Acute myocardial infarction and ischemic stroke due to blockage of blood vessels with clots are the two main causes of disability and mortality in modern society. 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 clot. For this, the thrombolytic agents, currently approved for clinical use are tPA, uPA, SK, anisolyted Pg-SK activator complex (APSAC) and recombinant tPA. The latest addition to this 'clot busters' list is staphylokinase, a Pg activator of bacterial origin. Recent clinical trials have established SAK as a potent and uniquely fibrin-selective thrombolytic agent. Being the smallest and fibrin-specific Pg activator, SAK is an ideal system for the development of new thrombolytic molecules.

Since SAK is naturally produced at low levels by various strains of *Staphylococcus sp.*, we therefore, developed expression system to produce large amounts of intact biologically active SAK for further structure-function studies. Intracellular expression of SAK under IPTG inducible T7 promoter results in accumulation of sufficiently large quantities of SAK inside the cells in soluble form. Purified SAK appears as a single 15.5 kDa band without any degradation product and fully retains its Pg activation properties. Use of IPTG for induction of recombinant proteins makes the system very costly for large-scale production; therefore, an alternative SAK expression system was generated with an objective to omit the regular use of IPTG for protein induction and to use this system for large-scale SAK production at high cell density culture. Gene encoding SAK was expressed under the control of a novel oxygen-sensitive OXY promoter, which is hyperinduced when oxygen level drops in culture. During the fermentation process, oxygen level of the growth condition rapidly falls below 5% of atmospheric oxygen and the reduced oxygen level in growth conditions automatically induces the OXY promoter resulting in high level expression of gene cloned under this promoter. Expression level of SAK under OXY promoter is comparable with SAK expression level under T7 promoter. Therefore, expression under OXY promoter may provide a comparatively less expensive

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system, for SAK production. In another expression system the ability of VHb (*Vitreoscilla* hemoglobin) to increase bacterial growth and production level of protein, when expressed in heterologous host was exploited in order to attain high cell density during recombinant SAK expression in *E. coli*. Although there are no significant differences in cell densities upon co-expression of SAK and VHb, the level of SAK production increases markedly.

Along with various procaryotic expression systems, *Pichia pastoris*, a faster, easier and less expensive eucaryotic expression system was tried for SAK expression. *P. pastoris* generally attains higher biomass, resulting in increased recovery of the cloned product. Unlike *E. coli*, *P. pastoris* expression system is free from endotoxins, therefore, feasibility of *P. pastoris* to produce SAK was explored. Expression of SAK in *P. pastoris* was confirmed by Western blot analysis and by analyzing Pg activation activity of yeast cell lysate. The expression however, was merely detectable on SDS-PAGE. Therefore, *E. coli* system was selected for expression and purification of SAK and its variants for structure-function characterization.

After establishing an efficient expression system, purified preparation of SAK protein was used for structure-function characterization. Although three-dimensional structure of SAK and its ternary complex with μPm have been resolved, the molecular mechanism whereby SAK brings about structural changes after association with partner Pg or Pm and interacts with free Pg to generate Pm activity is still largely unknown. From a mechanistic perspective, two distinct protein-protein interactions occur during the SAK-mediated Pg activation process. SAK generates an inactive 1:1 bimolecular complex with Pm, which requires removal of a decapeptide from the amino-terminus of SAK and the conversion of substrate Pg into Pm. SAK does not directly induce conformational changes in the active site residues but it alters substrate specificity of Pm indirectly by creating new subsite onto which substrate Pg docks for the enhanced presentation of the Pg activation loop towards the enzyme. The crystal structure of ternary complex μPm-SAK-μPm reveals only partial picture of SAK-Pm interactions owing to the absence of auxiliary domains (kringle structures) in μPm as compared to full length Pg/Pm molecule. We have

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demonstrated that SAK-Pm enzyme complex may result in additional interactions with the kringle domains of the substrate Pg via a charged loop region of SAK (SAK 90-loop) that may facilitate the interaction of substrate Pg during activation process. SAK 90-loop region is at a position totally distant from the protein-protein interaction sites in the three-dimensional structure of μPm-SAK-μPm ternary complex. Deletion of SAK 90-loop region results in 5-6-fold reduction in specific activity and significant attenuation of the Pg activation properties of the molecule. However, SAKΔ90 forms a 1:1 complex with Pm as well as displayed an amidolytic activity comparable to the native SAK-Pm binary complex. Moreover, progressive exposure of active site within the SAKΔ90-Pm complex clearly indicates that similar to native SAK, the SAK loop-deletion mutant is capable of generating activator complex with Pm. Therefore, the slow activation kinetics of Pg activation by SAKΔ90 may not be due to alteration in the activator complex formation with Pm rather the bimolecular complex formed may not be able to interact optimally with the substrate Pg. These results were further substantiated by slow cleavage of Pg into Pm by SAKΔ90-Pm activator complex. Furthermore, site-directed mutagenesis within the SAK 90-loop region revealed that substitution of Lys_{97} or Lys_{98} within this loop region with alanine causes similar lesion in substrate Pg activation by these SAK mutants. These results provide the evidence that Lys_{97} and Lys_{98} of the SAK 90-loop may be assisting in sequestering the substrate Pg. The orientation of substrate Pg with respect to SAK-Pm enzyme complex in a three-dimensional model of μPm-SAK-μPm complex indicates that SAK 90-loop might provide an ideal docking site for the kringle structure of substrate Pg. In an attempt to further evaluate the probable interaction of kringle structures and SAK 90-loop, competition between substrate Pg and kringle was examined during Pg activation by SAKΔ90-Pm activator complex and compared with that of native SAK-Pm complex. Kringle domains of Pg (K1+K2+K3+K4) effectively competes with Pg for interaction with SAK-Pm activator complex, whereas, SAKΔ90-Pm complex does not exhibit any significant change in Pg activation pattern in the presence of kringles. Conceptually, kringles could modulate the interaction of substrate Pg by providing more optimal docking

of substrate on SAK-Pm activator complex through additional interaction site(s) via their lysine binding sites. In conclusion, site-directed and loop-deletion mutagenesis of SAK has provided novel information on SAK-Pg interaction and provides the experimental evidence for the participation of SAK 90-loop region in facilitating the interaction(s) of substrate Pg with the SAK-Pm enzyme complex. Lys_{97} and Lys_{98} of SAK 90-loop region may be specifically involved in this process. These additional interactions between free Pg substrate and bimolecular SAK-Pm complex, thus may potentiate the SAK-mediated Pg activation process.

During Pg activation by SAK, high affinity intermolecular interactions between SAK and Pm are generated to create an activator complex, resulting in the creation of a new subsite onto which substrate Pg can dock-in optimally. In the next step, a transient binding of substrate Pg takes place during each catalytic cycle of Pg activation. Presentation of substrate Pg optimally on the activator complex is crucial for the Pg activation process and requires a number of intermolecular interactions and a topological complementarity between the energetically critical regions of SAK and Pg. 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, yet the basis of fine intermolecular interactions between SAK and Pg that occur to change the specificity of Pg, are not well understood at the molecular level. We have probed the role of SAK residues involved in modulating the interactions of activation loop of substrate Pg with the activator complex of SAK-Pm, using site-directed and molecular modeling approach.

Ternary complex of SAK with μPm indicates that sequence composition of SAK lining the active-site cleft of μPm may be crucial for proper positioning of activation loop of the substrate Pg in the activator complex. One of these regions spanning from Gly_{36}-Glu_{46}, lies near the active-site cleft. During activator complex formation extensive hydrogen bond network is generated between SAK and μPm that rearranges the positioning of His_{43} and Tyr_{44} making them protrude more within the active-site cleft of μPm. It has been

suggested that change in the positioning of His_{43} and Tyr_{44} due to intermolecular interactions between SAK and μPm alter the specificity of activator complex by narrowing down the boundaries of subsite, making it more suitable for the interaction of activation loop. Site-directed mutagenesis of SAK residues lining the active-site cleft of Pg reveals that substitution of His_{43} with hydrophobic residues results in drastic reduction in cofactor activity of SAK. In the crystal structure of μPm-SAK-μPm complex, the proposed role of His_{43} was merely to narrow down the active-site cleft of Pg. Had that been the case, substitution of His_{43} would not have such drastic change in Pg activation property of SAK. Occurrence of positively charged residue at position 43 in the reported natural variants of SAK, indicates that positively charged residue might be involved in some charge interactions with Pg. This hypothesis was supported by functional characterization of site-specific mutants in which His_{43} was replaced with lysine, phenylalanine or glutamic acid. Replacement of histidine with lysine also dose not make any distinct change in the functionality of SAK but substitution of histidine with hydrophobic or negatively charged residues causes a drastic reduction in Pg activation capability of SAK. These results provide strong evidence that a positively charged residue is required to maintain the functional integrity of SAK. Although histidine, arginine and lysine residues have different lengths of side chains, presence of these positively charged residue in natural variant as well as in SAK mutant have no effect on functional properties of SAK. Cofactor activity of SAK mutants carrying either of the positively charged residue 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.

Drastic change in Pg activation activity of SAK-Pm complex upon substitution of His_{43} with either hydrophobic or negatively charged residue strongly suggests that along with narrowing down the active-site cleft in enzyme Pg, His_{43} may be involved in direct charge interaction with the substrate Pg. This hypothesis is strongly supported by computational analysis of μPm-SAK-μPm complex using Insight II package. Structural analysis has shown that SAK residue at 43 position lies in vicinity of substrate μPm

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activation loop, at a distance and in an orientation, appropriate for electrostatic interactions with B7Glu (Glu_{554} of substrate Pg). Substitution of His_{43} of SAK with hydrophobic or negatively charged residue will abolish the favorable electrostatic interactions, resulting in reduced substrate Pg activation by activator complexes of these mutants with Pm.

The present study, thus establishes that the binding of SAK with Pm not only narrow down the active-site region but also anchors the substrate Pg in a position so that activation loop can be processed efficiently. 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.

SAK is the smallest known single domain Pg activator that exhibits close similarity with the α domain of multi-domain Pg activator, SK. SAK and α domain of SK perform similar function in a Pg activator complex through generating intermolecular contacts between partner Pg/Pm and substrate Pg, whereas β and γ domains of SK target Pg region different from where SK α and SAK bind. Unlike SAK, SK α domain is not fully functional as a Pg activator and requires integration of both β and γ domains to induce optimal Pg activation capability in full length SK molecule. We have examined the ability of different domains of SK to act synergistically with SAK during Pg activation. Isolated β domain or its two-domain combination with γ domain of SK increases the Pg activation potentiality of SAK dramatically, whereas γ domain of SK alone has no effect on Pg activation by SAK. Since β domain of SK carries high affinity binding site for Pg that does not overlap with SAK, it is possible that both β and βγ domains combine with the partner Pm along with SAK and restructure the activation complex to a conformation best suited for the Pg activation resulting in its enhanced catalytic activity. Surprisingly, in contrast to isolated domains, fusion of β and βγ domains along with SAK results in drastic reduction in Pg activation potential of SAK. Moreover, no secondary structure alterations were observed upon fusion of SK domains with SAK as CD spectrum of SAKβγ fully overlapped with that of SK.

Drastic difference in the behavior of free SK domains and their fusion with SAK towards the cofactor activity of SAK suggests that intermolecular interaction between Pg and isolated β and βγ domains of SK may be quite different. To get some insight into the procedure by which isolated SK β/βγ domains are facilitating the Pg activation by SAK, possible binding sites for β or βγ domains in Pg were analyzed from the earlier reports. Although crystal structure of SK and μPm complex shows only few intermolecular contacts among SK β and μPm in the activator complex, recent biochemical studies on SK have implicated that a positively charged surface exposed loop of β domain forms a critical interaction with kringle domain of substrate Pg. Addition of purified kringle domains of Pg inhibits the Pg activator activity of SAK in the presence of isolated SK domains to a greater extent as compared to cofactor activity of SAK and SAK fusion proteins. This suggests that instead of interacting with Pg kringle domains, SK β and βγ domains interacts largely with free kringle domains and therefore, fail to display their positive effect on Pg activation activity of SAK efficiently. Since free kringles can interfere with SAK as well as with isolated SK β domain, percentage inhibition of SAK cofactor activity is more pronounced when isolated SK domains are used. Low level of inhibition in case of SAK fusion protein by isolated kringle domains may be due to the inability of SAK and/or SK domains to interact efficiently with kringle domains of Pg. Interaction of isolated SK domains with kringle domains may restructure the Pg, making it a better substrate for SAK-mediated activation process. This hypothesis is supported by studies on μPg activation by SAK in the presence of isolated SK β and βγ domains. Isolated SK domains fail to alter the cofactor activity of SAK for μPg.

We have demonstrated that the enhanced Pg activation potential of SAK, in the presence of isolated domains of SK involves their interaction with Pg that at least in part, is mediated via the kringle structures. Extended intermolecular interaction between Pg and isolated SK domains presumably reconfigures Pg and enhances the substrate Pg activation by activator complex. Fusion of β and βγ domains of SK with SAK alters these favorable interactions of SK domains with Pg. Although all three domains of SK interact with

catalytic domain of Pg, β domain also possesses affinity for its kringle domains. Isolated SK α domain has a higher binding affinity for Pg than SAK, therefore it may be possible that in SK, strong interactions between α domain and catalytic domain of Pg are forcing the other two domains to bind next to α to the less favored catalytic domain of Pg, whereas, given a choice isolated β domain might prefer to interact with kringle domains of Pg. Since SAK binding to Pg is not as strong as that of SK α domain, there might be some constrain between SAK and β/βγ domains for binding to preferred sites on Pg after fusion resulting in drastically attenuated Pg activation activity of fusion. 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 of isolated SK domains to SAK fusion proteins.

In conclusion, the major findings and future implications of this study are:

- Novel intracellular hyper-production system for SAK has been established. Oxy promoter system can further be exploited for large-scale production of recombinant SAK.
- Site-directed and loop-deletion mutagenesis of SAK has provided the experimental evidence for the participation of SAK 90-loop region in facilitating the interaction(s) of substrate Pg with the SAK-Pm enzyme complex via kringle structure. Residues other than positively charged amino acids in 90-loop of SAK can further be subjected to site-directed mutagenesis to improve the kringle-mediated substrate specificity of SAK-Pm complex.
- Interactions of His_{43} with substrate Pg have been established by site-directed mutagenesis and is supported by computational structure analysis. Other residues of SAK surrounding the active-site cleft of Pm can be analyzed for their structure-function importance in SAK-mediated Pg activation.
- Interactions of isolated β and βγ domains of SK with Pg that at least, in part are mediated via the kringle structures results in enhanced cofactor activity of SAK. Although upon fusion with SK domains, SAK failed to activate Pg efficiently,

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yet attempts can be made to alter the site of fusion to improve the cofactor activity of SAK fusion protein.