Structural and Functional Analysis of Serine/Threonine Protein Kinase of *Staphylococcus aureus* Exhibiting Variations with Other Bacterial PknB

Abstract

*Staphylococcus aureus* expresses Serine-Threonine protein kinase (STPK) encoded by PknB gene in the extracellular filtrate and this enzyme is implicated in the expression of virulent factors, toxins and regulation of various metabolic pathways thereby creating anaerobic conditions which favours biofilm formation. Earlier we have cloned, sequenced (JN695616), expressed and characterized PknB gene of *Staphylococcus aureus*. The enzyme kinetics was established using synthetic peptide NLCNIPCSALLSSDITASVNCAK (stpks). The 3D structure of stpks was built using the amino acid sequence of stpks in Modeller 9v8. Further, stpks structure docked very close to the ATP binding site of STPK structure (PDB ID: 4EQM) with energy complex of -487.83Kcal/mol explaining the rapid transfer of γ-phosphate of ATP to its substrate therefore, proteins possessing STPK site would be highly vulnerable for phosphorylation by *S. aureus* PknB. Structural superimposition studies showed conspicuous variations were observed in STPK structure among bacteria as evidenced from the RMSD values which correlated with differences in the enzyme kinetics docking energy levels with substrate stpks. These differences indicate rate of phosphorylation among bacteria is variable which means factors contributing to pathogenesis are markedly different among bacteria.

Keywords: STPK; PknB; RMSD; ATP; Modeller9V8; Phosphorylation; Superimposition; Conspicuous; Pathogenesis

Abbreviations: STPK: Serine-Threonine Protein Kinase; RMSD: Root Mean Square Deviation; PIA: Polysaccharide Intracellular Adhesion; BHI: Brain Heart Infusion;

Introduction

Protein phosphorylation through PknB is one of the principal mechanisms by which the cell wall and polysaccharide intracellular adhesion (PIA) synthesis, functioning of various metabolic pathways, resistance to antibacterial agents and autolysis which are all required for biofilm formation. This pathogen survives in the human host mostly as biofilms contributing resistance to several antibiotics making *S. aureus* as a multidrug resistant strain. Further, in this organism PknB is actively present in the external secretions and along with phosphatases they interact with vast range of host tissue proteins aiding this pathogen to colonize in any anatomical locales in human host [1-9]. The biological roles of the STPKs vary in bacteria and are critically dependent on their substrate specificity. STPKs regulates stress responses, developmental processes, biofilm formation, cell wall biosynthesis, sporulation, metabolic nutrient acquisition and in the pathogenicity [10-17].

These kinases first demonstrated in the soil microorganism *Myxococcus xanthus* in 1991 [18]. However, with advent of bacterial genome sequence analysis presence of serine/threonine protein kinases (STPKs) in prokaryotes have been identified [19]. The number of STPKs varies among bacterial species for example *Mycobacterium leprae* contains 4 STPKs, *Myxococcus xanthus* contains 13 STPKs and as many as 24 in *Mycobacterium marinu*. The *M. tuberculosis* genome includes 11 STPK genes, namely PknA to PknL, and their domain organization indicates that only two enzymes PknG and PknK are cytosolic proteins and other nine STPKs are membrane bound proteins contains transmembrane domain which connects the N-terminal kinase domain inside the cell to a C-terminal sensory component outside the cell [20].

These STPKs contains 12 Hanks motifs in their catalytic domain which is the characteristic feature of their eukaryotic counterparts [21]. These enzymes consist three domains, first N-terminal catalytic domain having homology with its eukaryotic counterparts distributed in cytoplasm, second the transmembrane domain which anchors the enzyme to the plasma membrane and followed by third extracellular C-terminal PASTA (Penicillin-binding protein and Serine/Threonine kinase Associated) domain act as sensor [22]. This type of Hanks protein kinase was identified in the *S. aureus* PknB which has 30% sequence similarity with human STPK and 37% with PknB of *M. tuberculosis* [8,9]. In view importance of serine/threonine protein kinases in the regulation of metabolic pathways and pathogenesis in several bacteria including *S. aureus* the present study is aimed to understand the structural and functional characters of *S. aureus* PknB gene.
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Materials and Methods

Bacterial cultures and condition

Staphylococcus aureus ATCC 12600 was grown in modified Baird parkar agar plate, a single black shiny colony with distinct zone was picked and inoculated in Brain heart infusion (BHI) broth at 37°C. Thus, grown S. aureus culture was used for extraction of chromosomal DNA [23].

Amplification, sequencing and cloning of STPK gene from Staphylococcus aureus

The purified PknB PCR product was sequenced by dye terminating method at commercial sequencing facility of Xcelris Labs Ltd, Ahmedabad, India. The obtained sequences were analyzed and deposited at GenBank (http://www.ncbi.nlm.nih.gov/nuccore/JN695616). The STPK gene was cloned in the Sma I site of pQE 30 vector and the clone was named as PV 1. The pure recombinant enzyme was expressed using 0.75mM IPTG and purified by passing through nickel metal chelate agrose column as described earlier [8,9].

Serine/threonine protein kinase (Pkn B) assay

Culture was centrifuged at 10,000 rpm for 10min at 4°C and the supernatant was stored at -86°C for STPK enzyme assay which was carried out as described earlier Vasu & Prasad et al. [8,9].

Molecular modelling of stpks

The stpks structure was built using Modeller 9v8 tool and using the template human lysozyme PDB (ID: 1REX) and the structures were validated by PROCHECK [24].

Protein – protein docking

The molecular docking was carried out using Hex 6.3 software between S. aureus STPK, Mycobacterium tuberculosis, Streptococcus pneumoniae, Bacillus subtilis, Streptococcus agalactiae, Escherichia coli and Myxococcus xanthus STPK with stpks to predict the possible modes of interactions. To initiate the protein-protein docking STPK the structure was considered as receptor and synthetic peptide stpks as ligands. Both were loaded into HEX working environment and docking search was started by rotating the receptor and ligand about their centroids at their intermolecular distances. Both of them were assigned to Euler rotation angles and a six-dimensional search was carried out over full rotational ranges. An initial steric scan at N=18 was followed by a final Search at N=25, using steric contribution to the docking energy. Each orientation was evaluated using a steric and electrostatic correlation to order N for the significant increment of total docking times. High resolution final search correlation was performed using smaller distance increments for the fast low resolution steric scan phase for the rapid and finer coverage of search space at the final phase. This process was controlled by the distance range of 40, scan step of 0.8 and two sub steps using docking controls. The angular increments of 7.5 degrees and a twist angle of 5.5 degrees were used in the rotational search in each of ligand and receptor rotational angles. Newton-like energy minimization was applied and single molecular mechanics energy was calculated for each docking conformation using Lennard-Jones and hydrogen bond potentials, adapted from the OPLS force field parameters, along with an explicit charge-electrostatic contribution. Global rotational search and translational space scanning was done using spherical polar fourier transformations. This process can rank the output based on surface complementarities and electrostatic potentials [24].

Structural superimposition of S. aureus STPK with other bacterial STPK structures

The relative structural calculation studies to ensure the uniqueness and unpredictability of S. aureus STPK structure with other bacterial STPK structures were carried out using PyMOL program. The structural similarity score as the log-odds ratio of two sub structures using a scheme similar to Dayhoff’s amino acid substitution score. An alignment of superimposed structures and similarities were identified, scores and RMSD values for the following structures: the S. aureus STPK (PDB ID: 4EQM) was super imposed with other bacterial STPK such as Mycobacterium tuberculosis (PDB ID: 3ORI) were retrieved from PDB while Streptococcus pneumoniae (Built structure), Bacillus subtilis (Built structure), Streptococcus agalactiae (Built structure), Escherichia coli (Built structure) and Myxococcus xanthus (Built structure) STPK structures were built by using Modler9v8 method as mentioned earlier [25].

Results

Identification of STPK enzyme activity

The PknB gene in the clone PV 1 was sequenced (JN695616) and after confirming the sequence the expression of STPK in PV 1 clone was induced by adding 0.75mM IPTG and the recombinant proteins from the cytosolic fraction was purified by passing through nickel metal chelate agarose column. The STPK enzyme was identified by its ability to phosphorylate synthetic peptide stpks (NLCNIPCALLLSDITASYNCAK). The phosphorylated peptide stpks and enzyme were fractionated on Sephadex G-25 column, the autophosphorylated STPK appeared in the void volume while the peptide substrates appeared in the second elution peak. Thus, obtained phosphorylated proteins were treated with reagent-A and the intense blue coloured was measured at 820nm. Here, the ammonium molybdate in acidic condition specifically binds to the phosphate bound to the peptide substrates and enzymes. Since; we have purified the substrate and enzyme the reagent-A reacted only with the phosphate bound either to pure substrate or pure enzyme and the enzyme kinetics are shown in Table 1. These phosphorylated STPK and substrates fractionated in SDS- PAGE gel when immersed in reagent-A intense blue coloured bands were developed with decreased mobility of the proteins (Figure 1).

Structural and functional activities of STPK

The amino acid sequence of synthetic peptide was used to build three dimensional structure in Modeller 9v8 software using the human lysozyme PDB (ID: 1REX) as template. The predicted 3-D structure of stpks was validated through the PROCHECK and the resultant Ramachandran plot indicated 95% residues positive and negative bacteria.
were structurally in favoured region and 5% residues were in additional allowed regions indicating the predicted model was valid with good stereo chemical quality (Figure 2).

Table 1: Enzyme Kinetics of r PknB.

| Source of Enzyme                  | Enzyme Activity (µM/ml/min) | K_m (mM) | Vmax (µM/mg/min) |
|-----------------------------------|----------------------------|----------|------------------|
| r PknB (Substrate level)          | 0.157±0.07                 | 0.716±0.07 | 1.95±0.2        |
| r PknB (Auto phosphorylation)     | 0.32±0.08                  | 0.377±0.06 | 7.8±0.43        |

Values are the mean ±SD from three determinations.

The synthetic peptide stpks structure was docked with the STPK structure (PDB ID: 4EQM) (Figure 2) and the results showed that stpks is interacting with STPK structure very close to the ATP binding site. A list of 500 docked complexes was produced that are spatially having similar docking orientations. These conformations were grouped and ordered by energy. Among all the conformations the first conformation with lowest complex energy was chosen for the analysis purpose. There was an initial distance of 24.9Å among the centroids of the two molecules when loaded into the working environment. After the docking process the first docked conformation showed a distance of 18.4Å. The interacting residues were found to be 133D, 135K, 137G, 174Q, 175T, 275R, 277N, from STPK and N20, S18, S12, N1, T16, from stpks, the ligand interacting together by forming hydrogen bonds with the complex energy of -487.83Kcal/mol (Figure 2). These results clearly demonstrate stpks was bound close to the ATP binding site to induce catalysis validating the assay. Further, docking of stpks with STPK structure in other bacteria revealed similar docking with variation in energy complexes (Table 2 & Figure 2).

Structural comparison of S. aureus STPK with other bacterial STPK

The multiple sequence alignment of PknB in different bacteria revealed considerable variations (Figure 3). Thus, the S. aureus STPK structure was compared with other bacterial STPK structures. The S. aureus STPK structure showed close homology with Streptococcus pneumonia, Streptococcus agalactiae, Mycobacterium tuberculosis and Myxococcus xanthus, the RMSD values being 0.068 Å, 0.079 Å, 1.314 Å and 1.574 Å respectively however with, Escherichia coli and Bacillus subtilis, the structures showed extensive variations both in the domain and non-domain regions as evidenced from RMSD values 16.182 Å and 19.127 Å respectively. The identical regions are random throughout the alignment especially the structural superimposition of substrate binding regions showed much extent of variation and completely showing different conformations as indicated from the RMSD values (Figure 4).

Discussion

Reversible protein phosphorylation is one of the major regulatory mechanisms in bacteria which control the key metabolic pathways, cell wall biosynthesis, production of various toxins; expression of virulence factors, biofilm formation and antibiotic resistance. Typically, extracellular signals are translated into cellular responses by modulating the activity of a responsive protein depending on the phosphorylation of specific amino acid sites such as serine/threonine [1-9]. In earlier studies we have shown the functioning of isocitrate dehydrogenase is purely controlled through PknB and this has profound influence on the phosphorylation of specific amino acid sites such as serine/threonine [1-9]. In earlier studies we have shown the functioning of isocitrate dehydrogenase is purely controlled through PknB and this has profound influence on the phosphorylation of specific amino acid sites such as serine/threonine [1-9]. In earlier studies we have shown the functioning of isocitrate dehydrogenase is purely controlled through PknB and this has profound influence on the phosphorylation of specific amino acid sites such as serine/threonine [1-9].
The multiple sequence alignment showed distinct variations in the sequences between PknB in different bacteria [9]. These variations correlated with differences observed in enzyme kinetics and docking energies of substrate stkps with STPK structure (Table 2 & Figure 2). The structural superimposition studies exhibited large variations among the bacteria as indicated from the RMSD values (Figure 3 & Table 2) clearly explaining rate of phosphorylation in bacteria is different which probably has profound effect on their pathogenesis [20, 26-28].

The pathogenic bacteria show different rates of infectivity in the host primarily due to differences in the metabolic pathways and its regulatory mechanisms. Bacteria have placed its metabolic pathways according to the needs in any given niche which is largely controlled through phosphorylation by STPK [9,20,26-28]. Therefore, PknB functioning has profound role on the pathogenesis which is variable among bacteria.
Figure 3: Multiple sequence alignment of S. aureus STPK with other bacterial STPK protein sequences.
Figure 4: Superimposition of S. aureus STPK structure (green) with other bacterial STPK structures.

Table 2: RMSD values of S. aureus PknB with other bacterial PknB.

| S.No | Organism                  | $K_m$       | RMSD  | Docking Score   | Reference     |
|------|---------------------------|-------------|-------|-----------------|---------------|
| 1    | S. aureus                 | 0.377±0.06mM| -     | -487.83 Kcal/mol| In the present study |
| 2    | Mycobacterium tuberculosis| 40.9±15.4µM | 1.314 Å| -514.30 Kcal/Mol|               |
| 3    | Bacillus subtilis          | 61.3±1.7mM  | 19.127 Å| -429.5 Kcal/Mol | 11            |
| 4    | Streptococcus agalactiae   | 3.677±0.014mM| 0.078 Å| -481.27 Kcal/Mol| 32            |
| 5    | Streptococcus pneumoniae   | 1.43±0.29µm | 0.068 Å| -464.18 kcal/Mol|               |
| 6    | Escherichia coli           | -           | 16.182 Å| -525.67 Kcal/Mol|               |
| 7    | Myxococcus xanthus         | 45µM        | 1.574 Å| -472.48 Kcal/Mol| 31            |

Conclusion

Phosphorylation by serine/threonine protein kinase is the key mechanism which controls metabolic pathways and pathogenesis in pathogenic bacteria. In S. aureus it is involved in the phosphorylation large number of host human proteins influencing their function and thus paving way for its colonization and biofilm formation. This phenomenon is distinctly different in each organism the results of the present study is clear reflection which showed distinct structural variations correlated with differences in the docking energies for substrate stpks, enzyme kinetics and sequence among bacteria. Suggesting different phosphorylation mechanism prevails in bacteria which is aptly associated with differences in pathogenesis among bacteria.

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