4. STUDIES ON FUNCTION(S) OF 90-LOOP REGION (THR$_{90}$-GLU$_{100}$) OF STAPHYLOKINASE IN PLASMINOGEN ACTIVATION
Staphylokinase (SAK) forms a bimolecular complex with human plasmin(ogen) and changes its substrate specificity by exposing new exosites that enhances access of substrate plasminogen (Pg) to the Pm active site. SAK has no proteolytic properties of its own but it acts as a cofactor in the Pg activation process. Human Pg contains five kringle domains that serve as functional binding loci for other plasma proteins (Sugiyama et al., 1988). The activation of truncated Pg derivatives, miniplasminogen (miniPg carrying only kringle 5 and protease domain) and microplasminogen (μPg carrying only protease domain) is slower than the full-length Pg suggesting a role of kringle domains in the Pg activation process (Chang et al., 1998). The molecular mechanism by which these kringle structures modulate overall processing of Pg by various Pg activators is not very well known at present. The three-dimensional structures of SAK and μPm-SAK-μPm complex have been elucidated by X-ray diffraction studies (Rabijns et al., 1997; Parry et al., 1998). The heterotrimeric complex consists of two μPm molecules and one SAK molecule (Parry et al., 1998), SAK binds one μPm molecule in the proximity of its active site. Formation of this bimolecular complex presents a slightly concave surface onto which the second molecule of μPm docks in a substrate-like manner. Of the amino acids in SAK, 30% are charged and these amino acids including 20 lysine residues are critical for its Pg-activator activity. A number of SAK mutants (Silence et al., 1995; Schlott et al., 1997; Schlott et al., 1998) have been generated in order to investigate the determinants of SAK for Pg binding and activation. Site-directed mutagenesis of SAK indicated that its positively charged amino-terminal region may be involved in the interaction and/or stabilization of substrate Pg during the process of Pg activation (Rajamohan and Dikshit, 2000). Previous studies on alanine scanning mutagenesis of SAK (Silence et al., 1995) have indicated that the four clustered charged segments of SAK are important for its functional properties. Apart from the positively charged amino-terminus, two discrete segments of SAK spanning Glu44-Lys80 and Glu65-Asp69 form the core region of SAK and may be involved in Pg binding and activation. Another highly charged region of SAK (Lys96-Lys98) that significantly affect its functionality is a part of its surface-exposed loop structure. The three-dimensional structure
of the μPm-SAK-μPm complex (Parry et al., 1998) has indicated that this charged loop region of SAK is away from the interaction sites of SAK-Pm activator complex and substrate μPm.

Computer based modeling studies with kringle domain of Pg indicates that the surface-exposed positively charged residues of the SAK 90-loop may provide potential docking sites for the kringle domains of substrate Pg. The aim of present study was to probe the functional role of the positively charged loop region of SAK in the Pg activation process through site-directed and deletion mutagenesis.

4.1 Structural features of 90-loop region (amino acids Thr\textsubscript{90}-Glu\textsubscript{100}) of SAK

SAK is a single domain Pg activator that is folded into a compact and flattened structure consisting of a five-stranded β-sheet packed on a single 12-residue α helix (Rabijns et al., 1997). It exhibits significant three-dimensional structural similarity with the α domain of streptokinase (SK) including the similar β-grasp folding characteristic (Parry et al., 1998; Parry et al., 2000; Wang et al., 1998). Structure based sequence alignment of SAK with SK α domain indicates that SAK 90-loop region carrying several surface-exposed charged residues is unique in SAK structure. Protein modeling and structural overlay of SAK and SK α domain indicates that SAK 90-loop region is extended out as compared with the SK α domain loop region (Fig. 4.1a) and probably could be deleted without major structural perturbation. SAK 90-loop is hydrophilic in nature and contains four positively charged residues (Lys\textsubscript{94}, Lys\textsubscript{96}, Lys\textsubscript{97} and Lys\textsubscript{98}) at the tip and three negatively charged residues (Asp\textsubscript{93}, Glu\textsubscript{99} and Glu\textsubscript{100}) at the stem along with two aromatic residues (Tyr\textsubscript{92} and Tyr\textsubscript{93}).

The crystal structure of μPm-SAK-μPm ternary complex suggests that this loop region of SAK is away from the interface between SAK and μPm in the SAK-μPm enzyme complex and does not have any direct structural interaction with substrate μPm (Fig. 4.1b).
Fig. 4.1a) Superimposition of SKα domain (red) with SAK (black). The extended loop structure of SAK (SAK 90-loop) from 90-100 amino acid residues carrying a cluster of lysine residues at the tip of the loop, imparting distinctly positive electrostatic character to this loop, is shown.
Fig. 4b) The ternary complex of SAK and microplasmin. Position of SAK 90-loop with respect to SAK-μPm enzyme complex and substrate μPm (in red) is indicated by arrow. It is clearly seen that this loop makes no direct interaction with the substrate μPm. Orientation of the amino-terminus of the substrate μPm is indicated (Parry et al., 1998).
The orientation of the substrate μPm on the SAK-μPm enzyme complex suggests that it may be possible for the kringle domains of substrate Pg to make contact with this surface-exposed loop structure. Since the crystal structure of ternary complex of SAK and μPm provides only a partial picture of protein-protein interaction, owing to lack of kringle domains in μPm, computer modeling based on the SAK-μPm crystal structure and other available information including the position of substrate μPm relative to bimolecular complex of SAK-μPm docking of kringle on SAK 90-loop, etc. was carried out to look for the possible targets of the SAK 90-loop. The results indicated that it is feasible to dock kringle 5 of human Pg on the SAK 90-loop (Fig. 4.1c). A search for geometric and electrostatic complementary regions of both the protein structures indicated that Lys96, Lys97 and Lys98 of SAK 90-loop align and orient towards the cationic centers of kringle 5 and may be the probable target for kringle interaction. Considering the similarity between three-dimensional structure and basic conformation of the anionic center of the lysine-binding sites of kringle domains 1-5 of human Pg (Chang et al., 1998), it may be envisaged that any one of these structures of the substrate Pg could make the possible contact with the SAK 90-loop and facilitate optimal substrate positioning for processing by the SAK-Pm activator complex.

4.2 Deletion of 90-loop does not abolish the Pg activation function of SAK

Structural analysis of SAK and its superimposition on SK α domain indicated that SAK 90-loop region might be deleted without any major structural perturbation in overall conformation of SAK. To check this probability experimentally, nine amino acid residues covering Tyr92-Glu100 of SAK were deleted using overlap PCR method (Vallejo et al., 1995) and the resulting SAK mutant; SAKΔ90 was cloned and overexpressed in E. coli. The sequence of the deleted region of SAKΔ90 is given in figure 4.2a. On SDS-PAGE, SAKΔ90 migrates more quickly than native SAK, supporting the corresponding truncation
Fig. 4.1c) Stereo view of the manually docked complex of kringle 5 domain with the ternary complex of SAK and \( \mu \)Pm. The atomic center of the kringle 5 domain (red) carrying negatively charged patches, docks well with positively charged residues on the SAK 90-loop which contributes to the major positively charged surface of the SAK molecule. Position of specific region of kringle 5 with SAK 90-loop is shown.
in the SAK loop-deletion mutant (Fig. 4.2b). SAKΔ90 mutant was purified using SP-sepharose and hydrophobic interaction chromatography. A Pg-coupled radial caseinolytic assay (Fig.4.2c) of SAKΔ90 reveals that it is able to activate Pg. However, the diameter of its clearance zone indicated that its Pg activation capability is relatively lower than that of the native SAK.

4.3 Pg binding and activation properties of SAKΔ90

To examine the effect of loop deletion on the functional properties of SAK, the pattern of Pg binding and activation by SAKΔ90 was compared with that of native SAK. Purified SAK or its mutant protein (5 nM) was added to Pg (1.5 μM) in a quartz cuvette carrying 1.0 mM Chromozym PL in 50 mM Tris.HCl, pH 7.5, containing 0.1% BSA and 0.01% Tween 80. The change in absorbance was then measured as a function of time in a Shimadzu (UV-1601) spectrophotometer at 25°C. SAKΔ90 exhibits more than 60% decrease (64 IU/μg) in specific Pg activation activity as compared to full-length SAK (160 IU/μg). The catalytic amount of native SAK (5 nM) induced rapid activation of Pg to Pm resulting in nearly 90% of Pg activation within 6 min. In contrast, SAKΔ90 activates Pg into Pm slowly and reaches the level achieved by native SAK in about 15 min (Fig. 4.3).

4.3.1 Pg binding properties of SAKΔ90

Since SAKΔ90 exhibits reduced Pg activation property, its ability to bind Pg efficiently was examined by using radiolabeled-Pg. Purified SAKΔ90 preparation (1.5 ng) was suspended in binding buffer (50 mM Na₂CO₃/NaHCO₃, pH 9.5) and was immobilized on the microtitre plates or nitrocellulose strips. Blotted protein was air-dried and blocked with 1% BSA for 1 h at 4°C. Different concentrations of ¹²⁵-Pg, prepared by the Idogen method, were then added to the immobilized SAK proteins and incubated at 4°C for 1 h. Excess radioactivity was removed by repeated washing with the same buffer. The amount of labeled Pm bound with SAK was estimated by the amount of radioactivity (measured by
Fig. 4.2 Characteristics of loop-deletion mutant of SAK (SAKA90). a) Sequence of 90-loop region of SAK and SAKΔ90. Amino acid residues and nucleotide sequence of the SAK 90-loop region are shown. A small linker (shown in italics) was incorporated to facilitate the folding of the loop. b) Expression of SAKΔ90 in *E. coli* Lane 1: protein molecular weight marker; Lane 2: *E. coli* BL21 pET; Lane 3: *E. coli* BL21DE3 pETSAK; Lane 4: *E. coli* BL21DE3 pETSAKΔ90. The arrow indicates the position of SAKΔ90. c) Pg activation activity of SAKΔ90 as determined by the caseinolytic assay. Lane 1: SAK; Lane 2: SAKΔ90.
Fig. 4.3 Pg activation by SAKΔ90.
using γ-radiation counter) retained on the blot. BSA was used in place of SAK to check the background level of non-specific binding that was subtracted from the total level in order to determine specific Pm binding. The pattern of radiolabeled-Pg binding with immobilized native SAK and SAKΔ90 indicated that concentration dependent binding of I^{125}-Pg with SAK and SAKΔ90 has a similar trend in the formation of SAK-Pg binary complex. These results suggests that the ability of SAKΔ90 to generate stoichiometric complex with its partner Pg is similar to that of native SAK (Fig. 4.4).

4.3.2 Active-site exposure in SAKΔ90-Pm complex

To further check that the bimolecular complex of SAKΔ90 and Pm had not altered their active-site geometry, the ability of this mutant to expose active site was checked by monitoring the burst of p-nitrophenol release with equimolar mixtures of native SAK or SAKΔ90 and Pm after reaction with the active-site acylating agent (Chase and Shaw, 1969). Concentrated stock solutions of Pg and SAK-Pm complex were diluted in 0.1 M veronal buffer, pH 8.3 containing 0.1 M arginine to final concentration of 4.0 μM each. At different time points (0-20 min) after mixing with Pg and SAK-Pm or loop mutant of SAK (SAKΔ90)-Pm complex, NPGB was added to a final concentration of 100 μM. The concentration of active site was determined from ‘burst’ of p-nitrophenol using molar absorption coefficient of 16,700 M⁻¹cm⁻¹. Active-site exposure in the mixture of Pg and SAK-Pm occurs exponentially with an initial lag of 3 min whereas, in case of SAKΔ90-Pm, the corresponding value was about 4 min resulting in 98% of the active-site exposure (Fig. 4.5).

Overall results reveales no perceptible alteration in the functional properties of SAKΔ90 with respect to its ability to form a native SAK-like bimolecular complex with Pm and exposure of the active site within the activator complex.
Fig. 4.4 Pattern of binary complex formation by SAKΔ90 with Pg.
4.4 Catalytic efficiency of pre-formed SAKΔ90-Pm complex

Although SAKΔ90 exhibited native SAK-like ability to generate activator complex with its partner Pm, its Pg activation rate was 7-8 fold slower than that of wild type SAK. To explore whether this deficiency in Pg activation is due to the alteration in the interaction of SAKΔ90-Pm complex with the substrate Pg, the pattern of Pg activation by pre-formed complex of SAK-Pm was compared with SAKΔ90-Pm complex. For this experiment, equimolar mixtures of SAK or SAK mutants and Pm were pre-incubated in 50 mM Tris.HCl, pH 7.5 containing 0.1% BSA and 0.01% Tween 80 at 37°C for 5 min to generate activator complexes. These pre-formed activator complexes (5 nM) and generation of Pm was measured at 25°C for change in absorbance at 405 nm. Activation of Pg by pre-formed SAK-Pm complex occurs progressively with a brief lag phase followed by exponential phase (Fig. 4.6). The steady state kinetic analysis of SAK loop-deletion mutant indicates that the catalytic efficiency and Km of pre-formed SAKΔ90-Pm complex was about 50% lower and about 5-fold higher respectively, than the pre-formed SAK-Pm complex (Table 4.2).

4.5 Formation of ternary complex between Pm and SAKΔ90

To further compare the capability of ternary-complex formation with the activator complex formed with Pm and native SAK or SAKΔ90, the interaction of substrate Pg with the bimolecular complex formed with SAKΔ90 and Pm, a sandwich-binding assay was performed. Pm was immobilized on nitrocellulose strips or microplates and native SAK or SAKΔ90 protein was added to the immobilized Pm. Thereafter, I125-Pg was added at various concentrations. Immobilized bimolecular complex formed with SAKΔ90 and Pm exhibits more than 40% decrease in the interaction with the substrate Pg in the formation of ternary complex as compared to native SAK (Fig. 4.7).
Fig. 4.5 Time-course acylation of Pm active site in equimolar mixture of Pg with SAKΔ90-Pm complex.

Fig. 4.6 Pg activation by pre-formed complex of SAKΔ90 with Pm.
Fig. 4.7 Pattern of ternary complex formation by Pg with SAKΔ90-Pm complex.
4.6 Cleavage of substrate Pg to Pm by SAKΔ90-Pm complex

In order to compare the catalytic efficiency of SAKΔ90 with native SAK, cleavage of substrate Pg to Pm by equimolar complex of Pm with SAK and SAKΔ90 was monitored by SDS-PAGE. Equimolar amounts of SAK or SAKΔ90 and Pg were incubated at 25°C and aliquots were removed at different time points and subjected to 12% SDS-PAGE for monitoring the cleavage of substrate Pg (Fig. 4.8). Densitometric analysis of the protein profile indicated that more than 90% of Pg is converted into Pm within 5 min by SAK-Pm complex. Under similar conditions, nearly 25% of substrate Pg is cleaved into Pm in about 15 min by a pre-formed complex of SAKΔ90 and Pm.

4.7 Effect of kringle (K1+K2+K3+K4) domains on the activation of Pg by pre-formed complex of SAKΔ90 and Pm

Since Pg activator complex formed with the loop-deletion mutant of SAK (SAKΔ90) exhibited slower processing of the substrate Pg and the protein-modeling studies indicated possible interaction of kringle domain of substrate Pg with SAK 90-loop region, the pattern of Pg activation by pre-formed SAK-Pm complex was studied in the presence of isolated kringle domains (K1+K2+K3+K4) of human Pg. Separation of kringle domains from intact Pg was achieved by limited digestion of full-length Pg with pancreatic elastase. Cleaved kringle domains were isolated using Lysine-sepharose column chromatography. A time-course study on Pg activation by pre-formed SAK-Pm complex indicates that in the presence of 5 μM kringles, activation of Pg is inhibited by 30% which was further increased at 20 μM (Fig. 4.9a), indicating that the kringle domains are competing with full-length substrate Pg molecules for interaction with the SAK-Pm enzyme complex. Interestingly, pre-formed SAKΔ90-Pm complex exhibited very little difference in the Pg activation process and only 10% inhibition was observed in the presence of 20 μM of kringles (Fig. 4.9b), suggesting that the kringle domains are not effectively competing with the substrate Pg during activation via SAKΔ90-Pm enzyme complex.
Fig. 4.8 Cleavage of Pg by SAKΔ90. Lane M: Protein molecular weight marker; Lane 2: Pg; Lane 3: SAK; Lane 4-7: SAK-Pg incubated for 0, 1, 5, 10 min respectively; Lane 8: SAKΔ90; Lane 9-13: SAKΔ90-Pg incubated for 0, 1, 5, 10, 15 min respectively. The arrows indicate the Pg cleavage products.
Fig. 4.9a) Pg activation by SAK in the presence of kringle domains.

Fig. 4.9b) Pg activation by SAKΔ90 in the presence of kringle domains.
4.8 Site-directed mutagenesis of 90-loop region of SAK and functional properties of SAK mutants

With a view to further explore, whether the charged residues of SAK 90-loop structure provide any contribution to substrate Pg processing and activation, an attempt was made to delineate the probable target of 90-loop region of SAK by site-directed mutagenesis. Four SAK mutants, namely K$_{94}$A, K$_{96}$A, K$_{97}$A and K$_{98}$A were created by individually converting lysine into alanine. Out of these four SAK mutants, SAKK$_{94}$A and SAKK$_{96}$A exhibits Pg activator activity comparable with that of native SAK (Table 4.1) implying that these lysine residues may not be important for modulating the Pg activation function of SAK. In contrast, the other two mutants SAKK$_{97}$A and SAKK$_{98}$A exhibited about 4-5-fold reduction in their specific activities for Pg activation (Table 4.1). Additionally, these SAK mutants exhibits a 4-6-fold reduction in their affinity for the substrate Pg (Table 4.2), which is evident from their higher Km values for the substrate Pg. These observations clearly indicates that mutations in lysine residues of the 90-loop region of SAK has led specifically to a reduction in the ability of these SAK mutants to interact with substrate Pg.

Discussion

Although three-dimensional structure of SAK and its ternary complex with $\mu$Pm (Parry et al., 1998) have been resolved, the molecular mechanism whereby SAK brings about structural changes after association with partner Pg or Pm and interact 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 (Schlott et al., 1997; Schlott et al., 1998) and
Table 4.1 Functional properties of SAK mutants carrying alteration within 90-loop region. Underlined amino acid residues indicate substitution with alanine.

<table>
<thead>
<tr>
<th>SAK mutant</th>
<th>Sequence of the loop region</th>
<th>Specific activity (IU/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAK</td>
<td>Tyr Asp Lys Asn Lys Lys Glu Glu Thr</td>
<td>160</td>
</tr>
<tr>
<td>SAKK94A</td>
<td>Tyr Asp Ala Asn Lys Lys Glu Glu Thr</td>
<td>140</td>
</tr>
<tr>
<td>SAKK96A</td>
<td>Tyr Asp Lys Asn Ala Lys Glu Glu Thr</td>
<td>155</td>
</tr>
<tr>
<td>SAKK97A</td>
<td>Tyr Asp Lys Asn Lys Ala Lys Glu Glu Thr</td>
<td>49</td>
</tr>
<tr>
<td>SAKK98A</td>
<td>Tyr Asp Lys Asn Lys Lys Ala Glu Glu Thr</td>
<td>55</td>
</tr>
<tr>
<td>SAKΔ90</td>
<td>Gly Gly Ser Δ Thr</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 4.2 Kinetic constants for the Pg activation properties of equimolar complexes of SAK mutants and Pm. The results are mean±S.E.M of three determinations.

<table>
<thead>
<tr>
<th>Bimolecular complex</th>
<th>Km (µM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (s⁻¹ µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAK-Pm</td>
<td>2.5±0.12</td>
<td>2.0±0.09</td>
<td>0.80</td>
</tr>
<tr>
<td>SAKΔ90-Pm</td>
<td>14.4±0.20</td>
<td>1.9±0.050</td>
<td>0.14</td>
</tr>
<tr>
<td>SAKK97A-Pm</td>
<td>10.4±0.16</td>
<td>2.1±0.10</td>
<td>0.21</td>
</tr>
<tr>
<td>SAKK98A-Pm</td>
<td>11.8±0.18</td>
<td>1.8±0.10</td>
<td>0.16</td>
</tr>
</tbody>
</table>
the conversion of substrate Pg into Pm (Collen et al., 1993a, b; Parry et al., 2000; Grella and Castellino, 1997). SAK does not directly induce conformational changes in the active site residues but alters substrate specificity of Pm indirectly by creating new subsites onto which substrate Pg docks for the enhanced presentation of the Pg activation loop towards the enzyme (Rabijns et al., 1997). Precise molecular interactions which take place between SAK-Pm enzyme complex and the substrate Pg are far from clear at present. The crystal structure of the ternary complex μPm-SAK-μPm reveals only partial picture owing to the absence of auxiliary domains (kringle structures) in μPm as compared to full length Pg molecule. Recent biochemical studies (Arai et al., 1998) indicated participation of kringle structure in SAK mediated Pg activation. In the present study, it has been demonstrated that SAK-Pm enzyme complex may make an additional interaction 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 Pg activation.

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 (Fig. 4.2b). Retention of Pg activation capability in the SAK mutant lacking this loop region suggested that SAK loop-deletion mutant is still able to fold back and achieve its native SAK like conformation. SAK exhibits closest structural similarity with SK α domain and it has been suggested that SAK and SK α domain perform similar function(s) in the Pg activator complex (Parry et al., 1998). Structural overlay and amino acid alignment of SAK and SK α domain, based on their three-dimensional structural homology indicated that there are two distinct mobile regions that do not align when the structure of these two protein units are superimposed. One of these regions spans positions 44-64 in SK α domain, and another distinct region is the 90-loop region of SAK that is absent in SK α domain. Involvement of Asp41-His48 region of SK in interaction with the substrate Pg has recently been suggested on the basis of site-directed mutagenesis within this mobile region of SK (Kim et al., 2000). Deletion of SAK 90-loop region resulted in a 5-6-fold reduction in the specific activity of SAK and also generated significant reduction in the Pg activation properties of the molecule.
In principle, SAK could modify the specificity of the Pm active site by altering its conformation or by changing substrate Pg accession through altered docking of Pg on the SAK-Pm enzyme complex. SAKΔ90 formed a 1:1 SAK-Pm complex, which displayed an amidolytic activity comparable with that of the native SAK-Pm binary complex. Progressive exposure of active site within the SAKΔ90-Pm complex clearly indicated that the SAK loop-deletion mutant is capable of generating activator complex with Pm very similar to native SAK. Therefore, the slow activation kinetics of Pg activation by SAKΔ90 may not be due to alteration in the activator complex formation with Pm.

Deletion of SAK 90-loop led to a perceptible increase in Km for the substrate Pg. The slow kinetics of Pg activation by the pre-formed activator complex of SAKΔ90 and Pm suggested that this bimolecular complex is not able to interact optimally with the substrate Pg. These results were further substantiated by slow cleavage of Pg into Pm by activator complex of SAKΔ90 with Pm. Furthermore, site-directed mutagenesis within the SAK 90-loop region revealed that substitution of Lys97 or Lys98 with alanine within this loop region resulted in similar lesion in substrate Pg activation by these SAK mutants. These results provided the evidence that Lys97 and Lys98 of the SAK 90-loop may be assisting in the sequestering of substrate Pg. These results conform to those obtained in previous studies on alanine-clustered charge mutagenesis of SAK. Overall results, thus suggested that SAK 90-loop may not be very important in maintaining the functional conformation of SAK but may be involved in accession /interaction of substrate Pg via its positively charged residues, Lys97 and Lys98.

In an effort to determine the probable target of SAK 90-loop on substrate Pg, the orientation of substrate Pg with respect to SAK-Pm enzyme complex in a three-dimensional model of μPm-SAK-μPm complex was analyzed. It indicated that SAK 90-loop may provide an ideal docking site for the kringle structure of substrate Pg. Free Pg bound with the activator complex of the SAK-Pm with 3-fold higher affinity as compared to miniPg (Lijnen et al., 1993), suggesting that the presence of kringle structures are crucial for optimal interaction of substrate Pg. In an attempt to evaluate further 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 domain of Pg (K1+K2+K3+K4) effectively competed with Pg for interaction with SAK-Pm activator complex whereas SAKΔ90-Pm complex did 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. Biochemical studies on SAK have indicated that the lysine binding sites in K1+K2+K3+K4 kringle domain of Pg may play a role in the activation of Pg by SAK-Pm complex (Arai et al., 1998). In this process, SAK 90-loop may be the ideal target for this interaction owing to the presence of several surface-exposed clustered lysine residues. Molecular docking of kringle 5 on SAK 90-loop indicated that Lys97 and Lys98 are facing the anionic center of the kringle. A drastic decrease in Pg activation properties of SAK mutants SAKK97A and SAKK98A supports this structural prediction. Since the presence of kringle structure (K1+K2+K3+K4) effectively competed with full length Pg, any one of these kringle domains may possibly show an interaction with the SAK 90-loop region. The overall basic conformations of kringle domains are more or less similar, including kringle 5, which was tried to dock on the SAK 90-loop in modeling studies.

In conclusion, the present study on site-directed and loop-deletion mutagenesis of SAK has provided novel information on SAK-Pg interaction and 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. Lys97 and Lys98 of SAK 90-loop region might 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.