Chapter 5
Methionine aminopeptidase type 1c from 
M. tuberculosis: Role of N-terminal extension
Methionine aminopeptidase type 1c from *M. tuberculosis:*
Role of N-terminal extension

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MetAPs are involved in co-translational and irreversible removal of the initiator methionine residue soon after the nascent peptide emerges from the ribosome exit tunnel and before the commencement of protein folding (Giglione et al., 2004). Although, it is a ubiquitous and highly conserved process, how MetAPs interact with the ribosome is not yet clear. A school of thought is that the N-terminal extension present in MetAPs may be responsible for such an event. The existence of an unstructured short N-terminal extension in prokaryotic MetAP has been reported recently (Addlagatta et al., 2005); however, the functional relevance of this region is yet unknown.

As discussed in the Chapter 1, the first protein which interacts with the nascent polypeptide is Trigger Factor (TF), which builds a closed crevice around the exit tunnel. A recent report based on the E. coli, where methionine excision is preceded by the removal of formyl group from the initiator methionine by the peptide deformylase suggests that the emerging nascent polypeptide chain from the ribosomal exit tunnel first encounters the TF. TF forms a cradle that is open on several sides and serves as a passive router that channels the nascent chains first to PDF and finally to MetAP for the removal of the methionine present at the N-terminus (Bingel-Erlenmeyer et al., 2008). Thus, there is no direct interaction between the two proteins (PDF and MetAP) of prokaryotic NME pathway. Kerwar et al. (1971) have shown an association between the MetAP in rat brain and the ribosome; however precise interaction amongst two eukaryotic MetAPs (MetAP1b or MetAP2b) and the ribosome is not clear. In subsequent studies, it has been shown that MetAP1b in eukaryote has an N-terminal domain with zinc finger motifs which has been proposed to be important for its interaction with the ribosome (Vetro and Chang, 2002).

MtMetAP1c has ~ 40 residues long N-terminal extension and the amino acids at the position 14-17 (Pro-Thr-Arg-Pro) have been proposed to adopt polyproline II helix conformation (Addlagatta et al., 2005). It has been suggested that PXXP region can bind to SH3 protein motif. In fact ribosomal proteins that are present close to the exit tunnel, from where the nascent polypeptide chain emerges are known to have SH3 binding domain. Therefore, it is advocated that the MetAPs-ribosome interaction may be mediated by a complex between a PXXP motif on the MetAP and an SH3 domain on the ribosome. However, the presence of PXXP motif only in eukaryotic
MetAPs (absent in prokaryotic MetAP1a) suggests that this may not be the universal mechanism of interaction (Adlagatta et al., 2005).

In the earlier chapter, the methionine catalyzing activity of the MtMetAP1s has been compared but the role of N-terminal extension present in MtMetAP1c remains to be elucidated. In this chapter, the contribution of the N-terminal extension towards the functionality of the enzyme has been studied. Further, to study the MtMetAP1c interaction, ribosomal protein L24 has been cloned, expressed and purified. L24 has SH3 domain and is located adjacent to the tunnel from where the nascent polypeptide chain emerges from the large subunit of the ribosome. It has been proposed that MtMetAP1c can interact with ribosome by binding to the L24 protein through the PXXP motif present in its N-terminus region (Addlagatta et al., 2005). MtMetAP1c-L24 interaction has been studied by pull-down assay of the purified proteins and also following their co-expression in E. coli based expression system.

5.1 Role of N-terminal extension of MtMetAP1c in the enzyme activity

MtMetAP1c has a 40 amino acid long unstructured N-terminus. The presence of extension is not unusual among MetAP1s, however its contribution towards the enzymatic activity is not known (Zuo et al., 1995; Yang et al., 2001; Li et al., 2004). The role of N-terminal extension towards the functionality of this enzyme was evaluated. For this purpose, a series of N-terminal deletion mutants for MtMetAP1c were generated (Fig. 5.1A) and their enzyme activity was monitored (Fig. 5.1B). Surprisingly, compared to wild-type, the deletion constructs (Δ2-10 and Δ2-15) displayed drastic decrease in the enzymatic activity (Fig. 5.1B) advocating the importance of N-terminal residues in enzyme activity. Interestingly, even at 10 fold higher concentration, Δ2-10 and Δ2-15 exhibited only 30% activity and Δ2-20, Δ2-30 and Δ2-40 had no activity (Fig. 5.1B). These results suggest that the catalytic domain alone is not sufficient for the enzymatic activity of MtMetAP1c. This loss in activity could partially be due to loss in stability of the protein since analysis of its crystal structure reveals that the N-terminal extension present in MtMetAP1c wraps around the surface of its catalytic domain in contiguous crevice and its truncation would
Fig. 5.1 Deletion analysis of the N-terminal extension present in MtMetAP1c. (A) Schematic representation of the deletion constructs. Notations used: WT, wild-type; Δ, deleted residues. (B) Assessment of methionine aminopeptidase activity of deletion mutants. Methionine aminopeptidase activity assay was performed using indicated amount of wild-type and mutant proteins with 4 mM of Met-Ala-Ser as the substrate. Results expressed as the percentage of activity obtained with 125 ng of wild-type protein.
expose the hydrophobic residues, thereby destabilize the protein (Addlagatta et al., 2005).

In this direction, different deletion constructs were run on SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 5.2, upper panel). It is apparent that with the increase in the length of deleted region, there was a progressive loss in the purity of the mutant proteins (Fig. 5.2, upper panel). The authenticity of the purified proteins was confirmed by western blotting using anti-His antibody (Fig. 5.2, lower panel). The absence of any activity in Δ2-20, Δ2-30 and Δ2-40 can be attributed to the loss in purity of the protein (Fig. 5.2, upper panel, lanes 4-6). In fact, analysis of crystal structure of MtMetAP1c reveals that such deletion may result in an unstable/misfolded protein (Addlaggata et al., 2005). Far-UV and near-UV CD spectroscopic studies were performed for WT, Δ2-10 and Δ2-15 to examine if the alteration in the secondary and/or tertiary structure of the mutant proteins is responsible for the decrease in activity. The Far-UV CD spectra showed that the mutant proteins were folded like the wild-type protein (Fig. 5.3A) and there was little change observed in the near-UV CD spectra for the Δ2-15 mutant (Fig. 5.3B) which was probably because the deletion of these residues affected the tryptophan present at the amino acid position 20.

5.2 Importance of Val-18 and Pro-19 in MtMetAP1c enzyme activity

To understand the reason for the drastic decrease in the activity as well as purification of Δ2-20 mutant compared to Δ2-15 construct, sequences of some representative MetAPs with N-terminal extension from Gram positive bacteria were analyzed. The multiple sequence alignment revealed that Val-18 and Pro-19, two highly conserved residues are absent in Δ2-20 mutant (Fig. 5.4). To investigate the role of highly conserved Val-18 and Pro-19, point mutants replacing these two residues one at a time (V18A, V18G, P19A, P19G) or both at the same time (V18AP19A and V18GP19G) were generated using overlap extension method (Ho et al., 1989) as illustrated in chapter 2 (Fig. 2.1A). Primers used are mentioned in Table 2.4. On monitoring the activity it was observed that relative to wild type, V18A protein had ~30% activity while P19A and P19G exhibited ~65% and 35% activity respectively; however, V18G, V18AP19A and V18GP19G had no activity (Fig. 72).
Fig. 5.2 Authenticity of the deletion constructs. **Upper panel:** Coomassie stained SDS-PAGE gel of the purified deletion mutants. **Lower panel:** Western blotting using anti-His antibody of the mutant proteins for which the activity assay was performed.
Fig. 5.3 Effect of mutation on secondary and tertiary structure of the mutants. Far-UV (A) and near-UV (B) CD spectra of the wild-type (WT), Δ2-10 (10 del) and Δ2-15 (15 del) mutant proteins.
Fig. 5.4 Sequence alignment of MetAP1c from different gram positive bacteria. Notations used with accession number in parentheses: M tb-Mycobacterium tuberculosis (P0A5J2); M bovis-Mycobacterium bovis (P0A5J3); M avium-Mycobacterium avium (A0Q109); M marinum-Mycobacterium marinum (B2HJQ5); M paratuberculosis-Mycobacterium paratuberculosis (Q73VS7); M leprae-Mycobacterium leprae (Q9CBU7); S coelicolor-Streptomyces coelicolor (Q9RKR2); S scabies-Streptomyces scabies (C9ZHA6); R erythropolis-Rhodococcus erythropolis (C0ZY62); N farcinica-Nocardia farcinica (Q5YSA3); G bronchialis-Gordonia bronchialis (D0LBG0); C diphtheriae-Corynebacterium diphtheriae (Q6NGL5); C glutamicum-Corynebacterium glutamicum (Q6M437); B mcbrellneri-Brevibacterium mcbrellneri (D4YNZ0); L xyli-Leifsonia xyli subsp. xyli (Q6AFH6)
5.5A). On increasing enzyme concentration to 10-fold during assays, V18A, P19A and P19G mutant proteins displayed activities almost at par (78%-112%) with the wild-type. V18G, V18AP19A and V18GP19G proteins, on the other hand, hardly showed any activity (Fig. 5.5A). These results established for the first time, that Val-18 and/or Pro-19, the residues present in N-terminal extension (not in the catalytic domain) significantly contribute towards the enzymatic activity of MtMetAP1c.

Further, to check if alteration in the activity of the mutant protein could be due to structural changes, far-UV CD studies were performed. The CD analysis revealed that MtMetAP1c has higher negative mean residue ellipticity signal in the region of 200-210 nm than in the region of 220-225 nm (Fig. 5.5B). This feature is found when polyproline type II (PP-II) structure is present in a protein. PP-II structures are characterized by a much higher negative mean residue ellipticity signal in the region of 200-210 nm than in 220-225 nm range, which results in a significantly more intense negative band with a band minimum in the region of 205-208 than in the region of 222 nm. In the mutants V18A, P19A and V18AP19A although there was no reduction in the 220-225 nm band, but there was a significant reduction in the 205-208 nm band suggesting that these mutations alter the PP-II structure of the region. On the other hand, in the P19G mutant, there was reduction in the intensity of the 220-225 nm band without any significant change in the 205-208 nm band relative to what was seen with the V18A, P19A and V18AP19A mutants indicating that the PP-II structure is affected to the same extent with an additional reduction of the helical content. Strikingly, V18G mutant displayed a significant reduction in PP-II content as well as helical content. Thus, these results suggested the importance of these two residues (Val-18 and Pro-19) exclusively and mutually towards the enzymatic activity of MtMetAP1c presumably because of the structural alterations having occurred due to mutation at Val-18 and Pro-19.

5.3 Molecular dynamic simulations to assess the function of Val-18 and Pro-19

To gain an insight on how the Val-18 and Pro-19 of MtMetAP1c influence the activity of the enzyme, the Molecular dynamic (MD) simulation studies were carried out with the mutant proteins. The models for V18G, P19G, and V18GP19G were
Fig. 5.5 Role of Val-18 and Pro-19. (A) Effect of single and double mutants on the activity of the enzyme. The indicated amounts of wild-type and mutant proteins were taken to check the methionine aminopeptidase activity with 4 mM of Met-Ala-Ser as substrate. Results are expressed as the percentage of the activity obtained on taking 125 ng of wild-type MtMetAP1c. (B) Far-UV CD spectra of the different mutant proteins to monitor the effect of mutations on the secondary structure of MtMetAP1c.
Fig. 5.6 $C_\alpha$ RMSD plot as a function of time at 300K over a period of 20 ns. Simulations done for wild-type (WT) and the mutant (V18GP19G).
generated using \( M\text{i}M\text{etAP1c} \) (PDB ID-1YJ3) as the template and the mutations were generated using residue replacement editor in PYMOL. The structure of all the mutants and wild-type were energy minimized using LEap module of AMBER and stereo chemical properties were further validated by PROCHECK (Laskowski et al., 1996 and Case et al., 2006). The method for performing MD has been discussed in chapter2 section 2.2.5.2. Three mutant forms of \( M\text{i}M\text{etAP1c} \) (V18G, P19A, V18GP19G) were energy minimized in AMBER 9.0 using the PMEMD module with 8Å non-bonded cut off to avoid truncation of electrostatic interactions. Four MD simulations were performed in explicit solvent conditions to examine the effect of specific mutations on overall structure and on the interactions of amino acids lining the active site of the protein. The r.m.s. deviation plots for the wild-type and double mutant are depicted in Fig. 5.6. Overall fold of the mutant proteins remained same compared to that of the wild-type. However, in contrast to the wild-type, the side chain conformations of the residues forming substrate binding cavity underwent dramatic changes in mutant proteins (Figs. 5.7 and 5.8).

Analysis of wild-type trajectory snapshots over the time revealed that His-212 has a pendulum like motion within plane. In contrast, His-114 oscillates mostly around native coordinates within a range of 3 Å. However, its average movement was \( \sim 5\text{Å} \) since in some of the ensembles it moved drastically to about 9Å. Glu-238 displayed least movement, pointing towards its dual role in structural stability as well as in catalysis. Double mutant trajectories illustrated that His-212 moved randomly or lost the controlled motion (even went out of the plane in some ensembles). His-114, on the other hand, was displaced out of the centre to a distance of 4Å appearing to be locked in a particular coordinate system. As a result, it appears that compared to the wild-type the double mutant lost flexibility in its motion leading to loss in concerted action of peptide binding and further processing. It seems that the mutant moved vectorally out of the centre of geometry of the molecule, sideways to the \( \beta \)-sheet creating "openness" in the overall structure. The conformational change that initiated from the site of mutation (Val-18, Pro-19), shifted the following loop slightly moving helix \( \alpha 2 \), stereo-orienting His-114 and altering residue distance pair, changing \( \beta \)-sheet propensity to turn conformation. This dis-aligned the helices \( \alpha 3, \alpha 4 \) and the exclusion of residues 181-185 resulted in splitting of long helix \( \alpha 4 \). All these structural
Fig. 5.7 MD simulations. Upper panel: The snapshots at 5ns, 7.5ns, 10ns and 15ns for wild-type superimposed. The insets depicts the conformation at different intervals of three residues identified to be critical for the enzyme activity. Lower panel: V18GP19G snapshots at the time intervals similar to wild-type superimposed for 15 ns run. (Insets), depicts the highlighted regions of snapshots.
Fig. 5.8 MD simulations. Upper panel: Snapshots of single mutant (V18G, blue-grey) superimposed with double mutant (pink). Both overlap each other to maximum probability. In comparison to the wild type protein, the loop proximal to the site of mutation is out of plane of the active site. Lower panel: MD simulations snapshots of single mutant (P19A, yellow ribbon blue sticks) superimposed with wild type (blue-grey). Both overlap each other to maximum probability compared to the double mutant. The loop proximal to the site of mutation shows configurations similar to wild-type in 3-D space (in plane to the active site).
variations further propagated via long connecting loops, affecting both position and rotation of Glu-238. This destabilized the correct atom positioning as a consequence of which long β-sheet was formed, thus creating a mutant molecule in kind of a "locked" conformation which is unfavourable for the activity.

To establish the structure-activity relationship, the system with single mutants (V18G and P19A) using similar conditions of MD simulation was also assessed. Interestingly, as observed in biochemical studies, it was found that the single mutant V18G resembles the double mutant V18GP19G more closely in the stereochemistry around the active site (Fig. 5.8 upper panel). Like double mutant, it is apparent that similar kind of structural information was transmitted from the site of mutation to the catalytic triad in case of the single mutant and thus explains the loss of its enzyme activity (Fig. 5.8, upper panel). On the contrary, MD simulations of P19A appeared much similar to that of the wild-type but the presence of differential population of the ensemble at different time explains the reduction in the activity of the mutant protein (Fig. 5.8, lower panel). Thus, the results of our MD simulation studies ostensibly supported our biochemical observations.

5.4 Cloning of rplX for the purification of MtL24

Genomic DNA from M. tuberculosis H37Ra was used to PCR amplify rplX gene. The genomic DNA was isolated as described in chapter 2 (Section 2.2.4.1) and gene specific primers CK 211 with NdeI site and CK 212 with HindIII site were designed on the basis of published genome sequence (Cole et al. 1998). The sequence of the primers used is listed in Table 2.3. PCR amplification was carried out as mentioned in Chapter 2 (Section 2.2.1.7) using Herculase II fusion DNA polymerase (Strategene). The PCR sample containing template DNA, primers and enzyme exhibited the amplification of a single fragment of ~ 330 bp that corresponded with the size expected for rplX (Fig. 5.9 A, lane 2).

The ~ 330bp PCR product was gel purified and ligated at NheI/HindIII of pET-28c (Fig. 5.9 B). The resulting products were transformed into E. coli strain DH5α. Plasmid DNA was isolated from different clones and analyzed by restriction enzyme digestion. The construct (pET-rplX) was finally confirmed by DNA sequencing using an automated sequencer with T7 forward and reverse primers or
Fig. 5.9 Cloning, expression and purification of MtL24. (A) PCR amplification of rplX from M. tuberculosis genomic DNA. Lane 1: 100 bp ladder, lane 2: amplified product. (B) Cloning strategy. Cloning of the amplified rplX PCR product in the pET-28c expression vector (C) Purification of MtL24. Upper panel: Coomassie stained gel. Lane 1: uninduced, lane 2: induced, lane 3: Ni-NTA purified protein. Lower panel: MtL24 confirmed by Western blotting using anti-His antibody.
gene specific primers (Tables 2.3 and 2.5).

5.5 Expression and purification of L24 from *M. tuberculosis* as a histidine tagged protein

For the expression as His-tagged protein, pET-rplX DNA isolated from *E. coli* (DH5α) was transformed into *E. coli* strain BL21(DE3) and plated on LB plate containing kanamycin (50 μg/ml). 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 OD₆₀₀ of ~0.7. Cells were then induced with 0.4 mM IPTG for 12 h at 16°C and harvested (5000 x g/10min/4°C). The pellet was washed once with buffer (300 mM KCl, 50 mM Tris pH 8) before being resuspended in lysis buffer (300 mM KCl, 50 mM Tris pH 8, 20 mM imidazole, 100 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin). 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 50 mM Tris pH 8 containing 300 mM KCl and 20 mM imidazole. The purified protein was eluted with elution buffer comprising of 50 mM Tris pH 8 containing 300 mM KCl/250 mM imidazole. Imidazole was removed by dialysis at 4°C for ~14h (dialysis buffer: 50 mM Tris pH 8 with 75 mM KCl; buffer changed: 4 times). On Coomassie blue stained SDS-PAGE the affinity purified recombinant protein was identified and confirmed by Western blotting with anti-His antibody (Fig. 5.9C, upper and lower panels, lanes 3).

5.6 Cloning, expression and purification of GST-*MmMetAP1c*

The ~ 858bp PCR product obtained for *mapB*, as mentioned in chapter 4 (section 4.1) was digested with *BamHI/HindIII* and ligated at corresponding sites in pGEX-KG. The resulting product was transformed into *E. coli* strain DH5α. Plasmid DNA was isolated from different clones and analyzed by restriction enzyme digestion. The construct pGEX-mapB was confirmed by DNA sequencing using an automated sequencer with gene specific primers (Tables 2.3).
For the expression as GST-tagged protein, plasmid pGEX-mapB DNA isolated from *E. coli* (DH5α) was transformed into *E. coli* strain BL21(DE3) and plated on LB plate containing ampicillin (100 µg/ml). 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 OD₆₀₀ of ~0.7. Cells were then induced with 0.4 mM IPTG for 12 h at 16°C and harvested (5000 x g/10min/4°C). The pellet was washed once with buffer (75 mM KCl, 50 mM Tris pH 8) and resuspended in lysis buffer (75 mM KCl, 50 mM Tris pH 8, 100 mM PMSF, 1 µg/ml leupeptin and 1 µg/ml pepstatin). 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 glutathione sepharose affinity column, washed with 10 bed volumes of 50 mM Tris pH 8 buffer containing 75 mM KCl. The purified protein was eluted with elution buffer comprising of 50 mM Tris pH 8 containing 75 mM KCl/10 mM glutathione.

5.7 Interaction of MtMetAP1c with ribosomal protein

To determine the interaction between the MtMetAP1c and the ribosomal protein MtL24, *in vitro* pull down assay was performed using GST-MtMetAP1c and His-MtL24 as mentioned in chapter 2 section 2.2.4.3. Further, to elucidate the role of the PXXP motif (P₁₄T₁₅R₁₆P₁₇), its mutant GST-P₁₄A/T₁₅A/R₁₆A/P₁₇A was generated by site-directed mutagenesis as mentioned in chapter 2 section 2.2.1.8. The primers used for generation of the mutant are listed in Table 2.4. The purified protein bound to the glutathione beads was extracted using sample buffer and processed for Western blotting using anti-His monoclonal or anti-GST polyclonal antibody. As shown in Fig. 5.10 (lower panel, lanes 3 and 4) bands corresponding to His-MtL24 were detected on incubating the protein with GST-MtMetAP1c and GST-P₁₄A/T₁₅A/R₁₆A/P₁₇A in the presence of glutathione resin on probing with anti-His antibody. However, no band was visible for His-MtL24 in the presence of beads and the GST/beads alone indicating the specificity of interaction (Fig. 5.10, lower panel, lanes 1 and 2). The presence of the GST-tagged protein was confirmed by stripping and re-probing the same blot with anti-GST antibody (Fig. 5.10, upper panel, lanes 2, 3 and 4).
Fig. 5.10 *In vitro* pull-down assay for *MtMetAP1c* and L24 interaction. Purified GST-*MtMetAP1c* or GST was incubated with His-*MtL24* and glutathione beads. The proteins extracted from the beads after washing were probed with anti-GST antibody (*upper panel*) and anti-His antibody (*lower panel*). Bands in lane 2, 3 and 4 in *upper panel* corresponds to GST, GST-*MtMetAP1c* and GST-*MtMetAP1c-Mut*. Bands in lane 3 and 4 indicate His-*MtL24*. Notation used: GST-*MtMetAP1c-Mut* stands for GST-P14A/T15A/R16A/P17A.
Further, to validate the interaction co-expression approach was employed. As mentioned in chapter 2 section 2.2.4.4, pGEX-mapB or pGEX-P14A/T15A/R16A/P17A or pGEX only was cotransformed in the E. coli strain BL21(DE3) harbouring pET-rplX. The clones were cultured in LB broth, containing 50 μg/ml ampicillin and 25 μg/ml kanamycin, induced with 0.4 mM IPTG (16°C/12h) (OD$_{600}$ ~ 0.6) and the co-expressed proteins were further processed for purification using glutathione-sepharose affinity column. After purification, the protein samples were run on SDS-PAGE and transferred at 120 volts for 1h to nitrocellulose membrane. After electrophoresis, membranes were stained with Ponceau-S which showed the presence of the GST, GST-MtMetAP1c and GST-P14A/T15A/R16A/P17A proteins (Fig. 5.11, upper panel). The blot was then probed with anti-His antibody. As shown in Fig. 5.11 (lower panel) His-L24 co-purified with GST-MtMetAP1c and GST-P14A/T15A/R16A/P17A (lanes 3 and 4) but with GST-only no band for His-L24 was observed (Fig. 5.11, lower panel, lane 1). This further establishes that the interaction between L24 and MtMetAP1c is specific and the PTRP motif does not have any significant role in direct protein-protein interaction.

DISCUSSION

Compared to MtMetAP1a, sequence analysis revealed that the striking feature of MtMetAP1c is the presence of 40 amino acid long N-terminal extension. It has been suggested that this extension may be involved in the interaction of MtMetAP1c with the ribosome (Addlagatta et al., 2005). In ScMetAP1 and HsMetAP2 deletion of N-terminal extension did not significantly alter the catalytic efficiency of the enzymes (Zuo et al., 1995; Yang et al., 2001). However, such deletion in ScMetAP1 reduced its efficiency in rescuing the slow growth phenotype of a map mutant (Vetro and Chang, 2002). Interestingly, the zinc finger motif present in the N-terminal extension of ScMetAP1 was involved in its association with the ribosome (Vetro and Chang, 2002). In HsMetAP1, on the other hand, these sequences have been implicated for the interaction of the enzyme with substrates and inhibitors (Li et al., 2004). This led to exploration of the role of 40 amino acids long N-terminal extension present in MtMetAP1c towards the activity of the enzyme. A series of deletion mutants were constructed (Fig. 5.1A). The deletion of nine (Δ2-10) or fourteen (Δ2-15) amino
Fig. 5.11 Coexpression of GST-tagged MtMetAP1c and His-tagged MtL24. E. coli BL21(DE3) cells were co-transformed with different plasmid constructs, expressed and purified through glutathione sepharose affinity column. Upper panel represents the Ponceau-S stained blot. Lane 1, 2 and 3 indicates the presence of GST, GST-MtMetAP1c and GST-MtMetAP1c-Mut respectively. Lower panel shows immunoblot probed with anti-His antibody. Lanes 2 and 3 depicts the co-purified His-MtL24. Notation used: GST-MtMetAP1c-Mut stands for GST-P14A/T15A/R16A/P17A.
acids resulted in marginal activity of mutant proteins compared to that of the wild-type (Fig. 5.1B). On the other hand, as a consequence of removal of nineteen (Δ2-20), twenty-nine (Δ2-30) and thirty-nine (Δ2-40) amino acids, mutants displayed no activity even in carrying out enzyme assays with 10 fold excess of proteins (Fig. 5.1B). Intriguingly, the Coomassie stained SDS-PAGE analysis of the deletion constructs reflects that the reason for the loss of activity of mutants may be due to loss in purity of the protein, probably deleting these residues render the enzyme unstable/misfolded (Fig. 5.2, upper panel). The authenticity of the mutants was confirmed by Western blotting with anti-His antibody. Thus, all these results argued that the N-terminal extension is important for the catalytic activity as well as for the stability of MtMetAP1c protein, which is unlike the reports available with ScMetAP1, HsMetAP1 and HsMetAP2 (Zuo et al., 1995; Yang et al., 2001; Li et al., 2004).

Further, to evaluate if the structural changes in the proteins are responsible for the loss in activity in Δ2-10 and Δ2-15, the far-UV and near-UV CD analyses of the mutant proteins were performed. Far-UV and near-UV CD spectra revealed that the deletion of N-terminal extension upto 15 residue does not affect the folding of the protein (Fig. 5.3A). Since alteration in the purification profile as well as loss in enzyme activity of MtMetAP1c was observed in Δ2-20, Δ2-30 and Δ2-40, the logical postulation was that the amino acid residues between 15 and 20 of MtMetAP1c are crucial. To identify the critical residue(s) that are responsible for such behaviour of the protein, the sequence of MetAPs from different Gram positive bacteria with N-terminal extension were analyzed. This led to the identification of two conserved residues, Val-18 and Pro-19 in between amino acid 15-20 (Fig. 5.4). Point mutations at 18th and 19th residue of MtMetAP1c were generated on the basis of this observation (one at a time or together with type-to-type or drastic substitutions) and their effect on the activity of the enzyme was examined. While V18A and P19A were partially active, V18AP19A did not show any activity. On the other hand, P19G exhibited partial activity but neither V18G nor V18GP19G exhibited any activity (Fig. 5.5A). Further, to assess if these mutations resulted in any structural alterations, far-UV CD studies were carried out. The outcome of these studies indicated (Fig. 5.5B) that mutation of Val-18 or Pro-19 resulted in the reduction of the PP-II structural content (V18A, P19A, V18AP19A) of the protein. In the mutant P19A, PP-II as well as
helical content was reduced. However, the mutant V18G exhibited significantly higher reduction both in its PP-II and helical content. Thus, these results suggested the importance of these two residues (Val-18 and Pro-19) exclusively and mutually towards the enzymatic activity of MtMetAP1c as a result of structural alterations having occurred due to mutation at Val-18 and Pro-19. To uncover how these residues are able to influence the enzyme activity, MD simulations were executed with protein structures of wild-type, double (V18GP19G) and single mutants (V18G and P19A). The MD simulations displayed that in the double (V18GP19G) and single (V18G) mutants, the residues (His-114, His-212 and Glu-238) found to be critical for the activity of the enzyme in biochemical experiments exhibited significant differences in their movement on a 50 ns run (Figs. 5.7 and 5.8). These differences were clearly visible at 5 ns but to confirm results, simulations were performed up to 50 ns and it was established that the conformational changes that generated at the site of mutation (V18GP19G and V18G) propagated via connecting loops and helices to the enzyme active site. These changes resulted in random movement of His-212 instead of pendulum like motion of wild-type and even displaced the His-114 from the centre and locked it into a particular coordinate system. Glu-238 was least affected of the three residues monitored for any changes as a result of mutation. All these changes in the positioning of the amino acids would make the environment in the active site unfavourable for any activity. Although there was no alteration in the secondary structure of P19A compared to the wild-type (Fig. 5.5, lower panel), MD simulations indicated the presence of different ensemble population with time (Fig. 5.8, lower panel) and thus may affect the catalytic activity of the enzyme.

Further, these findings unequivocally emphasizes that N-terminal extension of MtMetAP1c contributes towards the functionality of the enzyme by regulating active site residues through "action-at-a-distance" mechanism and this presumably is the unique function of MtMetAP1c. Since Val-18 and Pro-19 are conserved residues throughout the Gram positive bacterial MetAP1s with N-terminal extension this mechanism of action may be true for others as well.

Finally, the assessment of the role of the PTRP motif present in MtMetAP1c challenges the long standing postulation about its involvement in the interaction of MtMetAP1c and ribosome. For this purpose, in vitro pull down assay was performed
with purified GST-MtMetAP1c and His-MtL24. The results of the assay indicated that both GST-MtMetAP1c and GST-P14A/T15A/R16A/P17A (alanine mutant for the PTRP motif) are able to interact with His-MtL24 (Fig. 5.10, lower panel, lanes 3 and 4). This interaction was specific since, His-MtL24 was not detected in the two negative controls used in this study (Fig. 5.10, lower panel, lanes 1 and 2). In addition to the in vitro pull down of the two purified proteins, interaction was confirmed by expression pull down assays. As depicted in Fig. 5.11 (lower panel, lanes 2 and 3) co-purification of His-MtL24 occurs with GST-MtMetAP1c or GST-P14A/T15A/R16A/P17A. The specificity of the interaction was confirmed by utilizing GST-His-MtL24 as a control (Fig. 5.11, lower panel, lane 1). Thus, these results clearly indicate that PTRP motif does not play any significant role in direct protein-protein interaction (MtMetAP1c-MtL24). In fact, MetAP1a involved in the NME pathway of Gram negative bacteria is devoid of PTRP motif. Thus, these results highlight the fact that this may not be the universal mechanism of MetAP-ribosome interactions.