Acute myocardial infarction and ischemic stroke due to blockage of blood vessels with fibrin clots are the two main causes of disability and mortality in cardiovascular diseases claiming million of lives every year. Other than surgical interventions to remove or bypass the blockage or the generation of collateral vessels to provide a new blood supply, the only treatment available is the administration of thrombolytic agents to dissolve the blood clots. The thrombolytic agents approved for clinical use include tPA, uPA, SK, anisoylated Pg-SK activator complex (APSAC) and recombinant tPA. But tPA and SK are the most effective thrombolytics currently available and widely used for treatment of thrombotic diseases. Although tPA is more efficient in terms of its thrombolytic potential but SK is more cost effective as compared to tPA due to its bacterial origin. The latest entry to the list of these ‘clot busters’ is another Pg activator of bacterial origin i.e. Staphylokinase (SAK). Being the smallest and fibrin specific Pg activator SAK is an ideal system for the development of a new thrombolytic molecule. The various clinical trials and animal studies have established the immense therapeutic potential of SAK as a clot buster. SAK is equally effective to tPA in arterial recanalization and results in less breakdown of plasma proteins because of its high fibrin specificity. Moreover, being a bacterial protein, it has the advantage of cost effectiveness which makes it an attractive choice to develop as a replacement for the currently used thrombolytics.

Pg activation by SAK-Pm complex is a highly complicated process that involves the participation of SAK as a cofactor, Pm as an enzyme with modified substrate specificity and Pg as a substrate. The activation process involves a sequence of events involving high affinity intermolecular interactions between SAK and Pm to generate the bimolecular activator complex onto which the substrate Pg can dock in optimally for its efficient activation. Subsequently, a transient binding of the substrate Pg takes place during each catalytic cycle of Pg activation. Formation of SAK-Pm bimolecular complex to generate an active site specific for activation loop of Pg and presentation of substrate Pg through exosites in proper orientation for catalysis in active site are the two crucial events of this cofactor mediated enzymatic process. Although crystal structure of ternary complex of SAK with μPm has provided some clues for structurally and functionally important regions of SAK and catalytic domain of Pg/Pm, yet the basis of the fine intermolecular interactions between SAK and Pm that occur to change the specificity of Pm, are not clearly understood at the molecular level. Through site
directed mutagenesis and molecular modelling approach we have probed the role of SAK residues that are involved in generating the specificity of the active site of bimolecular complex for activation of Pg. Moreover, the region of SAK, adjacent to SAK-Pm binary activator complex has been studied for its possible contribution in affecting the binary complex and subsequently ternary interactions with substrate Pg.

Ternary complex of SAK with μPm indicates that sequence composition of SAK lining the active site cleft of μPm may be crucial for positioning of activation loop of substrate Pg in the active site. The crucial region of SAK that lies at the interface of SAK-μPm binary complex near the active site of μPm spans from Gly36 to Glu46. During activator complex formation, extensive salt bridge and hydrogen bond network is generated between SAK and μPm that rearranges the positioning of His43 and Tyr44 making them to protrude more within the active site of μPm. It has been suggested that change in the positioning of His43 and Tyr44 due to intermolecular interactions at the interface alters the specificity of activator complex by narrowing down the subsites boundaries, making it highly specific for activation loop of Pg. Crystal structure analysis of the ternary complex of SAK and μPm through PyMol suggested that these two residues i.e. His43 and Tyr44 occupy a unique position at the interface near the active site pocket by establishing crucial contacts with Trp215 of μPm. Site directed mutagenesis of SAK residues lining the active site cleft of Pm revealed that substitution of His43 with a hydrophobic residue i.e. phenylalanine results in drastic reduction in cofactor activity of SAK. Role of His43 was proposed merely to narrow down the active site cleft of Pm. But according to this possibility, substitution of His43 with phenylalanine, would not have caused such drastic change in Pg activation property of SAK. Occurrence of positively charged at position 43 in the reported natural variant of SAK indicates that positively charged residue might be involved in some charge interactions with Pm. CAPTURE program was run to predict the possibility of cation-pi interactions of in silico mutants of SAK in which His43 was mutated to lysine or arginine. The positively charged virtual mutants of SAK were found to be involved in cation-pi interactions with Trp215 of μPm. This hypothesis was supported by functional characterization of site specific mutants of SAK in which His43 was replaced with lysine, phenylalanine or glutamic acid. Replacement of His43 with lysine does not make any distinct change in the functionality of SAK but substitution of His43 with a hydrophobic or negatively charged residue causes a drastic reduction in the Pg activation
property. These results provide strong evidence that a positively charged residue is required at this position to maintain the functionality of SAK. Although histidine, arginine and lysine residues have different lengths of side chains, presence of these positively charged residues in natural variant as well as in SAK mutant have no effect on functional properties of SAK. The cofactor activity of SAK mutants carrying either of the positively charged residues at position 43 remains largely unaltered indicating that instead of the length of side chain, the positive charge on the residue is a prerequisite for the optimal functionality of SAK.

Structure analysis of SAK-\(\mu\)Pm ternary complex revealed that Tyr44 of SAK may interact with Trp215 of \(\mu\)Pm strongly to form intermolecular contacts. Interatomic distances between the two participating residues and presence of a T-shaped geometry of interaction between the two aromatic rings of these residues suggested a strong possibility of a pi-pi interaction. Site directed mutagenesis of Tyr44 to phenylalanine does not hamper the cofactor activity of SAK while Pg activation ability of SAK gets reduced drastically on substitution of Tyr44 to alanine. The experimental data was further validated by molecular modelling studies. Energy minimization of various SAK mutants at 43rd and 44th position in ternary SAK-\(\mu\)Pm complex supported the occurrence of cation-pi interaction in SAKHis43-\(\mu\)PmTrp215 pair and pi-pi interaction in SAKTyr44-\(\mu\)PmTrp215 pair. Interestingly, lysine or arginine residue in SAKHis43 mutant establishes a new contact with Glu180 of \(\mu\)Pm to stabilize this intermolecular complex. But in case of SAKHis43Glu mutant, the negative side chain of this glutamate residue conflicts directly with negative side chains of Glu180 of \(\mu\)Pm. The structurally equivalent position to His43-Tyr44 pair of SAK in SAK-\(\mu\)Pm complex is occupied by a similar type of pair of basic and aromatic residue (Lys36-Phe37) in a domain of SK in SK-\(\mu\)Pm complex. This part of study establishes that binding of SAK with Pm not only narrows down the active site region by providing subsites but also participates in generation of intermolecular contacts for stabilizing the binary complex formation. Although intermolecular contact among SAK and \(\mu\)Pm was altered even in functional mutants of SAKHis43 but local pi-pi interaction between SAKTyr44 and \(\mu\)PmTrp215 was maintained in all functional mutants of SAK and may be critical for the specificity switch and/or Pg activation. Overall results, thus, provide a new insight into the molecular mechanism by which His43 and Tyr44 residues of SAK, residing at the interface of SAK-Pm bimolecular complex may generate crucial interactions with the
partner Pm and modulate the functionality and/or specificity of SAK-Pm activator complex.

The structural comparison of SAK-μPm complex with that of SK-μPm reveals that a domain of SK makes similar type of intermolecular contacts with μPm near its active site as that of SAK. In the interface region of SAK with μPm spanning from Gly36 to Glu46, the zone of hot spots for making intermolecular contacts lies from Ser41 to Glu46. SAKSer41-μPmGln177 and SAKGlu46-μPmArg175 interactions in SAK-μPm complex are well conserved in SK-μPm complex at structurally equivalent positions in SK i.e. Ser34 and Glu39 to establish similar contacts with μPm. His43 and Tyr44 of SAK establish cation-pi and pi-pi interactions with Trp215 of μPm to generate the specificity switch at the active site of μPm in the binary activator complex. Pro42 and Val45 of SAK are present on the flanking sides of His43-Tyr44 pair of SAK and lie at the junction of residues participating in direct contacts with μPm i.e. Ser41 and Glu46. Unbound SAK differs from the bound one in SAK-μPm complex suggesting the participation of various residues in adapting structural changes during protein-protein interactions in SAK-μPm binary complex formation. Presence of Pro42 and Val45 at the crucial junctions i.e. Ser41-His43 and Tyr44-Glu46 region respectively may play a crucial role in the structural orientation of these residues for optimal interaction with μPm at the interface. Site directed mutagenesis of Pro42 and Val45 of SAK region validates their requirement in maintaining the structural integrity of this region of SAK to participate in binary complex formation. Replacement of Pro42 with leucine and Val45 with tyrosine or phenylalanine in order to mimic the structural topology of SK residues present at structurally equivalent positions disrupts the functionality of SAK. Energy minimization studies of in silico mutants of SAK at Pro42 and Val45 positions suggest their role in the structural regulation of His43-Tyr44 pair in generating the specificity switch at the active site of μPm. Energy minimized coordinates of the bimolecular complexes of these virtual mutants show the loss of cation-pi interaction between His43 of SAK and Trp215 of μPm. These studies suggest that these residues play an important role in modulating the specificity switch generated by His43-Tyr44 pair with Trp215 of μPm through cation-pi and pi-pi interactions respectively.

In an attempt to characterize the region of SAK adjoining SAK-Pm binary interface, residues from Phe47 to Lys50 were targeted through site directed mutagenesis. Crystal structure of SAK-μPm ternary complex shows that Glu46-Pro48 region of SAK
Overview

juxtaposes to Glu5-Gln7 region of substrate μPm. The role of Glu46 of SAK to interact
with Arg175 of μPm for making binary activator complex is already well established.
So, Phe47-Lys50 region of SAK, which is present at the junction of SAK-μPm interface,
may play a crucial role in Pg binding and activation as well. Site directed mutagenesis of
these residues abrogated the Pg activation capability of SAK. Biochemical and
biophysical data to characterize the binary or ternary interaction of SAK with μPm
suggests that this region of SAK plays a dual role in the Pg activation process. As shown
in the crystal structure, the substrate μPm interacts with SAK at this junction during its
catalysis. All the mutants of this region except Ile49Val show their inefficiency in
making 1:1 binary activator complex with Pg and proper exposure of the active site of
their bimolecular complexes.

Overall, these site-directed mutagenesis and molecular modelling studies
suggest that the structural arrangement of the various residues at the interface region of
SAK with μPm is well organised and highly specific to generate the bimolecular
complex for the activation of substrate Pg. His43 and Tyr44 pair of SAK interacts with
Trp215 of μPm through cation-pi and pi-pi interactions for generating the active site
specificity of this binary complex for substrate Pg. The interactions of this pair for
generating the specificity switch in the bimolecular complex are modulated by two
flanking residues on both sides i.e. Pro42 and Val45. Phe47-Lys50 region of SAK plays
a dual role in binding with Pm for generation of binary activator complex and then
interacts with the substrate for its optimal presentation for the catalytic cleavage of the
activation loop of Pg in the active site. The elucidation of this mechanism would provide
a better understanding of the cofactor activity of SAK and may eventually be translated
into general concept on how to modulate the substrate specificity of Pm and other serine
proteases.

The second aspect of the present study is to employ various strategies for
improving the thrombolytic potential of SAK. PEGylation has been a well known
approach for improving the therapeutic potential of proteins. SAK being a small protein
utilises its most of the residues in cofactor activity as a Pg activator. Thus, site specific
PEGylation at any non essential position in SAK can provide a chance to increase its
plasma half life and reduce antigenicity to develop it as better thrombolytic molecule.
Addition of one or two cysteines at N- and/or C- terminus and then cysteine specific
PEGylation increases the temperature stability, plasma half life and decreases its
protease susceptibility and immunogenicity. The PEGylated derivatives of SAK show more stability in terms their temperature and protease susceptibility. These derivatives were equally active to native SAK when kept at 37 °C for different time points. However, PEGylated SAK molecules showed higher resistance to temperature at 65 °C. Native SAK was not able to activate Pg after 1-2 hr of incubation at 65 °C while activation potential of PEGylated SAK molecules gradually decreased from 2-12 hr. The half life of various SAK derivatives increased significantly in the range of 10 min to 3 hr. Similarly, the immunogenicity of these derivatives decreased from 70% to 30% as compared to native SAK. The interesting aspect of the study is to use cysteine specific PEGylation without altering the core amino acids which may affect the structural and functional properties of SAK. The relative increase in the improvement of the properties of various PEGylated derivatives of SAK depends upon number and size of PEG molecules attached. Moreover, C-terminal PEGylation SAK is more advantageous in terms of PEGylation efficiency and purification.

In addition to PEGylation, the other strategy to improve the thrombolytic potential has been employed by generating chimera of SAK with β domain of SK in order to enhance the Pg activation capability. Previous studies in our lab have demonstrated that addition of β or βγ domain separately in the reaction mixture can increase the Pg activation process by SAK. Surprisingly, in contrast to isolated domains, fusion of β or βγ domains with SAK results in reduction in Pg activation potential of SAK. Since SAK binding to Pg is not as strong as that of SKα domain, there might be some constrain between SAK and β domain for binding to preferred sites on Pg resulting in drastically attenuated Pg activation ability of chimera. Therefore, linkers with different length and composition were used in order to reduce the possibility of domain hindrance with each other. Initially, linker between α and β domains of SK was used to fuse SAK with β domain, but chimera was less efficient in Pg activation. In other attempts, the linkers of 20 amino acids were used and composition was also varied to achieve the increase in Pg activation potential of SAKβ fusion protein. Even the linkers already reported in literature for C-terminal fusion of SAK were used but the enhancing effect of β domain on Pg activation potential of SAK could not be achieved. By engineering the linker region between SAK and SK domains in SAK fusion proteins, it might be possible to transfer the enhanced cofactor activity of SAK imparted by the presence isolated SK domains to SAK fusion proteins.
In conclusion the major findings and the future implications of this study are:

- Site directed mutagenesis of SAK has provided the experimental evidence for the participation of His43 and Tyr44 residues of SAK in generating the crucial intermolecular contacts for making binary activator complex and generating the specificity in the active site of μPm for activation loop of Pg for its efficient cleavage. The specificity switch generated by His43-Tyr44 pair of SAK is modulated by Pro42 and Val45 present on flanking sides of this pair. Replacement of Pro42 and Val45 with other residues affects SAKHis43-PmTrp215 interaction negatively that leads to defect in binary complex along with impaired specificity. Structure analysis and molecular modelling studies along with experimental data highlights Ser41-Glu46 as hot spot zone of interaction for making binary activator complex. Moreover, adjoining region at the junction of this interface i.e. Phe47-Lys50 is actively involved in SAK binding with Pg/Pm for making binary complex and then interaction with substrate Pg for its activation. Site directed mutagenesis of the other residues of interface of SAK with Pm and their adjoining region may provide a better understanding of binary and ternary interactions of SAK with Pg/Pm. Moreover, other regions of SAK can be explored through biophysical and biochemical characterization for identifying the substrate binding sites that are not explained through crystal structure of SAK with truncated version of Pm.

- Addition of cysteine at the terminus by site directed mutagenesis and its cysteine-specific PEGylation increases the thrombolytic potential of SAK by increase in its plasma half life and reduction in the immunogenicity. Antigenic epitopes within the molecule can be targeted for PEGylation in order to have better SAK derivatives with reduce antigenicity without affecting its Pg activation potential. Although SAK chimeras with β domain of SK failed to activate Pg efficiently, yet attempts can be made to engineer the linker properties to improve the cofactor activity of SAK fusion protein. Moreover, the fusion of SAK with other proteins or domains with anticoagulant properties can really improve the thrombolytic potential of SAK by overcoming the problem of reocclusion in patients.