SUMMARY AND CONCLUDING REMARKS

Phosphorylation is one of the most prevalent post translational event used in transmittance of the signals in all living organisms. Initially, it was believed that the two component system responsible for histidine and aspartate phosphorylation is only present in prokaryotes while Ser/Thr or Tyr kinases are restricted to eukaryotes. However, with bacterial genome sequencing, it became evident that Ser/Thr phosphorylation is also a major event influencing prokaryotic cellular physiology. The presence of two component system along with the eukaryotic-type Ser/Thr kinases in bacteria hinted at some specialized functions being performed by these proteins. At present, there are numerous reports documenting that Ser/Thr kinases through phosphorylation control myriad of biological processes within bacteria, ranging from cell division and growth, antibiotic resistance, transcription control to virulence. Also, many pathogenic bacteria were reported to possess Ser/Thr kinases. One such example is *Mycobacterium tuberculosis* that differs from other strains within its species by evolving diverse tools to subvert host immune system. There are eleven eukaryotic-type Ser/Thr kinases and one cognate phosphatase that control several processes in this pathogen.

In this context, the present study is focussed on PknA, an essential eukaryotic-type Ser/Thr kinase in *M. tuberculosis*. Our lab has been associated with PknA since its characterization as an active Ser/Thr kinase. The most stimulating observation which paved a way for future studies was that expression of PknA leads to elongation of *E.coli* cells which was also confirmed in *M. smegmatis* and *M. bovis* by another group; thus, confirming its putative role in cell division. As the key molecule involved in bacterial cell division is FtsZ so its regulation by PknA was assessed. The phosphorylated FtsZ showed decreased GTPase and polymerization activities. One of the other interacting partners of PknA was found to be MurD, a ligase involved in peptidoglycan synthesis. Subsequently, structure function studies of PknA were in progress. PknA being a transmembrane receptor comprises of a catalytic domain along with a juxtamembrane domain in its cytosolic region and an extracellular domain for sensing the environmental signals. Previous studies from lab showed indispensability of all domains for functionality of PknA. However, what role each domain has towards PknA kinase activity remained unknown. Therefore, an effort to have a deep insight into domain function was made. The catalytic domain (1-277 amino acids) consists of all conserved

Hank's subdomains required for catalytic function, therefore it was expected to exhibit kinase activity. But, catalytic domain was found to be inactive. However, upon addition of entire juxtamembrane domain to the catalytic domain, autophosphorylation as well as transphosphorylation activities were exhibited. These findings became a theme of the present study as it became intriguing to understand whether whole juxtamembrane region was required for kinase function and how it contributes towards attainment of catalytic function by PknA. For this, a series of mutants of PknA catalytic domain were constructed. All these mutants were examined for their autophosphorylating abilities by *in vitro* kinase assay. Interestingly, the initial six residues from the N terminal of the juxtamembrane comprising of 1-283 amino acid region (designated as PknA-283) showed autophosphorylation while all other mutants from 1-277 (PknA-277) till 1-282 (PknA-282) were unable to exhibit autophosphorylation. Further, the catalytic properties of PknA-283 and PknA-338 were compared which included their requirement for divalent cations, effect of inhibitors on their kinase functions and their efficiency to phosphorylate substrate protein. It was found that PknA-283 was able to display auto- and trans- phosphorylation in a manner similar to PknA-338. Being non-phosphorylatable in nature, contribution of the six residues was a bit intriguing. To investigate their role in PknA-283 activity, all the six residues were replaced by alanines leading to the generation of PknA-283A mutant. PknA-283A was utilized for assessing its autophosphorylating ability and surprisingly, it was found to have retained the activity to a substantial extent. This mutant was also tested for its ability to transphosphorylate FtsZ, the natural substrate of PknA and it could transphosphorylate the same. These findings highlighted the need of the precise length for exhibiting the kinase function. Still, insight into the role of the six remained unexplored. As per the reports on other kinases, presence of the juxtamembrane generally, provides stability to the kinase structure, thus influencing its kinase activity. Therefore, to determine whether these six residues contribute to the secondary structure of the kinase protein, CD analysis was done. It was observed that in comparison to secondary structure of PknA-283, PknA-277 in CD analysis showed a remarkable difference, thereby indicating that the addition of the six residues contribute to the secondary structure. Another supporting clue that was obtained was that the secondary structure of PknA-283A which exhibited significant autophosphorylation as well as transphosphorylation activity resembled the

secondary structure of PknA-283. Altogether, these results for the first time establish that only six residues of PknA juxtamembrane N-terminus are needed along with its catalytic domain for exhibiting the full kinase activity and they help the kinase to attain the best conformation for efficient catalysis to occur.

PknA has been found to be predominantly phosphorylated at threonines compared to serine as per the mass spectrometric analyses done by two separate groups. But, identification of a phosphorylated residue cannot confirm its role towards the activity of the protein as phosphorylation at a site can be involved in either positive or negative regulation. Therefore, our next aim was to identify the threonines important for PknA activity. PknA-283 depicts autophosphorylation activity signifying that the residues within this region are participating in the kinase function. For this, the fragment between the activation loop till the last 283rd amino acid of PknA was chosen to identify the participatory threonines. It was found that this region contained five threonines and one of them, Thr-224 was the only common residue found in the mass spectrometric analyses of separate groups. To investigate role of these threonines, they were individually substituted for alanines except for the ones in the activation loop. For Thr172 and Thr174, a double mutant was created. The double mutant of activation loop threonines was designated as T172A-T174A (lie in TQT motif) and the single mutants as T180A, T224A and T252A (position of the replaced threonines is designated by the numeral) were created. All these threonine mutants were tested for their ability to autophosphorylate and transphosphorylate. Interestingly, all the threonines except T252A exhibited highly decreased autophosphorylation and transphosphorylation activities. Since, the decrease in activity of the PknA-283 threonine mutants could be an affect of absence of charge which was earlier provided by the phosphoryl group, to mimic presence of the negative charge, aspartate mutants of all the threonines (T172D-T174D, T180D, and T224D) except Thr252 were generated and their autophosphorylation activities were examined. The presence of charge did not enhance the autophosphorylation efficiency of aspartate mutants. It was observed that in case of T180A/T180D mutants there was a drastic reduction in autophosphorylation activity. Further, to investigate whether these mutants had any effect on transphosphorylation ability, myelin basic protein, an exogenous substrate was tested and it was found that transphosphorylation abilities of all the aspartate mutants were also drastically reduced.

These findings clearly suggested that all these threonines are required for efficient functioning of PknA-283. The transphosphorylation activities of alanine mutants of PknA-283 towards its natural substrate, FtsZ were also monitored. It was observed that compared to wild type PknA-283 all others (T172A-T174A, T-180A, T-224A) could barely phosphorylate FtsZ, suggesting that these mutants had nearly complete loss of function. Also, transphosphorylation of FtsZ by threonines mutations was tested *in vivo* by taking *E.coli* as a heterologous host. For this, FtsZ was co-expressed along with full length PknA and its threonines variants. The purified FtsZ protein was examined for its phosphorylation status by anti-phosphothreonine antibody. As observed previously, the PknA threonine mutants were deficient in their ability to phosphorylate FtsZ. The FtsZ protein was found to be phosphorylated only in presence of the active PknA whereas no phosphorylation was seen in FtsZ co-expressed with threonine mutants.

As expression of full length active PknA in *E.coli* elongates the cells and inactive PknA, K42N-PknA is devoid of any such phenotypic effect, it was intriguing to know the effect of the threonines mutations on *E.coli* morphology. For this, threonine mutations in full length PknA (T172A-T174A-PknA, T180A-PknA, and T224A-PknA) were transformed into DH5α cells and examined for morphological change in the cells. Interestingly, a remarkably distinct phenotype was observed in the cell morphology of the cells bearing threonines mutants. These cells were unable to exhibit a significant change in cell morphology compared to the filamentous cells harbouring active PknA. A careful analysis of these results, to unravel how each threonine contributes towards PknA activity was done. The reduction in kinase activity due to mutations at Thr172-Thr174 could be a consequence of alteration in the interactions activation loop makes for the kinase to achieve active conformation. Interestingly, rather than activation loop mutant, it was Thr-180 mutant which led to $\geq 90\%$ loss of activity. The literature survey documented this residue to be a part of highly conserved GT motif. The threonine in this motif is implicated in hydrogen bond formation with catalytic aspartate of the HRD motif. Therefore, threonine mutation at this position might have led to loss of interaction, further causing complete loss of kinase activity. Even though threonine of GT motif was found to be phosphorylated in PknE, but the presence of negative charge at Thr-180 in PknA-283 was also not sustained leading to loss of PknA activity. Thr-224 was the only other residue besides activation loop threonines whose replacement with

alanine demonstrated a significant decrease in both auto and transphosphorylation activities. It lies in the αG helix which is reported to be involved in substrate binding as well as in autophosphorylation of PknB. In addition, both PknB and PknL, two other eukaryotic-type Ser/Thr kinases which share clade with PknA have conserved serine at the position of Thr-224 in PknA. All together, these results signify that apart from activation loop threonines, Thr-224 serves as an important regulatory residue in PknA.

Dimerization has been proposed to be a general activation mechanism for Ser/Thr kinases. Apart from phosphorylation of the regulatory sites, no additional regulatory mechanism for PknA is yet known. Hence, it was enticing to explore whether oligomerization has role to play in PknA function. To investigate this, gel permeation studies were done. Two separate fractions, one of 42kDa ±2 kDa and another of 92 kDa ±5 kDa were resolved during gel permeation studies, corresponding to a monomer and presumably a dimer. The identity of these proteins was verified by anti-His antibody. Interestingly, these two fractions showed a different migratory pattern with ~92 kDa fraction running slightly slower than ~42 kDa fraction on coomassie gel. Literature has several reports where many other mycobacterial kinases PknD, PknH and PknL were found to have multiple bands which were established as different phosphorylation isoforms of the same protein. This information indicated towards a possibility of different phosphorylation isoforms in PknA-283 and hence both the populations were probed with anti-phosphothreonine antibody. A very intense signal was seen in ~92 kDa fraction while comparatively lower intensity signal was visible in ~42 kDa fraction, indicating a remarkable difference in their phospho-occupancy. Further, autophosphorylation activity of both these fractions was assessed. Surprisingly, negligible autophosphorylation activity was observed in the ~92kDa fraction while the ~42 kDa fraction demonstrated robust autophosphorylation activity. The contrasting results of western blot and activity assay could be an outcome of saturated phosphosites in the ~92 kDa fraction because of which no autophosphorylating activity was visible in this population. Therefore, it seemed logical to examine whether it could transfer the phosphate to the substrate. To analyze this, transphosphorylation activities of both ~92 kDa and ~42 kDa fractions were assessed. Though the ~42kDa fraction exhibited the transphosphorylation activity towards the exogenous substrate, the ~92 kDa fraction was found to be devoid of it. The presence of highly phosphorylated ~92 kDa fraction in an

tive form suggested that it could be a regulatory mechanism of the protein to shut f when there was no active phosphatase as in case of *E.coli* system. To verify this mption, PknA-283 was co-expressed along with specific Ser/Thr phosphatase and ıer examined for its gel permeation profile. The outcome was interesting as a single nct population of ~38 kDa was observed. This population was examined for its ity. It was found to be capable of autophosphorylation activity. This observation ved that ~92 kDa fraction was actually an outcome of excessive or multiple site phorylation. To verify our results, an approach to mutate activation loop threonines taken. The rationale behind this approach being, that PknA's activation loop onines (as known in other RD kinases) must be the primary site of phosphorylation, efore these might influence the phosphorylation on other sites in the protein. The ation loop mutant was checked for its gel permeation profile. A fraction equivalent 8 kDa was eluted. This finding indicated towards role of the activation loop phorylation in the formation of ~92 kDa fraction. But, it still remained unclear ther the phosphorylation at activation loop also has some role. To analyze this, a ble aspartate mutant (T172D-T174D) which mimiced the negative charge of two phorylated threonines was utilized for examining gel permeation profile. And, this there were two fractions, one eluting at the molecular weight equivalent to ~92 kDa another at the size of ~ 42 kDa. These results were in consensus with our hypothesis ıe need of charge at the activation loop. All together the results pertaining to the lation of PknA suggested that once a kinase monomer attains excessive charge or omes hyperphosphorylated, they associate together to form a dimer in such a manner the threonines participating in phosphotransfer are buried inside with no access to substrate. Thus, our studies provide evidence for the first time that erphosphorylation of PknA might be its autoregulatory mechanism when sphatase becomes inactive.

However, the long established concept for kinase activation involves their erization. To investigate that whether dimerization triggers PknA-283 activation, ıcible dimerization system was utilized. This system was used because several orts proved that kinase domains formed very weak dimers and forceful dimerization required to study its influence on kinase activity. Our results depicted enhancement utophosphorylation activity of both phosphorylated and unphosphorylated PknA-283

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mechanism of PknA to shut off its activity in absence of active phosphatase. Since unphosphorylated kinases are very slow in catalysis, allosteric regulation by dimerization is implicated in their activation. Whether PknA-283 is also regulated by dimer formation, inducible dimerization system was used which showed that the trigger for PknA-283 to get activated is provided by the dimerization. Thus, all together, this study gives new insights into how PknA molecule gets regulated.

Though the residues participating in the kinase function have been deciphered, the mechanism behind their functioning remains to be explored. Structural analysis of PknA would be the most effective tool to answer these queries.