The role of poly-P, the linear chains of several ortho-phosphate residues, in bacterial stress response is well known. The principal enzyme responsible for its synthesis is polyphosphate kinase (PPK). Interestingly, the same enzyme is also involved in the mobilization of poly-P to NTPs. Therefore, PPKs are among the crucial bacterial enzymes whose importance is beyond any doubt, especially in pathogens where its direct or indirect association in virulence has often been led to its consideration as a drug target. In this context, the present study has been focused on *M. tuberculosis*, where two genes encoding polyphosphate kinases, *ppk1* and *ppk2*, are reported. While the intra-cellular life of *M. tuberculosis* within macrophages has been shown to be regulated by mPPK1, mPPK2 acted synergistically with nucleoside diphosphate kinase (NDK) in maintaining intracellular NTP pools under stressed conditions. In fact, DNA-based aptamer(s) for PPK2 that inhibited the catalytic activity of the enzyme have also been developed. However, importance of different amino acid residues performing series of events by the *M. tuberculosis* PPK has not yet been elucidated in detail. The work embodied in this thesis therefore, deals with the structure-function analyses of PPK1 from *M. tuberculosis* in context of poly-P and ATP syntheses reactions mediated by it. In addition dimerization aspect which is crucial for all PPK1 mediated activities has also been addressed.

The *ppk1* gene was PCR amplified as 2.2 *kb* fragment using *M. tuberculosis* genomic DNA. Amplified fragment was cloned in pET-28c and the expression of the recombinant His-tagged protein (pET-mPPK1) was monitored upon IPTG induction. Cell lysate and Ni-NTA purified protein on resolving in 10% SDS-PAGE yielded a band of 86 ± 0.13 kDa (Mean ± S.D., n=4) which is very close to the calculated molecular mass of mPPK1. Integrity of the fusion tag was confirmed with anti-His antibody. Furthermore, protein was also recognized with the anti-sera raised against the recombinant protein. This recombinant protein (hereafter referred as mPPK1) was used throughout the study. mPPK1 exhibited considerable similarity in its nucleotide derived amino acid sequences when compared to its counterpart in E. coli. Based on this sequence conservation, the different domains of mPPK1 are outlined. These are N-terminal (N-domain, amino acid residues 1-155), head (H-domain; amino acid residues 156-376) and catalytic (C1and C-2 domains; amino acid residues 378-558 and 559-742

respectively) domains. Experiments were than planned to evaluate the importance of different amino acid residues or a stretch of amino acid residues within the domains.

The poly-P synthesis mediated by PPK1 involves two sequential steps. These are NTP-binding to PPK1 as well as autophosphorylation of the protein followed by poly-P biosynthesis. Step-wise analyses of these events with mPPK1 were performed. Expectedly, His-tagged mPPK1 protein used in this study exhibited Mg^{2+}/Mn^{2+} dependent autophosphorylation activity. The enzyme showed optimal activity within the temperature range of 25°C-37°C. C1 and C2 domains of PPK1 exhibited structural similarity with that of the PLD family of proteins for which oxyanions are known to be inhibitors. Interestingly, among different oxyanions (molybdate, vanadate and tungstate), all of them showed the inhibition of mPPK1 autophosphorylation although at varied concentrations.

Despite its ATPase activity, mPPK1 does not exhibit any of the typical nucleotide binding motifs. However, a concomitant decrease in autophosphorylating ability of mPPK1 during pre-incubation with increasing concentrations of FSBA, a non-hydrolysable ATP-analogue was noticed, which strongly argued that the ATP binding is necessary for the initiation of autophosphorylation reaction. In *E. coli* PPK1, where X-ray crystal structure of the protein has already been solved, it is well documented that adenine ring of the ATP binds to N-terminal domain of the protein. Since N-terminal domain of bacterial PPK1s are exhibiting ~76% similarity between *E. coli* and *M. tuberculosis*, some of these residues (Phe-63, Ile-87 and Asn-91; numbering based on mPPK1 amino acid sequences) already known to be involved for such interactions were mutated. As expected, none of the mutant proteins, like F63A, I87A and N91A, displayed any autophosphorylation ability, thereby suggesting the importance of the N-terminal domain of mPPK1 in ATP binding as reported for *E. coli* protein. Furthermore, pre-incubation with different NTPs/dNTPs also inhibited the ^{32}P-labeling of mPPK1, which argued their possibility of being substrates for autophosphorylation reactions.

Phosphoryl-protein linkages are pH dependent. Analysis of effect of pH on mPPK1 phosphorylation revealed that it can withstand alkaline pH of as high as 14. Such an observation strongly argues in favor of the notion that mPPK1 is a histidine kinase. Subsequent loss of the autophosphorylation signal of mPPK1 upon heating for

increasing time periods suggested inclusion of mPPK1 under histidine kinases. Available literature indicated that both His-491 and His-510 are involved in autophosphorylation of mPPK1. To have an insight on this aspect, both His residues at 491 and 510 positions were substituted with either Ala or Gln. Expectedly, none of the His-491 mutant proteins (H491A and H491Q) exhibited any autophosphorylation activity. Similarly autophosphorylation assay using same amount of protein as has been used for wild-type did not yield any activity with H510A. Surprisingly, H510Q mutant protein displayed autophosphorylation, which is quite comparable to that of the wild-type. Thus, it seems the effect is amino acid specific and His-510 has no role in autophosphorylation of mPPK1.

The autophosphorylation of the protein is followed by its poly-P synthesis ability and it is the last step of the forward reaction mediated by the mPPK1. Since these activities are inter-linked, it was observed that the mutations of mPPK1 compromised for autophosphorylation also reflected in its poly-P synthesizing ability. ATP binding mutants also revealed highly compromised for poly-P synthesis activity thus establishing the essentiality of ATP binding for poly-P synthesis by mPPK1. Additionally, increasing concentrations of either H480A or H480Q showed the activity comparable to wild type and thus nullified the involvement of His-480 in mPPK1 mediated forward reaction. However, neither H491A nor H491Q mutants showed any activity even at higher protein concentrations indicating the indispensability of His-491 for mPPK1 mediated forward reaction. Poly-P synthesis by H510A was found to be highly compromised as compared to wild-type. However, as expected, replacement of His-510 by glutamine (H510Q) did not have any effect on the poly-P synthesis. These findings were further substantiated by the kinetic analysis of the histidine mutants. H510A mutant protein showed an increased K_m value for ATP (~3 fold) compared to that of the wild-type, which suggests that decreased affinity for the substrate presumably affected the catalytic efficiency of the enzyme for the poly-P synthesis. The difference in the behavior among H510A and H510Q mutants is also indicative of the fact that His-510 *per se* does not have any contribution in poly-P synthesizing ability of mPPK1 but mutations depending on the amino acid substitution affects the activity of the protein. On the other hand, His-491 is the phospho-histidine residue of this kinase, which is critical for the functionality (both autophosphorylation and poly-P synthesis) of the mPPK1.

PPK1 catalyzes the ATP synthesis from poly-P as a part of its reverse reaction. In general, nucleoside diphosphate kinase (NDK) is a ubiquitous enzyme, responsible for maintenance of NTP pools in prokaryotes. It catalyzes the phosphorylation of various NDPs by utilizing NTPs as phosphate donor and thus generates NTPs. Since PPKs catalyze the ATP synthesis from poly-P as a part of its reverse reaction, they are often been referred as auxiliary NDK. PPK1 in *E. coli* and *P. aeruginosa* is known to catalyze formation of different NTPs/dNTPs utilizing poly-P as phosphate donor. Assessment of the ability for the synthesis of different NTPs/dNTPs revealed that mPPK1 synthesizes almost all NTPs and dNTPs albeit with varied efficiency. At this juncture, it is intriguing to mention that in some microorganisms PPK2 has been assigned to perform auxiliary NDK function. Utilization of poly-P by *M. tuberculosis* PPK2 for the synthesis of ATP or GTP has also been investigated. However, the experimental conditions used in the present study, it was observed that the mPPK2 can synthesize only ATP, which is at par with the properties of single domain PPK2 as it is in *M. tuberculosis*.

The behavior of the His residue mutants was also monitored on the ATP synthesizing ability of mPPK1 using poly-P20 as the substrate. As has been observed in poly-P synthesis, H491A or H491Q proteins hardly depicted any ATP generating capability. Surprisingly, both H510A and H510Q proteins displayed a significant decrease in catalytic efficiency compared to that of the wild-type for ATP synthesis. Kinetic analysis of the enzymatic activity of H510A protein revealed that there is ~31 fold increase in K_m and ~ 4 fold decrease in k_{cat} values compared to that of the wild-type, which resulted in overall ~ 145 fold decrease in catalytic efficiency of the mPPK1 enzyme for ATP synthesis. Unlike poly-P synthesis activity of mPPK1, catalytic efficiency of the H510Q enzyme for ATP synthesis is reduced by ~2 fold compared to that of the wild-type and obviously this has happened due to decrease in k_{cat} value. Thus, the results for the first time ascertained that His-510 in addition to His-491 contributes in reverse reaction mediated by mPPK1 to generate ATP.

The elution profile of mPPK1, based on the experimental set up used in the present study, revealed two peaks corresponding to monomeric and dimeric population of the mPPK1 protein besides void volume peak (oligomeric population) in molecular sieving chromatography. As expected, eluted samples from both the peaks when

resolved in SDS-PAGE exhibited single band corresponding to its monomeric molecular mass. However, in autophosphorylation assay it was found that dimeric but not monomeric population was active. This observation corroborates well with earlier reports that mPPK1 is active as a dimer. Importantly, it was noticed that both the population must be in equilibrium (in the present experimental condition dimeric population is ~2.5-fold to that of monomer) so that the protein is active. Interestingly, in H510A protein monomeric population was significantly increased compared to that of the wild-type. Thus, the results argue that His-510 of mPPK1 has the ability in contributing towards maintenance of monomer-dimer ratio, which has never been reported for any PPK1.

The role of different residues of the carboxy-terminal domain of mPPK1 was also investigated. Among PPK1s characterized to date, X-ray crystal structure of *E. coli* protein has already been determined. In fact, both *E. coli* and *M. tuberculosis* PPK1 amino acid sequences exhibited considerable (68%) similarity. Therefore, *E. coli* PPK1 structure was used for designing different mutants of mPPK1 and the effect of mutations were monitored on the poly-P as well as ATP synthesizing abilities of the protein. For this, alanine scanning mutagenesis was performed through the C1 and C2 domains of mPPK1. As expected, it was noticed that the mutations of amino acid residues involved in binding of adenine/ribose moieties of ATP affected both the activities. Furthermore, the mutations of the amino acid(s) interacting with phopho-His (His-491) residue exhibited loss in both the activities. On the other hand, the mutations in residues very likely to be involved in proper positioning of phosphate group of ATP or ADP disrupted only the NTP synthesizing function of mPPK1. Interestingly, despite being conserved residues it was observed that the mutation of Arg-431, Tyr-524 and Arg-624 of mPPK1 behaved differently than its *E. coli* counterpart. This species specific behavior is not unusual and therefore demands structure determination of mPPK1 to unravel the mystery. However, it seems that the amino acids involved in ploy-P and NTP syntheses reactions are distinct and well crafted for specific functions.

Another important aspect of PPK1 is its dimerization which is decisive for all its activities. Autophosphorylation with the alanine mutants of the predicted (based on *E. coli* crystal structure) residues (F269A, K385A and D419A) and deletion mutant Δ268-273 revealed that F269A and K385A behave like wild-type and thus ruling out

the possibility of their involvement in enzyme dimerization. However, autophosphorylation studies with the alanine mutants of Phe-176 and Arg-230 residues in the head domain of mPPK1, which were earlier reported to be involved in PPK1 dimerization showed complete loss of the activity. To monitor if these mutations affected the secondary structure of mPPK1, Far-UV CD spectra of different mutants were monitored. Although D419A or Δ268-273 proteins did not show any gross changes compared to the wild-type, secondary structure of F176A and R230A mutant proteins exhibited a significant alteration. Since PPK1 is active only as a dimer, it was presumed that their mixtures are very likely to complement the activity of the wild-type at least to a certain extent. To test this hypothesis, Ni-NTA purified proteins from each of these mutants were mixed together in equal amount (1:1) (D419A+ Δ268-273 or F176A+R230A) or with a functionally inert mutant of mPPK1, H491A (D419A+H491A or Δ268-273+H491A or F176A+H491A or R230A+H491A). Needless to say that secondary structure of this mutant (H491A) did not show any significant change. Activity assays (autophosphorylation, poly-P synthesis or ATP synthesis) with these samples when compared to wild-type, exhibited gain in function for all combinations except for F176A+R230A, F176A+H491A and R230A+H491A. Thus this result, argued in favor of intermolecular association of mPPK1 for its functionality and displayed role of Asp-419 and Δ268-273 in dimerization. To confirm this aspect, gel permeation chromatography was carried out with D419A and Δ268-273 mutant proteins using Sephacryl S200 column. Analysis of the elution profiles of wild-type mPPK1, F176 and R230 revealed two major peaks, one in the void volume (multimeric fraction), another at 172 kDa and a minor peak at 86 kDa. On the other hand, both D419A and Δ268-273 showed a major peak at ~86 kDa (and a minor peak at 172 kDa). Kinase assay of different peak fractions from column elutes indicated that monomeric population as exhibited by D419A and Δ268-273 mutant proteins are devoid of any activity. Thus, it is apparent that Asp-419 of C-1 domain interacts with "LFPGME" of H domain resulting in dimerization in an asymmetric fashion.

Finally, the results of the present study gleaning a wealth of useful information about the structure-activity relationship governing the forward or reverse reactions mediated by mPPK1 and unequivocally established that several amino acid residues are required either for poly-P or ATP synthesis but not both activities of the protein. Additionally, the residues from H and C-1 domains involved in dimerization of mPPK1

are identified. Since PPK1 is much conserved throughout the phylogeny, it seems that results presented here have broader implications and not solely restricted to *M. tuberculosis* only.