Protein synthesis involves three sequential steps namely, initiation, elongation and termination. Although, the process exhibits overall similarity between prokaryotes and eukaryotes subtle differences do exist at translation initiation step. In eukaryotes, except in organelles (mitochondria and chloroplasts), the first amino acid incorporated in the process of polypeptide synthesis has been shown to be methionine. On the other hand, in prokaryotes the process has been reported to be initiated with N-formyl-methionyl-tRNA leading to the formylation of all nascent polypeptides at the amino-terminal end. Since N-terminal peptidases have been found to be unable to utilize formylated peptides as substrates, their removal has been a mandatory step in prokaryotes for mature polypeptide synthesis (Solbiati *et al.*, 1999). The peptide deformylase enzyme (PDF) catalyzes the deformylation of N-formylmethionine of the nascent polypeptide chains in the cytoplasm of prokaryotes, and therefore, its importance has long been envisaged (Adams and Capecchi, 1966; Ball and Kaesberg, 1973; Meinnel *et al.*, 1993b). In the advent of genome sequencing, the putative gene encoding peptide deformylase (*def*) has already been reported throughout the eubacterial lineage including pathogens (Mazel *et al.*, 1997).

The *def* gene has already been reported to be essential in several bacteria (Mazel *et al.*, 1994; Margolis *et al.*, 2000, 2001) and is currently being considered as a target for developing antibacterials (Giglione *et al.*, 2000a). In this context, the work presented in this thesis is focused on PDF of *M. tuberculosis* (mPDF). It is well known that emergence of drug resistant strains of *M. tuberculosis* together with the AIDS epidemic caused rapid flourishing of tuberculosis in recent years and as a result there was considerable human mortality worldwide (Bloom and Murray, 1992; Pozniak, 2001; Reid *et al.*, 2006). To overcome the situation there is an urgent need to develop novel drug intervention strategies. To achieve this objective, identification of drug target is a prime requirement. Interestingly, inhibitors of PDF have already been reported to affect bacterial growth but as such have no toxic effect on human cell lines (Nguyen *et al.*, 2003; Serero *et al.*, 2003). Furthermore, a recent work by Cynamon *et al.* (2004) indicated that a PDF inhibitor, BB3497, inhibited the growth of *M. tuberculosis* in culture. However, no report is yet available on the nature of this

protein. This study is therefore deals with detailed biochemical characterization of mPDF enzyme.

The *def* gene was PCR amplified from *M. tuberculosis* strain H37Ra using primers designed on the basis of sequence of the pathogenic strain H37Rv (Cole *et al.*, 1998) and cloned in pUC19. Sequencing of the construct (pUC-PDF) indicated absolute identity at the nucleotide level between the *def* from virulent (H37Rv) and avirulent strains (H37Ra) of *M. tuberculosis*. Following sub-cloning in pET vector (pET-PDF), it was expressed as a histidine-tagged fusion protein utilizing an *E. coli* based expression system. Overexpression of mPDF in *E. coli* strain BL-21(DE3) resulted in the accumulation of the recombinant protein in the pellet fraction. Therefore, to obtain pure active mPDF, it was solubilized using 3 M urea and 2% triton-X-100. This was followed by extensive dialysis (14 h at 4°C) of the supernatant fraction against 20 mM phosphate buffer (pH 7.4). The mPDF was then subjected to Ni-NTA affinity chromatography for purification as a His-tagged protein. Finally, recombinant protein was eluted in elution buffer (20 mM phosphate buffer, pH 7.4 containing 300 mM NaCl, 250 mM imidazole and 10 µg/ml of catalase). The expressed and purified protein had an additional 19 amino acids (HHHHHHSSGLVPRGSH from the vector) including that of the poly-histidine region (six residues) at the amino-terminal end. SDS PAGE as well as Western blot analysis of the over-expressed and purified protein using anti-his tag antibody revealed the molecular mass of 31 ± 1.4 kDa (Mean ± SD, n = 7), which was higher compared to the expected 22.6 kDa.

The native PDFs from various microorganisms have already been identified as either Fe^{2+} or Zn^{2+} containing metalloprotease. Therefore, to identify the metal ion in mPDF, protein samples were subjected to atomic absorption spectroscopy, which revealed that the enzyme from *M. tuberculosis* contains iron in its catalytic core. To determine whether the eluted protein was enzymatically active, the deformylation ability of mPDF was assessed with different substrates (N-formyl-Met-Ala, N-formyl-Met-Leu-Phe and N-formyl-Met-Phe) in TNBSA assay. Among the substrates tested,

maximum activity was obtained with N-formyl-Met-Ala and therefore, it was used in subsequent studies.

Iron containing PDFs have often been shown to loose their enzyme activity due to conversion of Fe^{+2} to Fe^{+3} form. To prevent such oxidation, oxygen-scavenging enzymes were used during elution of the protein as well as in assays. In fact, in the experimental conditions used, omission of catalase during elution resulted in an enzymatically inactive protein. The need of these carrier proteins (catalase or BSA) in assays for mPDF activity was also monitored. Results showed that presence of catalase and/or BSA was mandatory to obtain catalytic activity mPDF. These findings were in agreement with the previous reports for other bacterial PDFs and thus, implicated the importance of metal ion in the catalytic core of mPDF.

The mPDF protein was able to exhibit deformylase activity at broad pH range (6 to 9.5). Compared to other bacterial PDFs, where monovalent cation (K^+/Na^+) increased the rate of deformylation, mPDF exhibited inhibition in its enzyme activity beyond 30 mM concentration of K^+ /Na^+. On the other hand, except Fe^{+2} no other divalent cations (Co^{+2}, Ca^{+2}, Mg^{+2}, Mn^{+2}, Ni^{+2}, Zn^{+2}) showed any significant effect on the mPDF enzyme activity. Assessment of catalytic parameters indicated that mPDF was an active enzyme with K_{cat} of 5.0 ± 0.3 s^{-1} (considering molecular mass of 22.6 kDa), although the catalytic efficiency (K_{cat}/K_m value of 1220 ± 6.0 $M^{-1}s^{-1}$) was significantly lower than that of the reported value for its *E. coli* counterpart. Inhibitors, like EDTA and PEG have been reported to inhibit the bacterial deformylase activity either by chelation of divalent cations in the catalytic core or binding to the active site. Among different inhibitors tested, 1,10-phenanthrolene was found to be potent compared to EDTA or PEG. As has been observed with other PDFs, the enzyme activity of mPDF was also found to be inhibited by a naturally occurring antibiotic actinonin, however, the K_i value was 19.7 ± 5.1 nM, which was high compared to its *E. coli* counterpart.

Although conversion of Fe^{+2} to Fe^{+3} by environmental oxygen resulted in inactivation of the deformylation ability of *E. coli* PDF, the enzyme activity of mPDF

was very stable at 30°C with a half-life of 4.1 ± 0.7 h. To rule out the possibility that this was an experimental artifact during refolding of the protein, the mPDF was expressed by co-transforming pET-PDF with pKY206 chaperonin vector in *E. coli* strain BL21(DE3). The overexpressed mPDF was found to be in soluble fraction and subsequently purified using a Ni-NTA column. The mPDF obtained in this way exhibited similar enzyme activity profile (half life = 4.4 ± 0.5 h). Interestingly, pre-incubation with oxidizing agent like H_2O_2 had no significant effect on the enzyme activity and thus, despite sharing some common properties with other bacterial homologues, mPDF seems to be distinct from known iron containing peptide deformylases.

Bacterial PDFs were broadly categorized into two classes, namely type I (gram-negative) and type II (gram-positive). Comparison of mPDF with well-characterized representatives belonging to both type I and type II classes revealed the presence of three highly conserved motifs (I: GXGXAAXQ, II: EGCLS and III: QHEXXH where X is any hydrophobic residue). The cysteine residue present in motif II and histidines in motif III of other bacterial PDFs were known to be involved in metal ion coordination and thus, essential for enzyme activity. To determine the role of these amino acid residues of mPDF, point mutants were generated through site-directed mutagenesis using PCR based approach. The overexpressed mutant proteins were subsequently purified and assessed for their deformylation abilities. Unlike wild type, all the mutants hardly displayed any deformylase activity. Thus, similar to other bacteria mutational studies emphasized the importance of cysteine and histidine residues in the conserved regions of mPDF in exhibiting its deformylation ability.

Available literature indicated that extended carboxy-terminal end is the characteristic of gram-negative bacteria. However mPDF sequence also possessed such extended carboxy-terminal end (amino acid residues 182-197 specifically "PGLSWLPGEDPDPFGH"), which is atypical for the gram-positive bacteria. Besides this, like other gram-positive bacteria (type II class), mPDF possessed insertions (amino acid residues 74-85 specifically "MTARRRGVVINP"), between

conserved motifs I and II. To evaluate the contribution of these regions towards enzymatic activity of the mPDF, deletion mutants spanning insertion region (IR: deletion of amino acid residues 74-85 or ID: deletion of N-terminal six amino acid residues of IR) and extended C-terminal end (TD, where amino acid residues 182-197 were removed) were generated. Subsequently, these mutants were expressed as his-tagged proteins and purified through Ni-NTA resin. However, the purified mutant proteins (IR, ID and TD) did not show any enzyme activity.

Loss in enzyme activity has often been correlated with its stability. The enzymatic stability, especially in Fe^{+2} containing PDFs, is a perplexing issue. Presumably it depends on the sequence variation in PDFs since amino acid side chains that are metal ion ligands could affect the rate of Fe^{+2} oxidation. It has been observed that both the deletion mutants (ID and TD) of mPDF were enzymatically inactive. Have these mutations affected enzymatic stability of mPDF rather its deformylase activity as such? Therefore to address this question, the mutant proteins (ID or TD) were mixed either with each other (ID+TD/TD+ID) or with the wildtype (WT+ID/WT+TD) at the ratio of 1:4 after denaturation with urea. Following refolding and subsequent purification through Ni-NTA resins, their deformylation abilities were assessed as the function of increasing concentration of total proteins. Among them, "co-folded" protein in the combination of TD+ID displayed marginal deformylation ability compared to wildtype, suggesting the complementation of these mutants towards enzymatic activity. This was further apparent with WT+ID or WT+TD, where higher level of enzymatic activity was noticed compared to the corresponding amount of wildtype protein present in the combination. In contrast to WT+ID and WT+TD, the C106S mutant (mutation at the metal ion coordinating Cys at motif II of mPDF) when mixed with wildtype (WT:C106S:: 1: 4; named as WT+C106S) showed enzyme activity at the level corresponding to the amount of wildtype protein present in the mixture. These results argued that the region containing the residues deleted in both ID and TD mutants were critical for the mPDF enzyme activity as well as enzymatic stability of mPDF. This was further evident from the altered half-life of the deformylase activity with WT+ID or WT+TD

compared to the wildtype alone. These results further suggested that the mPDF was catalytically active as a multimer and it was therefore logical to presume that 'hetero-monomeric" units of wildtype and ID/TD exhibited cooperativity among themselves for rendering enzymatic activity of "co-folded" proteins. The multimeric nature of mPDF and deletion mutants (ID and TD) was evident by dynamic light scattering studies and gel exclusion chromatography.

Despite the presence of Fe^{+2} at its metal binding core, pre-incubation of mPDF with oxidizing agent, like H_2O_2, had no significant effect on its deformylating ability. This seems to be an important observation considering the fact that *M. tuberculosis* has to cope up with oxidative stress for its survival within the host as a successful pathogen. To know further on this aspect, effect of H_2O_2 on the enzyme activities of WT+ID and WT+TD proteins were monitored. While the WT+ID exhibited significant decrease in the deformylase activity, the wild type or WT+TD had no effect. Therefore, it was concluded that at least N-terminal six amino acids (residues 74-79 specifically "MTARRR") of insertion region had contribution in exhibiting resistance to oxidizing agents like H_2O_2.

Interestingly, analysis of insertion region of mPDF revealed the presence of three consecutive arginines (R77, R78 and R79), typical of different mycobacterial species (*M. tuberculosis*, *M. bovis*, *M. avium*, *M. smegmatis* and *M. leprae*). Moreover, interaction of oxygen with side chain of arginine has already been established. Therefore, based on these observations it was hypothesized that arginine residues present in the insertion region could play a crucial role in preventing oxidation of Fe^{+2} in mycobacterial PDFs. To have an insight on this aspect in greater detail, point mutations were created substituting three arginines with lysine (one at a time, R77K or R78K or R79K). The alterations in any of these arginines of mPDF affected the enzyme turn over rates. Compared to Arg-77 and –78, Arg-79 was more sensitive to mutations. Mutation of Arg-79 residue also drastically affected the enzyme stability as well as its resistance to oxidizing agents, like H_2O_2. Circular dichroism (CD) spectroscopy of the mutant proteins (R77K or R78K or R79K) indicated a structural alteration of mPDF, which further substantiated their

contribution. Interestingly, incubation of *M. smegmatis* culture with an antisense phosphothiorate modified oligonucleotide directed against this conserved insertion region of mycobacterial PDFs exhibited growth inhibition. Furthermore, molecular modeling of mPDF based on available structures suggested that these arginines are not in the vicinity of the enzyme active site. They are likely to be surface exposed and presumably facilitated interaction of monomers to form dimer for enzyme activity. Thus these results emphasized the possibility of designing inhibitors of mPDF without involving active site of the enzyme and such inhibitors would definitely be mycobacteria specific.