SUMMARY

A distinctive feature of protein synthesis in prokaryotes and eukaryotic organelles (mitochondria, chloroplast) compared to eukaryotes is the involvement of an additional step of formylation of methionyl-tRNAMet in translation initiation. Therefore, N-formylated methionine has been reported as the first amino acid to incorporate at the amino terminal end of nascent polypeptide chains. This methionine is often not retained and is subsequently excised from nascent polypeptide chain by specialized hydrolytic machinery and the process of methionine cleavage is known as N-terminal methionine excision (NME) pathway. It includes two successive enzymatic reactions, first reaction is hydrolysis of formyl group from methionine by the enzyme, peptide deformylase (PDF) followed by subsequent cleavage of this exposed methionine by methionine aminopeptidase (MAP). In fact, removal of the formyl group of the first methionine, is mandatory for its subsequent excision in the process for the maturation of the polypeptide chain and therefore, importance of PDF enzyme as an antibacterial drug target is well known.

The *def* gene in bacteria encodes for PDF which comprises of three highly conserved motifs (I: GXGXAAXQ, II: EGCLS, III: QHEXXH where X is any hydrophobic residue). X-ray and NMR structures from different bacterial PDFs were solved in recent years. The metal atom in this metalloprotease has been reported to be tetra/penta-hedrally coordinated with the cysteine in motif II and two Histidines in motif III. In its naturally occurring form either iron (Fe^{2+}) or zinc (Zn^{2+}) is present in different bacterial PDFs. Iron containing *Escherichia coli* PDF was reported to be very labile because of oxidation of Fe^{2+} to Fe^{3+} by atmospheric oxygen. This extreme sensitivity of the Fe^{2+} ion of PDF to environmental oxygen prevented its isolation and biochemical characterization for a long time. To stabilize the enzymatic activity of bacterial PDFs, variety of oxygen scavangers (catalase, superoxide dismutase) are used. Also several laboratories have grown bacteria in defined media replacing Fe^{2+} with either Ni^{2+} or Co^{2+} in order to generate stable PDF variant. Interestingly, enzymatic activity of iron containing PDF from *S. aureus* did not show any oxygen sensitivity. Also PDF from *M. tuberculosis* (mPDF) was found to be an iron containing enzyme and exhibiting

resistance to oxidizing agents, like H_2O_2. Structure-function analysis as well as molecular dynamics simulation studies revealed that insertion sequence together with glycine in its conserved motif III was attributed to modulate the active site of mPDF enzyme at 'action-at-a-distance' mode for providing H_2O_2 resistance.

To know whether such an observation is universal and to explicate if these properties are particular to intracellular bacteria, PDF from another intracellular pathogen, *Salmonella typhimurium* was studied. Available genome sequence of *S. typhimurium* also indicated the presence of putative *def* open reading frame. However there is no report yet available on sPDF enzyme as such. PDF from *M. tuberculosis* (mPDF) has already been characterized with protein preparation from inclusion bodies by using urea solublization method. In this study, attempt to get solublized mPDF were successful. In this context, the present study focuses on the PDF from two intracellular pathogens *M. tuberculosis* and *S. typhimurium*. The values of different kinetic parameters obtained for mPDF were higher than that reported earlier (Saxena and Chakraborti, 2005a), this is presumably due to difference in the method used in purification of protein. Further, *def* gene was isolated and cloned from an intracellular gram negative pathogen, *S. typhimurium* which at the amino acid level exhibits ~100% amino acids sequence identity with *E. coli* PDF (EcPDF). Following its over-expression as histidine-tagged protein (sPDF) in *E. coli*, its enzyme activity was assessed using formyl-Met-Ala as the substrate. The recombinant iron containing sPDF protein was found catalytically active and exhibited similar biochemical characteristics that of its counter-part from *M. tuberculosis*. Contrary to the earlier reports with *E. coli* PDF, sPDF enzyme at 4°C was as stable as was reported for mPDF.

Analysis of kinetic parameters revealed that both sPDF and mPDF proteins in the presence of catalase (an enzyme that prevents oxidation) displayed very close values for K_m and k_{cat}/K_m. However, in the absence of catalase, the values obtained for kinetic parameters of sPDF and mPDF were very different. As reflected in the K_m values, there was ~4 fold decrease in enzyme-substrate affinity for sPDF but it was unaffected in mPDF. On the other hand, enzyme turnover rate is compromised in both the cases; the magnitude of effect was drastic in sPDF (~ 53-fold as opposed to ~7-fold in mPDF when

mpared between the values obtained in the presence and absence of catalase). rthermore, in surrogate sPDFs, where iron is replaced with either Ni^{2+} or Co^{2+}, reased enzyme activity in the presence of catalase presumably point towards the fact it sPDF protein as such is sensitive to oxidative stress.

In this study, the issue of modulation of enzymatic activity of iron containing PDFs response to oxidative stress was addressed. Comparison of enzymatic activities in ponse to pre-incubation with oxidizing agent like H_2O_2 also posed a differential effect ong sPDF and mPDF. While 10 µM of H_2O_2 completely inhibited the sPDF enzyme ivity, in mPDF it was in the range of ≥100 mM. Interestingly, both mPDF and sPDF teins contain iron at their metal binding core, they exhibit considerable amino acid uence homology as well as presence of conserved sequence motifs and they are ucturally super imposable on each-other. Therefore, it argues that if iron oxidation us is the sole factor in determining differential H_2O_2 sensitivity between sPDF and DF, it would have affected the functionality of both the proteins in similar fashion. is it suggested that the apoprotein itself is sensitive to oxidation.

Since cysteine in a protein is known to be easily and instantly oxidized in response to O_2 treatment, both the proteins, sPDF and mPDF were analysed in this aspect. uence analysis of sPDF reveals the presence of two cysteines, while in mPDF they three in numbers. Alignment of both sPDF and mPDF amino acid sequences ealed that only one cysteine is conserved in both of them (Cys-90 of sPDF and Cys- of mPDF). This conserved cysteine is reported to be essential for the functionality acterial PDF enzymes and in fact, its most conservative substitution (C90S for sPDF 106S for mPDF) did not yield any enzymatically active protein. However, being the (Fe^{2+}) coordinating residue in PDFs, it is postulated that on H_2O_2 exposure metal oxidation ultimately leads to over-oxidation of cysteine, which in turn converted to inic or sulfonic forms and as a consequence the enzyme becomes inactive. stitution of the other cysteine (Cys-130) with serine in sPDF neither showed any ificant alteration in the activity of the mutant protein (C130S) nor H_2O_2 sensitivity ile compared to the wild-type. Among other non-conserved cysteines of mPDF, ation at Cys-59 to Ser yielded a protein (C59S), where enzymatic activity as well as

H_2O_2 sensitivity patterns of which was also indistinguishable from the wild-type. Surprisingly, C68S mutant of mPDF although did not show any significant change in its enzyme activity but exhibited ~32-fold decrease in its IC_{50} value for H_2O_2 compared to that of the wild-type. Such an observation is very intriguing because in C68S mutant of mPDF, where Cys-106 is intact exhibited modified H_2O_2 sensitivity and therefore, argues the possibilities of the involvement of non-conserved cysteines in the process.

It was observed that the corresponding amino acid of Cys-130 of sPDF is methionine (Met-145). Swapping cysteine for methionine (M145C) in mPDF, however, resulted in a deficient mutant, which showed a drastic decrease (~267-fold) in H_2O_2 sensitivity as determined by calculating IC_{50} values. MALDI analysis of trypsin digested fragment containing Cys-145 of M145C protein also indicated its increased susceptibility to oxidation. This observation therefore, suggested that presence of Met-145 at this position is crucial in mPDF for its H_2O_2 sensitivity. Taken together of the independent inferences from M145C and C68S mutants of mPDF, a double mutant, C130M-V63C, of sPDF was generated which though showed ~5-fold deficiency in the enzyme turnover rate, but there was no change in affinity for the substrate or secondary structure. Interestingly, compared to the wild-type C130M-V63C exhibited ~30-fold increased IC_{50} for H_2O_2. To know the oxidation state of cysteines in sPDF, NBD-Cl assay showed predominance of oxidized (Cys-SO-NBD) population of the protein in response to H_2O_2 treatment. On the other hand, H_2O_2 was unable to affect oxidized (Cys-SO-NBD) and reduced (Cys-S-NBD) populations in C130M-V63C mutant protein. Comparison of crystal structures of PDF enzymes from *M. tuberculosis* and *S. typhimurium* (derived from *E. coli*) revealed that Cys-106, Met-145 and Cys-68 of mPDF corresponds to Cys-90, Cys-130 and Val-63 of sPDF respectively. As expected, conserved metal ion co-ordinating Cys-106 of mPDF and Cys-90 of the sPDF are quite superimposable. Interestingly, Cys-68 is in a loop at close proximity (4.5 Å) to Met-145 of mPDF and flexible enough to have interaction between them.

Bacterial PDFs are categorized in two groups, type I comprising of all PDFs from gram negative bacteria while type II includes PDFs from all gram positive bacteria. BLAST search using sPDF as query sequence with non redundant data base revealed

that all the type I PDFs are having two cysteines aligning with Cys-90 and Cys-130 positions of sPDF. On the other hand, similar search with mPDF as query sequence identified all type II PDFs, majority of them are having three cysteines (varies from 3-5) aligning with Cys-68 and Cys-106 of mPDF; also Met-145 is very conserved. Thus it is tempting to speculate that besides the conserved metal coordinating Cys-106; they are crafted in such a way that Met-145 and Cys-68 cross-talk with each-other to exhibit characteristics to withstand high level of stress upon treatment with H_2O_2. This is at least reflected in the *M. tuberculosis* physiology, where the bacterium copes up oxidative stress efficiently to be a successful pathogen. Although the mechanistic details enduring oxidative stress by mPDF is not clear at this moment, role of methionine in protection against oxidation and in endogenous anti-oxidant defense of proteins have already been reported. Further studies in this direction would unravel how oxidation/reduction of methionine, which is a reversible process, could regulate the event. Nonetheless, this study present here firm evidence for the first time that bacterial PDFs might display disparity in sensitivity towards H_2O_2, because of the involvement of non-catalytic amino acids such as cysteine/methionine in mPDF or type II PDFs.

Regarding the functionality of PDF, it has already been shown that deformylation occur cotranslationally on ribosomes. Class I PDFs which are present in all Gram-negative bacteria, including *E. coli*, are characterized by a carboxy-terminal α-helical extension. Whereas, class II PDFs are present in gram positive bacteria, including *M. tuberculosis* have insertion sequences at N-terminal end. Earlier reports have shown that *E. coli* PDF interacts with ribosomes directly with via its C-terminal extension which is dispensable for its deformylase activity. On the other hand how class 2 PDF interacts with ribosome is not known yet. Infact C-terminus of mPDF is even longer than class I PDFs and is indispensable for enzymatic activity. Interaction of PDF and ribosome was extrapolated for PDFs (sPDF and mPDF) from intracellular pathogens (*S. typhimurium* and *M. tuberculosis*). Ribosomes sedimentation assay showed interaction of both sPDF and mPDF with the ribosomes of the respective pathogen. Furthermore, ribosomal proteins, L17, L22 and L32 are reported to be involved in such interaction with class 1 PDF. To know PDF interactiong proteins of ribosome, these

ribosomal proteins were cloned, expressed and purified from *S. typhimurium* and *M. tuberculosis*. Study with sPDF and mPDF showed their interaction with respective L17, L22 and L32 ribosomal proteins reflecting that the proteins involved in interaction with both the PDFs are same.

Further work was carried out to know about the ribosome-interacting region of mPDF. Analysis of mPDF structure indicated that the C-terminal end of mPDF does not form a regular structure and C-terminus of mPDF residues 166-177 aligns well with that of the EcPDF. The speculated amino acids of mPDF when mutated showed deformylation activity but the interaction with ribosome was unaffected. Sequence analysis of class 2 PDFs revealed the presence of a conserved stretch comprising of Asp-Pro-Phe-Gly-His at C-terminal end spanning amino acid residues 193-197. Further analysis of amino acid sequence of mPDF through *PrDOS* server predicted residues from 189-197 to be disordered indicating that these residues are flexible and hence may be involved in ribosome interaction. Among them since Phe-195 was reported to be interacting with the active site of the protein. Therefore, His-197 was thought to be the candidate for mutational analysis. Activity assay revealed that H197A although enzymatically active but the residue was found to be essential for interaction with the ribosome as these results showed that PDF-ribosome interaction is universal, and beside *E. coli* PDF, among these two pathogenic bacteria *S. typhimurium* and *M. tuberculosis*, PDFs interact with ribosome through C-terminaus. Although the mechanistic aspect this regard needs to be deciphered but it is well known in the literature that histidines are capable of maintaining similar interactions.

CONCLUDING REMARKS

Peptide deformylase is an essential bacterial enzyme involved in deformylation of the N-formyl group of nascent polypeptide chains during protein synthesis. Cloning and expression of this iron containing metallo-protease from *Salmonella typhimurium* (sPDF) and *Mycobacterium tuberculosis* (mPDF) as histidine-tagged protein was carried out. Although both the enzymes exhibited optimal *in vitro* activity in the presence of

oxidation preventing agent like catalase, it was noted that sPDF and mPDF displayed differential sensitivity in response to H_2O_2, an oxidizing agent. The IC_{50} value for H_2O_2 was considerably high in mPDF compared to that of the sPDF. Since cysteine in a given protein is easily and instantly oxidized in response to H_2O_2 treatment, focus of study was put on this aspect to account for such a discrepancy. Analysis of the amino acid sequences of both the proteins revealed that sPDF has two (Cys-90, Cys-130) and mPDF is having three (Cys-59, Cys-68, Cys-106) cysteines. Among them both Cys-90 of sPDF and Cys-106 of mPDF, co-ordinate iron. Interestingly, mutation of Cys-68 in mPDF (C68S) displayed altered H_2O_2 sensitivity. Sequence alignment revealed that Cys-130 of sPDF corresponds to Met-145 of mPDF. Swapping the Met with Cys in mPDF, M145C protein displayed a drastic decrease in IC_{50} value for H_2O_2. Taken together of these independent inferences, a double mutant of sPDF (C130M-V63C) exhibited increased IC_{50} for H_2O_2. This observation strongly insinuate the involvement of Met-130 (replacement of Cys-130) by acting as intraprotein antioxidant in regulating deformylation ability of the double mutant protein in response to H_2O_2 treatment. Although the mechanistic details enduring oxidative stress by mPDF is not clear at this moment, role of methionine in protection against oxidation is well known. Further studies in this direction would unravel how oxidation/reduction of methionine, which is a reversible process could regulate the event. Nonetheless, this study present here firm evidence for the first time that bacterial PDFs might display disparity in sensitivity towards H_2O_2, because of the involvement of non-catalytic amino acids such as cysteine/methionine in mPDF or type II PDFs. However, the exact mechanism needs to be elucidated. Further studies in this direction would unravel how intraprotein antioxidant defense by oxidation/reduction of methionine, which is a reversible process, could regulate the event.

Deformylation occur cotranslationally on ribosomes and Class I PDFs interacts with ribosomes directly via its C-terminal extension which is dispensable for its deformylase activity. On the other hand C-terminus of mPDF is indispensable for enzymatic activity. Interaction of PDF and ribosome was extrapolated for sPDF and mPDF. Ribosome sedimentation assay showed interaction of both sPDF and mPDF

with the ribosome. Furthermore, ribosomal proteins L17, L22 and L32 were found to be interacting with sPDF and mPDF as reported earlier for class 1 PDF. Analysis of mPDF structure indicated that the C-terminal of mPDF does not form a regular structure and residues 166-177 aligns well with ribosome interacting C-terminus of EcPDF. The speculated amino acids of mPDF when mutated showed deformylation activity but the interaction with ribosome was unaffected. Sequence analysis of class 2 PDFs revealed the presence of a conserved stretch comprising of Asp-Pro-Phe-Gly-His at C-terminal end spanning amino acid residues 193-197. Further analysis of amino acid sequence of mPDF through *PrDOS* server predicted that these residues are flexible and hence may be involved in ribosome interaction. Among them Phe-195 was reported to be interacting with the active site of the protein. Therefore, His-197 was thought to be taking part in ribosome interactions. It was observed that H197A mutant protein was enzymatically active but the residue was essential for interaction with the ribosome as shown by the ribosome sedimentation assay. These results showed that PDF from *S. typhimurium* and *M. tuberculosis* interacts with ribosome through C-terminaus. Although the mechanistic aspect in this regard needs to be deciphered but reports suggested that histidines are usually capable of maintaining similar interactions. Although this interaction study is suggestive, it is the beginning. This is an important issue to resolve in future.