The samples containing template DNA, primers and enzyme exhibited the amplification of desired fragments. As expected, amplified mapA and mapB genes were ~ 801bp and ~ 858bp respectively, in size (Fig. 4.1 A and Fig. 4.1 B).

The ~ 801bp and ~ 858bp PCR products were digested, gel purified and ligated at Nhel/HindIII and Ndel/HindIII sites of pET-28c respectively (Fig. 4.1C). The resulting products were transformed into E. coli strain DH5α. Plasmid DNA was isolated from different clones and analyzed by restriction enzyme digestion. The constructs used in this study (pET-mapA as well as pET-mapB) were finally confirmed by DNA sequencing using an automated sequencer with T7 forward and reverse primers or gene specific primers (Tables 2.3 and 2.5). It is worth mentioning at this juncture that nucleotide as well as nucleotide derived amino acid sequences of both (mapA and mapB) exhibited 100% identity between M. tuberculosis strains H37Ra (non pathogenic) and H37Rv (pathogenic). This is expected as these are the house keeping genes.

4.2 Expression and purification of MtMetAP1a and MtMetAP1c from M. tuberculosis as a histidine tagged protein

For the expression as His-tagged proteins, plasmid DNA of pET-mapA and pET-mapB isolated from E. coli strain DH5α was transformed into strain BL21 (DE3). They were selected on LB kanamycin (50 µg/ml) plates. One such colony of
Fig. 4.1 Cloning of *mapA* and *mapB*. (A) PCR amplification of *mapA* from *M. tuberculosis* genomic DNA. Lane 1: 100 bp ladder, lane 2: amplified *mapA*. (B) PCR amplification of *mapB* from *M. tuberculosis* genomic DNA. Lane 1: 100 bp ladder, lane 2: amplified *mapB*. (C) Cloning strategy. Cloning of the amplified *mapA* and *mapB* PCR products in the pET-28c. *mapA* cloned at *Nhe*I/*Hind*III and *mapB* at *Nde*I/*Hind*III site in pET-28c.
each plasmid was used for subsequent studies. An overnight culture (≈ 14 h at 37°C) of a single colony harbouring the expression plasmid was re-inoculated in fresh media using 1% inoculum and grown to an OD$_{600}$ of ≈ 0.7. Cells were then induced with 0.4 mM IPTG for 12 h at 16°C and harvested (5000 x g/10 min/4°C). The pellet was washed once with buffer (50 mM Tris pH 7.5 containing 150 mM NaCl) and resuspended in lysis buffer (Table 2.1). Cells were lysed by sonication (duration 10 min; cycle of 10 sec on, 15 sec off) followed by centrifugation at 13000 x g for 30 min at 4°C. The supernatant fraction was further loaded on a Ni-NTA affinity column, washed with 10 bed volumes of 100 mM Tris pH 7.5 containing 300 mM (for *MtMetAP1a*) or 200 mM (for *MtMetAP1c*) NaCl and 20 mM imidazole. The purified protein(s) was eluted with elution buffer comprising of 100 mM Tris pH 7.5 containing either 300 mM NaCl/250 mM imidazole (for *MtMetAP1a*) or 200 mM NaCl/150 mM imidazole (for *MtMetAP1c*). Imidazole was removed by dialysis at 4°C for ≈ 14 h (dialysis buffer: 100 mM Tris pH 7.5 with either 300 mM NaCl for *MtMetAP1a* or 150 mM NaCl for *MtMetAP1c*; buffer changed: 4 times). On Coomassie blue stained SDS-PAGE, the affinity purified recombinant proteins revealed molecular masses of 37.3 ± 1.7 kDa ($n = 4$) and 39.1 ± 1.2 kDa ($n = 4$) for *MtMetAP1a* and *MtMetAP1c*, respectively (Fig. 4.2A and Fig. 4.2B, left panels, lane 3). Western blotting with anti-His antibody recognized the fusion protein (Fig. 4.2A and Fig. 4.2B, right panels, lanes 2 and 3).

### 4.3 Biochemical Characterization

#### 4.3.1 Assessment of *MtMetAP1s* activity

MetAP activity was determined employing a colorimetric assay performed at 30°C in a microtiter plate by monitoring the absorbance of oxidized o-dianisidine at 440 nm (Ben-Bassat *et al*., 1987). For this, an enzyme coupled assay system was used with increasing concentration of protein being incubated with the reaction mixture as mentioned in Chapter 2 (Section 2.2.3.2) and 4 mM substrate. The activity of the enzyme was monitored in an ELISA plate reader for 2-15 min. The values obtained were corrected by subtracting the blank readings (no significant difference was noticed when assays were carried out with all ingredients except either substrate or protein) and calculated as μmol of product released by using the extinction
Fig. 4.2 Expression and purification of *MtMetAP1a* and *MtMetAP1c*. (A) Purification of Histidine tagged *MtMetAP1a*. *Left panel*: Coomassie stained gel of *MtMetAP1a*. *Right panel*: immunoblot probed with anti-His antibody. *Lane 1*: uninduced; *lane 2*: induced; *lane 3*: Ni-NTA purified His-tagged *MtMetAP1a*. (B) Purification of *MtMetAP1c*. *Left panel*: Coomassie stained gel. *Lane 1*: uninduced; *lane 2*: induced; *lane 3*: Ni-NTA purified *MtMetAP1c*. *Right panel*: *MtMetAP1c* confirmed by Western blotting using anti-His antibody. Arrow heads in *upper and lower panels* indicate *MtMetAP1a* and *MtMetAP1c* respectively.
coefficient of oxidised o-dianisidine as 10580 M⁻¹ cm⁻¹ (Frottin et al., 2006). A linear increase in the activity was observed with the increasing protein concentration (Fig. 4.3A for MtMetAP1a and Fig. 4.3B for MtMetAP1c). For the subsequent experiments 5000 ng and 125 ng of protein was used for MtMetAP1a and MtMetAP1c respectively.

### 4.3.2 MtMetAP1s activity and different substrates

MetAPs exhibit differences in the activity depending on the penultimate residue (Sherman et al., 1985). Since MetAPs cleave the terminal methionine cotranslationally, peptides of different length were utilized as substrates. The activity of the two mycobacterial MetAP1s was monitored in the presence of Met-Gly (dipeptide), Met-Ala-Ser (tri-peptide) and Met-Gly-Met-Met (tetra-peptide). As shown in Fig. 4.4, at least a tripeptide is the prerequisite for their activity. In fact both the enzymes showed almost negligible activity with the dipeptide Met-Gly. MtMetAP1a and MtMetAP1c displayed maximum activity with Met-Gly-Met-Met (Fig. 4.4A) and Met-Ala-Ser (Fig. 4.4B) respectively. The specificity of the two enzymes for the terminal methionine residue was confirmed by using Gly-Gly-Ala as the substrate and as expected both the enzymes were unable to hydrolyze it (Fig. 4.4A for MtMetAP1a and Fig. 4.4B for MtMetAP1c).

### 4.3.3 MtMetAP1s at different pH

In order to examine the effect of pH on the MetAP activity both the mycobacterial enzymes were diluted in buffers of different pH (3, 5.5, 7, 7.5, 8 and 10) and used for the assay. It was observed that MtMetAP1a is active at a broad pH range of 3-10 (Fig. 4.5A) while MtMetAP1c exhibited pH optimum at 7-8 (Fig. 4.5B).

### 4.3.4 MtMetAP1s at different temperature

The effect of different temperatures on the enzymatic activity of MtMetAP1a and MtMetAP1c was also monitored. For this purpose, both the enzymes following incubation (10 min) at different temperatures (4°, 30°, 37° and 50°C) were assayed for their enzyme activities. The maximum activity for both the MtMetAPs was noticed at 30-37°C. Interestingly, compared to the activity at 30°C, MtMetAP1a retained ~85%
Fig. 4.3 Effect of increasing concentration of protein on the enzyme activity of MtMetAP1a and MtMetAP1c. MetAP activity monitored with increasing protein concentration. Activity represented as μmoles of methionine released/min. (A) depicts MtMetAP1a and (B) MtMetAP1c respectively.
Fig. 4.4 Methionine aminopeptidase activity with different substrates. (A) represents MtMetAP1a and (B) MtMetAP1c. Enzyme activity of the two methionine aminopeptidases was determined using different substrates (4 mM) and 5000 ng (MtMetAP1a)/125 ng (MtMetAP1c) of purified proteins. Results with different substrates are expressed as percentage maximum of the activity obtained on using MGMM for MtMetAP1a and MAS for MtMetAP1c. Notations used: MG, Met-Gly; MAS, Met-Ala-Ser; MGMM, Met-Gly-Met-Met; GGA, Gly-Gly-Ala.
Fig. 4.5 Effect of pH on the methionine removal capability of (A) MtMetAP1a and (B) MtMetAP1c. MetAP activity of the two MtMetAP1s was determined using 5000 ng (MtMetAP1a) or 125 ng (MtMetAP1c) of purified proteins incubated in buffers of different pH as depicted on X-axis. The substrates used were 4 mM of Met-Gly-Met-Met for MtMetAP1a and Met-Ala-Ser for MtMetAP1c respectively. The activity was calculated in percent to that obtained at pH 7.5.
of its activity at 50°C as opposed to only ~15% by MtMetAP1c (Fig. 4.6A for MtMetAP1a and Fig. 4.6B for MtMetAP1c). This led to the postulation that alteration in activity might be occurring due to structural alterations in both the proteins as a result of heating. This possibility was explored by incubating them at 50°C and comparing their far-UV CD spectra between 190-250 nm. As shown in Fig. 4.7A (left panel) MtMetAP1a retained its secondary structure on heating at 50°C while MtMetAP1c was unfolded (Figs. 4.7A left and right panels). These findings, therefore, establish the adaptive ability of MtMetAP1a to high temperature compared to that of the MtMetAP1c. Further, MtMetAP1a and MtMetAP1c were heated in the thermoelectric cell of the CD spectrophotometer at a fixed rate of 1°C/min and the thermal denaturation curves were recorded at 208 nm. Tm of MtMetAP1a and MtMetAP1c was found to be 53.6°C and 42.7°C respectively (Figs. 4.7B left and right panels). These results demonstrate that MtMetAP1a is more thermostable than MtMetAP1c.

4.3.5 Determination of kinetic parameters

In order to determine kinetic parameters, purified enzymes (5000 ng for MtMetAP1a or 125 ng for MtMetAP1c) were incubated with increasing concentrations (0.75-8 mM) of substrate (Met-Gly-Met-Met for MtMetAP1a and Met-Ala-Ser for MtMetAP1c) and their activity was monitored at 440 nm. The rate of the reaction for both the proteins when plotted as the function of substrate concentrations yielded typical Michaelis-Menten curves (Fig. 4.8A for MtMetAP1a and Fig. 4.8B for MtMetAP1c). $K_m$ and $k_{cat}$ values obtained from Lineweaver–Burk plot are depicted in Table 4.1. Thus, the determination of the catalytic parameters indicated that both MtMetAP1a and MtMetAP1c are active enzymes with $k_{cat}$ of ~12 s$^{-1}$ and ~9 s$^{-1}$ respectively (considering a molecular mass of ~29.5 kDa for MtMetAP1a and ~33.1 kDa for MtMetAP1c). Strikingly, using same substrate (Met-Ala-Ser), the catalytic efficiency of MtMetAP1c was found to be ~180 fold higher compared to that of the MtMetAP1a (Table 4.1).
Fig. 4.6 Effect of temperature on *MtMetAP1*s. The two *MtMetAP1*s were incubated at the indicated temperatures for 10 min and then their enzyme activity was monitored using 5000 ng enzyme and 4 mM Met-Gly-Met-Met for *MtMetAP1*a or 125 ng enzyme and 4 mM Met-Ala-Ser for *MtMetAP1*c. The enzyme activity represented in percent to that observed at 30°C. (A) and (B) represents *MtMetAP1*a and *MtMetAP1*c respectively.
Fig. 4.7 Effect of temperature on the secondary structure of MtMetAPIs. (A) The secondary structure of MtMetAPIa and MtMetAPIc was monitored at 25°C and 50°C. Far-UV CD spectra monitored at 25°C and 50°C depicted by solid squares and triangle respectively. (B) Temperature unfolding of MtMetAPIa and MtMetAPIc was monitored by CD spectroscopy at 208 nm. X-axis represents temperature in °C.
Fig. 4.8 Kinetic analysis of methionine aminopeptidase activity of MtMetAP1s. Kinetic analysis of methionine excision ability of MtMetAP1a (A) was carried out by using 5000 ng of purified protein with increasing concentration of Met-Gly-Met-Met as substrate. The reaction was monitored for 15 min. (B) 125 ng of purified MtMetAP1c was incubated for 5 min with increasing concentration of Met-Ala-Ser as substrate to determine the kinetic parameters of MtMetAP1c. The activity expressed as μmoles/min/mg protein.
Table 4.1 Kinetic parameters of MtMetAPs

Enzyme assays were carried out as described under ‘Materials and Methods’ following the incubation of purified MtMetAP1a (5 μg/reaction) or MtMetAP1c (125 ng/reaction) proteins with different concentrations (0.75-8 mM) of Met-Ala-Ser (MAS) or Met-Gly-Met-Met (MGMM) as substrate. $K_m$ and $V_{max}$ values were calculated from Lineweaver-Burk plots, and the results are expressed as Mean ± SD. For calculating $k_{cat}$ values, the molecular mass of recombinant enzyme(s) was considered as 29.5 kDa (for MtMetAP1a) or 33.1 kDa (for MtMetAP1c).

<table>
<thead>
<tr>
<th>System</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtMetAP1a (MAS)</td>
<td>3.6±0.63</td>
<td>11.76±1.84</td>
<td>3.38±0.09</td>
<td>4</td>
</tr>
<tr>
<td>MtMetAP1c (MAS)</td>
<td>3.27±0.91</td>
<td>2147±126</td>
<td>695±196</td>
<td>4</td>
</tr>
<tr>
<td>MtMetAP1a (MGMM)</td>
<td>2.21±0.78</td>
<td>9.35±1.53</td>
<td>4.80±2.58</td>
<td>3</td>
</tr>
</tbody>
</table>
4.3.6 **MtMetAPIs and different inhibitors**

Since MetAPs are known as dinuclear metalloproteases, the effect of EDTA, a metal ion chelator on the activity of the enzyme was monitored. As expected, metal ion chelator affected the activities of both the enzymes; however, compared to MtMetAP1c enzyme activity of MtMetAP1a was inhibited even at lower concentrations of EDTA (Fig. 4.9A for MtMetAP1a and Fig. 4.9B for MtMetAP1c).

Leucine aminopeptidase inhibitors like amastatin and bestatin have been shown to inhibit MetAP activity by binding to its metal centre (Lowther and Matthews, 2002). As shown in Fig. 4.9, amastatin affected both MtMetAP1a and MtMetAP1c activities, magnitude of inhibition of enzyme activity varied considerably (compared to untreated control ~80% decrease in MtMetAP1a as opposed to ~45% in MtMetAP1c with 100 μM). Bestatin, on the other hand, had no effect on the enzyme activity of both the proteins in the concentration range tested in our experimental conditions.

### 4.4 Structure-Function Analysis

#### 4.4.1 Sequence analysis of the two MtMetAPIs and molecular modelling of MtMetAP1a

The crystal structure of MtMetAP1c (PDBID-1YJ3) has already been resolved (Adlaggata et al., 2005). On the other hand, the structure of MtMetAP1a is not available as yet. BLAST search of MtMetAP1a amino acid sequence against PDB database did not result in any solved structure that had high sequence similarity to it. Since both MtMetAP1a and MtMetAP1c have functional similarity and are considered as paralogs, their amino acid sequences were aligned using CLUSTAL X programme (Fig. 4.10). The alignment yielded an identity score of 41% and the aligned regions were well spread across the entire sequence having significant score of 34. The sequence alignment of MtMetAP1a and MtMetAP1c depicted presence of an N-terminal extension in MtMetAP1c and also several conserved residues in them (Fig. 4.10). Using CLUSTAL X, sequence alignment of MtMetAP1a (structure based) with that of MtMetAP1c was done followed by the structure modelling as mentioned in chapter 2 section 2.2.5.1. PROCHECK validation of the model depicted 97.6% residues in the most favoured regions of Ramachandran plot. MtMetAP1a was expected to have conserved 'pita-bread' folded structure (Figure 4.11). The
Fig. 4.9 Effect of different inhibitors and chelator on MtMetAPs activity. The two mycobacterial MetAP1s were preincubated for 15 min at room temperature with the indicated amount of the inhibitors or chelator and then the activity assay was performed. The enzyme activity plotted as the percent of that observed in the untreated control. (A) represents MtMetAP1a and (B) MtMetAP1c respectively.
Fig. 4.10 Sequence alignment of the two mycobacterial methionine aminopeptidases was performed using Clustal X. Gaps in the sequences were introduced for optimum alignment. Asterisk and dots denote, identical and similar amino acids, respectively. Residues highlighted with black represent the 40 amino acid long N-terminal extension present in MtMetAP1c and those with grey are the mutated amino acids in the two MtMetAPs.
Fig. 4.11 Structural comparison of the two MtMetAP1s. Structural alignment of MtMetAP1a (orange) with respect to MtMetAP1c (slate green). Residues in purple blue (sticks) represent MtMetAP1c active site residues His212, His-114, Glu-238. Residues in hot pink (sticks) are MtMetAP1a active site residues His-88, His-193, Glu-219. Residue in yellow is Tyr-183 (MtMetAP1a) and in grey is Phe-202 (MtMetAP1c). The differences in secondary structure elements are shown in purple blue (MtMetAP1c) and red (MtMetAP1a).
monomeric protein molecule consists of central antiparallel β-sheet (β3 and β4) along with spiral twisted, irregular strands (β5 to β12) onto which rest of the structure packs. Two pairs of α-helices (α1 to α4) pack on the either side of the central β-sheet to provide major structural shield to the residues involved in catalysis. For consistency, the four helices from N- to C-terminus were numbered as α1 (49-62), α2 (24-41), α3 (131-148) and α4 (155-171). Both these elements contributed ~45% to the protein molecule and rest of the ~55% comprised of regulatory loop regions interspersed. An N-terminal extension lies across the surface of the catalytic domain, which is devoid of any secondary structural element. The total surface area of the molecule came out to be ~10648 Å. Electrostatic surface potential calculations revealed that there is lesser percentage of hydrophilic regions around the active site. Moreover, the active site residues were spread more in space, in an L-shaped manner (more centred in C-shape in MtMetAP1c).

4.4.2 Identification of the critical residues for the activity of MtMetAP1s

On the basis of structural alignment, His-88, His-193, Asp-106, Asp-117, Glu-219, Glu-250 and Trp-236 were identified as the residues lining the perimeter of the MtMetAP1a active site. Besides these, Tyr-183 also appeared to be lining the active site. Catalytic site residues on the model were mapped as His-88, His-193 and Glu-219 (hot pink) based on MtMetAP1c (blue sticks) structure (Fig. 4.11). All these residues were expected to affect the MtMetAP1a activity. Similarly, based on crystal structure of MtMetAP1c the most likely homologous residues included His-114, His-212, Asp-131, Asp-142, Glu-238, Glu-269, Phe-202 and Trp-255. Thus it appeared very likely that, the active site residues responsible for the enzyme activities are essentially conserved in both the proteins.

4.4.3 Generation of mutants

Site-directed mutagenesis was carried out by overlap extension method (Ho et al., 1989) as illustrated in chapter 2 (Fig. 2.1A) using pET-mapA or pET-mapB as the template. For each mutation, two external and two internal primers were used. For mapA the external primers used were CK74 (HindIII site is incorporated) and CK140 (NheI site is incorporated). On the other hand, for mapB external primers used were
CK64 (Ndel site is incorporated) and CK65 with (HindIII site is incorporated). The internal primers used in this study are listed in chapter 2 (Table 2.4). Base mismatches for the desired mutations were incorporated in the internal primers. PCR conditions were kept same as used for amplification of wild type mapA or mapB (section, 2.2.1.7). After secondary PCR, the amplified products were gel purified, digested with Nhel - HindIII (pET-mapA) Ndel - HindIII (pET-mapB) followed by ligation to pET-28c vector digested with either Nhel/HindIII or Ndel/HindIII. Finally, the ligated products were transformed into E. coli strain DH5α. The mutations were confirmed by sequencing.

4.4.4 Assessment of MetAP activity of the mutants

To gain an insight on their contribution towards the enzyme activity, several point mutants were generated (H88A, H193A, D117A, E219V and W236L for MtMetAP1a and H114A, H212A, D131A, E238A, W255L for MtMetAP1c). Interestingly, none of the mutants demonstrated any activity even on taking 10-fold excess protein compared to that of the wild-type (Figs. 4.12 and 4.13). In fact, mutating W236L in MtMetAP1a and W255L in MtMetAP1c led to complete loss in activity (Figs. 4.12 and 4.13), which is in contrast to the report available with E. coli (Chiu et al., 1999). Expression of all the mutants was authenticated by Western blotting using anti-His antibody (Insets in Figs. 4.12 and 4.13). To examine the effect of mutation on the secondary and tertiary structure of the MtMetAP1s, the CD spectral studies were performed. Far-UV CD spectrum of the mutant proteins of MtMetAP1a revealed that H88A, D117A, E219V and W236L show reduction in helical content. The trend of the data in the regions of 200-210 nm suggests that the band with negative mean residue ellipticity at lower wavelengths has a reduced signal strength owing presumably to the conversion of some helical regions to less ordered structures (Fig. 4.14A). Interestingly, mutant H193A shows greater helicity than the wild-type protein. Near-UV CD spectra of the mutants of MtMetAP1a reveal that the changes observed in the secondary structure of the proteins is not reflected to the same extent in their tertiary structure (Fig. 4.14B). This could owe to a greater degree of association of the aromatic residues with chiral structures (helices/sheets) in mutants D117A and E219V despite the overall reduction in helical content. Far UV
Fig. 4.12 Identification of the critical residues for \textit{MtMetAP1a} activity. Methionine aminopeptidase activity of different point mutants was monitored at two different concentrations as indicated in the graph. Enzyme activity for the indicated amount of wild-type and purified mutants of \textit{MtMetAP1a} was monitored using 4 mM of substrate, Met-Gly-Met-Met and expressed as the percentage of the activity observed with the wild-type. \textit{Inset:} The authenticity of the mutant proteins was confirmed using anti-His antibody.
Fig. 4.13 Identification of the critical residues for MtMetAP1c activity. Methionine aminopeptidase activity of different point mutants was monitored at two different concentrations as indicated in the graph. Enzyme activity for the indicated amount of wild-type and purified mutants of MtMetAP1c was monitored using 4 mM of substrate, Met-Ala-Ser and expressed as the percentage of the activity observed with the wild-type. Inset: The authenticity of the mutant proteins was confirmed using anti-His antibody.
Fig. 4.14 Effect of mutation on the structure of *MtMetAP1a*. (A) Far-UV CD spectra to monitor the effect of mutation on the secondary structure of the protein. (B) To monitor the effect of the mutations on the tertiary structure of the protein near-UV CD spectra analysis was done.
CD spectral studies of wild type MtMetAP1c and its mutants revealed that as a result of mutation in D131A although there is no significant reduction in the mean residue ellipticity in the 200-210 nm range but it decreased in the 220-225 nm range (Fig. 4.15A). On the other hand, in H114A and E238A reduction in band intensity was observed at both 200-210 nm and 220-225 nm. In H212A and W255L the band intensity in the 200-210 nm as well as in 220-225 nm was further reduced (Fig. 4.15A). Changes were observed in the near-UV CD spectra of the mutant proteins indicating change in the aromatic environment of protein as a result of mutations (Fig. 4.15B).

**DISCUSSION**

MetAP1s represent a unique class of metalloprotease that catalyzes the co-translational removal of N-terminal methionine residue in the process of protein synthesis (Sherman et al., 1985). Since MetAP action in both prokaryotes and eukaryotes is mandatory during nascent protein synthesis, the importance of this enzyme has long been realized. Though the presence of two MetAP1s was known with the availability of the *M. tuberculosis* genome sequence (Cole et al., 1998), both of them (*MtMetAP1a* and *MtMetAP1c*) are active has been shown recently (Zhang et al., 2009; Olaleye et al., 2010; Lu et al., 2010; Lu and Ye 2010 and this study). However, it is still not known whether they are merely complementing each other for their functionality and therefore redundant within the genome or presence of each enzyme is because of the specific needs. Recently, it has been reported that *MtMetAP1a* knock down caused ~75% viability of mycobacteria while elimination of *MtMetAP1c* resulted in 95% survival (Olaleye et al., 2010). Since this enzyme from different sources has been considered as a potential drug target, identification of inhibitors against these enzymes, particularly for *M. tuberculosis* *MtMetAP1a* is in progress (Olaleye et al., 2010). However, the initial step of structure-activity relationship between these two proteins has not been addressed in detail. As an initial step for carrying out systematic study of these two enzymes from *M. tuberculosis*, biochemical analysis of MetAP1s was performed to elucidate subtle differences between them in their characteristics.
Fig. 4.15 Effect of mutation on the structure of *MtMetAP1c*. (A) Far-UV CD spectra to monitor the effect of mutation on the secondary structure of the protein. (B) To monitor the effect of the mutations on the tertiary structure of the protein near-UV CD spectra analysis was done.
To determine if the enzymes have any substrate preferences, dipeptide (Met-Gly), tripeptide (Met-Ala-Ser) and a tetrapeptide (Met-Gly-Met-Met) were used. Among the substrates, it is apparent that a tetra-peptide like Met-Gly-Met-Met is the preferred substrate for \( Mt\text{MetAP1a} \) while the tri-peptide like Met-Ala-Ser was cleaved more efficiently by \( Mt\text{MetAP1c} \) (Figs. 4.4A and 4.4B). Both \( Mt\text{MetAP1a} \) and \( Mt\text{MetAP1c} \) did not show any activity with the di-peptide Met-Gly (Figs. 4.4A and 4.4B). This indicates that probably the minimum requirement for the efficient hydrolysis by the mycobacterial MetAP1s is a tripeptide.

Further, assessing the activity of the two enzymes at different temperatures revealed that \( Mt\text{MetAP1a} \) is active even at 50°C while \( Mt\text{MetAP1c} \) hardly retains any activity (Figs. 4.6A and 4.6B). To have a better understanding of the plausible reason for the difference in the behaviour of the two \( Mt\text{MetAPs} \) at higher temperature, their far-UV CD spectroscopic studies were carried out 25°C and 50°C. Interestingly, it was observed that with increase in temperature \( Mt\text{MetAP1a} \) was able to retain its secondary structure but \( Mt\text{MetAP1c} \) was unfolded (Fig. 4.7A, left panel for \( Mt\text{MetAP1a} \) and Fig. 4.7A, right panel for \( Mt\text{MetAP1c} \)). This may explain the drastic loss in the enzymatic activity of \( Mt\text{MetAP1c} \) at 50°C. It also suggested that \( Mt\text{MetAP1a} \) is structurally more stable at high temperature compared to \( Mt\text{MetAP1c} \).

To examine whether \( Mt\text{MetAP1a} \) is structurally more stable than \( Mt\text{MetAP1c} \), thermal unfolding of the two enzymes was carried out at a fixed rate and the structural change with the increase in temperature was monitored at 208 nm in a CD spectrophotometer. \( T_m \) of \( Mt\text{MetAP1a} \) was calculated to be 53.6°C while that of \( Mt\text{MetAP1c} \) was 42.7°C (Fig. 4.7B, left and right panel). Thus, it is apparent that \( Mt\text{MetAP1a} \) is more thermostable than \( Mt\text{MetAP1c} \). Interestingly, this finding also corroborates well with reduction in the activity of \( mapB \) promoter at the high temperature as observed in Chapter 3 (Fig. 3.10). It seems that \( Mt\text{MetAP1c} \) is unable to retain any structure at high temperature and hence, would not be able to perform its function.

The kinetic analysis of \( Mt\text{MetAP1c} \) revealed that it has \( \sim \)180 fold enzyme turnover rate with the same substrate compared to \( Mt\text{MetAP1a} \) (Table 4.1), although they have different substrate preferences (Fig. 4.4A for \( Mt\text{MetAP1a} \) and Fig. 4.4B for \( Mt\text{MetAP1c} \)). Monitoring of the promoter activity of the two genes encoding these
proteins revealed that mapA promoter is at least 9 fold more active than the mapB promoter (Chapter 3). This finding is also supported by a recent report which indicates higher transcript level of MtMetAP1a compared to MtMetAP1c in different phases of mycobacterial growth (Zhang et al., 2009). However, the kinetic results presented in this study suggest that under in vivo conditions even at low protein level MtMetAP1c enzyme may have higher activity. In fact, this possibly explains the reason for ~75% viability of mycobacteria in mapA-knock down mutant as observed by Olaleye et al. (2010).

To have an insight into the cause of such differential behaviour of the two enzymes, their sequences were analyzed and structure of MtMetAP1a was modelled (Figs. 4.10 and 4.11). The analysis of sequences and the model divulged that the active site residues in both the MtMetAP1s were essentially same, though their arrangement in space was different. Furthermore, these amino acids were crucial because any alteration in them (for both the enzymes) yielded an inactive protein (Fig. 4.12 for MtMetAP1a and Fig. 4.14 for MtMetAP1c). Thus it is logical to presume that this difference in the arrangement may be responsible for the difference in their activity/functionality. The far-UV and near UV studies on the wild-type and mutant proteins of MtMetAP1a and MtMetAP1c revealed that as a result of mutations there are changes in both secondary and tertiary structure of both the enzymes (Figs. 4.14 and 4.15).

In this chapter, it has been established that the two MtMetAP1s have some similar as well as unique features. Further, the residues critical for the activity of the two mycobacterial MetAP1s have also been identified but the role of the unstructured 40 residues long N-terminal extension present in MtMetAP1c remains to be elucidated. Another aspect that remains to be unravelled is the role of N-terminal extension in the interaction of MtMetAP1c and ribosomal proteins.