6. BIOCHEMICAL CHARACTERIZATION OF COFACTOR ACTIVITY OF STAPHYLOKINASE IN THE PRESENCE OF STREPTOKINASE DOMAINS
Two bacterial protein cofactors, staphylokinase (SAK) and streptokinase (SK) have been of interest because, as plasminogen activators, they have proven to be useful clinically in dissolving blood clots that cause heart attacks. The structures of microplasmin ($\mu$Pm) bound to the three-domain molecule, SK (Wang et al., 1998) and the single domain SAK (Parry et al., 1998) were solved almost simultaneously. Comparison of the structures reveals that SK essentially surrounds the catalytic domain of Pm whereas much smaller SAK contacts only one side (Fig. 6.1). The contacts of $\mu$Pm with SAK are similar to those made by $\alpha$ domain of SK (Esmon and Mather, 1998). However, due to multi-domain nature of SK, its interactions with $\mu$Pm are more extensive and complex in comparison to SAK. In addition, SAK also shares the same $\beta$-grasp folding with SK $\alpha$ and $\beta$ domains.

Although, SAK and SK display basic similarities in their function as a cofactor, certain key differences exist in their molecular mechanism of Pg activation. SK can form activator complex with Pg as well as Pm, whereas SAK forms activator complex only with Pm (Reddy and Markus, 1972; Collen et al., 1993b). Also, the generation of Pg activation potential in SAK proceeds via Pm-mediated removal of the 10 amino-terminal residues of SAK (Schlott et al., 1997; Schlott et al., 1998, Rajamohan and Dikshit, 2000). It has been observed that SAK binds with lower affinity to Pm (Lijnen et al., 1994; Sakharov et al., 1996) and the SAK-Pm activator complex possesses a relatively lower catalytic efficiency as compared to SK-Pg/Pm complex (Cederholm-William et al., 1979; Collen, 1980). Additionally, SAK-Pm complex, but not the SK-Pg/Pm complex is inactivated by $\alpha$2-antiplasmin (Sakharov et al., 1996; Lijnen et al., 1991; Lijnen et al., 1992; Sakai et al., 1989; Silence et al., 1993a) and unlike SK, Pg binding to fibrin degradation products dramatically enhances SAK binding and Pg activation (Silence et al., 1993a, b).

The structure determination of ternary complex of $\mu$Pm with SAK and binary complex of $\mu$Pm with SK, together with molecular modeling and mutagenesis studies have provided novel insight into the molecular aspects of cofactor mediated Pg activation. In a model generated for the $\mu$Pm-SK complex, Loy et al., (2000) suggested that a distinct function can be assigned to each domain of SK. Whereas SAK and SK $\alpha$ domain assist in
Fig. 6.1 Ribbon diagrams of SAK-μPm structure (left) and SK-μPm structure (right). μPm are shown in blue, the cofactor domains are in green, yellow and gold with central helix in white (Parry et al., 1998; Wang et al., 1998).
proper substrate pre-orientation and presentation to the active site of the enzyme and SK β domain provides a further substrate anchoring site that also modulates the interaction of Pm with macromolecular inhibitors. SK γ domain has been proposed to participate in the binding and activation upon complex formation.

Recently, a two-domain streptokinase (SUPA) has been isolated from Streptococcus uberis (Johnsen et al., 1999). SUPA exhibits two domain structure carrying similarity with α and β domains of SK. The major structural difference between SK and SUPA is apparently the lack of γ domain. These three bacterial Pg activators display increase in their molecular size as well as Pg activation potential by addition of one domain unit. Unlike SUPA and SK, SAK activates Pg without assistance of additional domains. The α domain of SK which is structurally homologous to SAK binds Pg with 10-fold higher affinity but display several fold reduced Pg activation capability (Loy et al., 2000). When α domain combines with β and γ domains in full length SK, it displays highly efficient Pg activator activity. The Pg activation capability of two-domain SUPA is intermediate of SK and SAK suggesting a domain cooperation in the Pg activation process. Thus, it becomes logical to test the contribution of individual domains of SK on Pg activation characteristics of SAK. The present study addresses the question whether it is possible to generate progressive increase in cofactor activity of SAK by synergistic effects of SK domains.

6.1 Structural and functional comparison of SAK with SK domains

Sequence homology between individual SK domains and SAK is beyond recognition using conventional sequence alignment methods. However, upon three-dimensional structure-based sequence alignment, considerable structural homology is observed between SK α and β domains and SAK (Fig. 6.2) (Wang et al., 1999b). The validity of this alignment is reinforced by its correlation with the functions of these domains, i.e. SK α domain and SAK perform similar functions in Pg activator complex
SAK (1) SSFBKGYKKGD DA SYFEPGPYLMVNVTVGD GKNELL
SKα (1) IAGPEWLLDRPSVNNSQLVYSVAGTVE GTNQDI
SKβ (146) YKEKPIONQASKSDVEYTVQFTPLNPDDDFRPG
SPHVEFPIKP GTTLTK
SLKEFEDLTSPAHGKTQEQLSPKSKPFATDSGAMPHKLEK
LKDTKLKTLA IGDITTS
SAK (58) EKIEYYVEWADATAYKEFRLVELDPSAKIEVTYYDKN
SKα (77) ADLKKIAEQLIAANVHSNDDYFEDFASDATITDR
SKβ (197) QELLAQAQS1INKTHPGYTIYERDSSIVTHD
SAK (95) KKEETKSFPIT EKGFVYPDLOSEH IKNPGF
SKα (113) NGKVFADKDSVTLPQVPQEF
SKβ (228) NDIFRTILPMDQEFTYRMKNERQAYEINKKSLNEEINNTD
SAK (126) NLITKVVEIKK
SKα (136) LESGHVRVRP
SAKβ (269) LISEKYVYLKKGKPYD

Fig. 6.2 Amino acid sequence alignment of SAK, SKα and SKβ domain based on three dimensional structural homology. Conservative residues are highlighted. This figure was produced using the program ALSCRIPT (Wang et al., 1999).
while SK β domain does not. For example, SAKGlu36 and SK αGlu39 are found to be critical for interacting with PgArg719 in the complex formation, however, topologically equivalent position (residue 185) in SK β has leucine residue that would abolish the favorable electrostatic interaction. Even the inter-β-strand region of SK domains and SAK showed some level of similarity. Although, individual α and β domains of SK are structurally homologous to SAK but their Pg activation potential is very low. But the full length SK α, β and γ domains show synergistic effect and display very high Pg activation activity. Such a close structural and functional equivalence between α domain of SK and SAK prompted us to characterize the effect of β and γ domains of SK on Pg activation properties of SAK.

6.2 Plasminogen activation by SAK in combination with single or two domain component of SK

In order to study the effect of individual domains of SK on Pg activation activity of SAK, β and γ domains of SK were overexpressed individually, as well as in combination. Pg activation profile of SAK was determined in the presence of these SK domains to evaluate the potentiality of SK units for modulating the Pg activation function of SAK.

6.2.1 Cloning, expression and purification of streptokinase domains

DNA fragments encoding β and γ domains of SK were amplified from plasmid vector carrying recombinant gene encoding full length SK using PCR as mentioned in material and methods and cloned in pBS KS+ plasmid vector. Authenticity of cloned fragments was established by DNA sequencing. For the overexpression of β, γ and βγ domains of SK, DNA fragments encoding the respective domains were subcloned in pET9b and transformed into E. coli BL21DE3. To check overexpression, single colonies of recombinant E. coli cells were inoculated in LB supplemented with kanamycin and induced
with IPTG when OD$_{600}$ was 0.3-0.5 and allowed to grow further for 8-10 hours. Expression of cloned SK domains was monitored by SDS-PAGE analysis. Expression of SK β, γ and βγ domains under T7 promoter results in accumulation of 8-10% of β and γ and more than 25% of the βγ recombinant protein inside the cells (Fig. 6.3).

The SK domains were purified from the recombinant *E. coli* cells to near homogeneity (Fig. 6.3). SK β domain was purified using DEAE-sepharose. Briefly, after 7-10 hours of induction, cells were harvested and resuspended in 20 mM Tris.HCl, pH 7.5. Cell lysate was prepared by ultrasonication. After centrifugation of cell lysate at 12,000 rpm for 1 h, clear supernatant was used for recovery of β domain. DEAE–sepharose column was equilibrated with 20 mM Tris.HCl, pH 7.5 and protein was eluted using 30 mM NaCl in Tris.HCl, pH 7.5. For the purification of SK γ domain, the cell lysate was first passed through 35 kDa cut-off filter and the filtrate was then concentrated using 10 kDa cut-off filter. Concentrated supernatant was used for further studies. SK βγ domain was partially purified by using DEAE–sepharose. Protein was eluted at 90 mM NaCl in 20 mM Tris.HCl, pH 7.5. Partially purified protein was concentrated using 10 kDa cut-off filter and further purification was carried out with Sephadex G-200 gel filtration column using 20 mM Tris.HCl, pH 7.5.

### 6.2.2 Pg activation by SAK in presence of SK domains

Individual SK domains show no significant intrinsic Pg activation activity (Fig. 6.4). To check the effect of these domain on Pg activation property of SAK, 5 nM of SAK and 20 nM of Pg were mixed with 5 μM of various SK domains (β, γ and βγ) in a quartz cuvette carrying 1.0 mM Chromozyme PL in 0.1 M phosphate buffer, pH 7.5 containing 0.1% BSA and 0.01% Tween 80. The change in absorbance at 405 nm was then measured as a function of time in a Shimadzu (UV-1601) spectrophotometer at 25°C. No significant change in Pg activation pattern of SAK is observed in the presence of SK domains (Fig. 6.4). These results indicate that either individual SK domains do not affect Pg activation by SAK or
Fig. 6.3 Expression and purification of SK domains. Lane 1: Protein molecular weight markers; Lane 2: *E. coli* BL21DE3 pET; Lane 3: *E. coli* BL21DE3 pETβγ; Lane 4: purified βγ; Lane 5: *E. coli* BL21DE3 pETβ; Lane 6: purified β; Lane 7: *E. coli* BL21DE3 pETγ; Lane 8: purified γ. The arrows indicate the purified proteins.

Fig. 6.4 Pg activation activity of individual SK domains and SAK in the presence of SK domains.
interact slowly and may need more time to exert their effect on SAK-mediated Pg activation.

6.2.3 Pg activation by SAK in presence of pre-formed complex of Pg and SK domains

Since SK domains do not facilitate Pg activation by SAK when added directly to reaction mix, therefore, pre-complex of 20 nM of Pg was formed with molar excess of SK domains at 37°C for 5 min. To this Pg-SK-domain complex, 5 nM of SAK was added and Pg activation was studied by monitoring change in absorbance at 405 nm as described in previous experiment. A concentration dependent increase in Pg activation by SAK is observed with pre-formed complex of Pg and SK β domain. Addition of 5 μM of β domain brings about nearly 50% increase in Pg activation and a slight reduction in lag time (Fig. 6.5a). In contrast, addition of γ domain of SK dose not show any significant change in Pg activation by SAK (Fig. 6.5b). When individual β and γ domains were used simultaneously to form pre-complex with Pg, the Pg activation pattern by SAK was similar to that observed in the presence of β domain alone (Fig. 6.5c). These observations suggests that γ domain might not be interacting effectively to generate activator complex with Pg and SAK. Surprisingly, in contrast to individual β and γ domains, when fused βγ domain was used to form pre-complex with Pg, a sharp increase in Pg activation by SAK was observed with increasing concentration (Fig. 6.3d). SAK shows up to 60% increase in its Pg activation capacity and lag period was reduced to zero min in the presence of Pg-βγ pre-complex suggesting that integration of β and γ domains together is able to enhance the Pg activator activity of SAK better than individual domains. To further check the effect of SK domains on active-site exposure in substrate Pg by SAK-Pm complex, time course study on generation of active site was performed. Equimolar mixture of SAK-Pg (1.5 μM) in presence of 3-fold molar excess of SK β or βγ domains was mixed with p-nitrophenol-p'-gunaidinobenzoate (100 μM) in 0.1 M Veronal buffer, pH 8.3. The concentration of active site was determined from the burst of p-nitrophenol using molar absorption coefficient of
Fig. 6.5 Pg activation by SAK in the presence of increasing concentrations of SK β (a), γ (b), β+γ (c) and βγ (d) domains after pre-incubation with Pg.
16,700 M⁻¹ cm⁻¹. A distinct reduction in lag time is observed in the presence of SK domains as shown in figure 6.6.

6.2.4 Determination of steady state kinetic parameters of Pg activation by SAK in the presence of SK domains

Since β and βγ domains of SK facilitate Pg activation property of SAK, further experiments were carried out to establish the mechanism of this positive effect exerted by these SK domains. To calculate the steady state kinetic constants of Pg activation by SAK in the presence of SK β and βγ domains, equimolar mixtures of SAK (1.5 μM) and Pg (1.5 μM) were pre-incubated along with 3-fold molar excess of SK β or βγ domains in 0.1 M phosphate buffer, pH 7.5 containing 0.1% BSA and 0.01% Tween 80 at 37°C for 5 min to generate the activator complex. These pre-formed activator complexes (5 nM) were mixed with different concentrations of Pg (1-10 μM) and 1.0 mM Chromozym PL. The change in absorbance at 405 nm was measured as a function of time in Shimadzu (UV – 1601) spectrophotometer. The overall activation pattern obeys Michaelis-Menten kinetics and Km of SAK-Pg complex in the presence of SK β or βγ domains are 1.47 and 1.3 μM respectively (Table 6.1). Catalytic efficiency of SAK is improved by the presence of SK domains as indicated by kcat values in Table 6.1.

Cleavage of substrate Pg to Pm by SAK was monitored by SDS-PAGE to further compare the catalytic efficiency of SAK in the presence of SK β and βγ domains. For this experiment, equimolar amounts of SAK and Pg (1.5 μM each) were incubated with 3-fold molar excess of SK β or βγ domains at 15°C in 50 mM Tris.HCl, pH 7.5. At each time point, a 7.5 μl aliquot was removed and analyzed for the conversion of Pg into Pm by 12% SDS-PAGE. Densitometric analysis of the protein profile indicates that almost 100% Pg was converted to Pm by SAK in presence of SK β and βγ domains in less than 5 min, whereas SAK alone shows only 50% Pg to Pm conversion in 5 min (Fig. 6.7).
Fig. 6.6 Time-course acylation of Pm active site in equimolar mixtures of Pm with SAK in the presence of SK domains.
Fig. 6.7 Cleavage of Pg by complexes of Pg with SAK in the presence of purified SK domains. Lane M: Protein molecular weight marker; Lane 2: Pg; Lane 3: purified SAK. Lane 4-7: SAK-Pg reaction mixtures incubated for 0, 1, 2, 5 min respectively; Lane 8: purified SAK+β; Lane 9-12: SAK+β-Pg reaction mixtures incubated for 0, 1, 2, 5 min respectively; Lane 13: β+Pg incubated for 5 min; Lane 14: purified SAK+βγ. Lane 15-17: SAK+βγ-Pg reaction mixtures incubated for 0, 1, 2 min respectively. Lane 18: βγ+Pg incubated for 2 min. The arrows indicate the Pg cleavage products.
<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>
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<tr>
<td>SAK-Pm</td>
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<td>0.8</td>
</tr>
<tr>
<td>SAK+β-Pm</td>
<td>1.5±0.10</td>
<td>2.9±0.08</td>
<td>1.99</td>
</tr>
<tr>
<td>SAK+βγ-Pm</td>
<td>1.3±0.12</td>
<td>3.2±0.12</td>
<td>2.46</td>
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Table 6.1. Kinetic constants for the Pg activation properties of equimolar complexes of SAK and Pg in the presence of SK β or βγ domains. The results are mean±S.E.M. of three determinations.

6.2.5 Effect of α2-antiplasmin on activator complex formed between SAK, Pg and isolated SK domains

One of the properties that distinguish SAK from SK is the inhibition of SAK-Pm activator complex by α2-antiplasmin unlike SK-Pm activator complex that retains its functionality in its presence. Therefore, role of SK domains was examined in the interaction of SAK-Pm complex with α2-antiplasmin. 5 nM of Pg was incubated with SAK (final concentration of 5 nM) in presence of 3-fold molar excess of β or βγ domains of SK for 5 min at 25°C and then incubated with increasing concentrations of α2-antiplasmin (0 – 10 μM) for 10 min at 25°C. Inhibition of the proteinase activity of binary complex of Pm with various proteins was measured by adding assay buffer containing 1.0 mM Chromozym PL and 1.5 μM Pg. Pre-formed complex between SAK-Pm and isolated β domain of SK displays inhibition by α2-antiplasmin very similar to that of native SAK-Pm complex (Fig. 6.8). However, activator complex formed between SAK-Pm and βγ domain exhibits partial resistance towards inhibition by α2-antiplasmin (Fig. 6.8).
Fig. 6.8 Residual Pg activation activity of SAK supplemented with SK domains in the presence of increasing concentrations of α2-antiplasmin.
6.3 Generation of chimeric SAK carrying β and γ domains of SK

The presence of SK β and βγ domains in-trans facilitates the Pg activation property of SAK indicating that various SK domains can be fused with SAK to generate a better Pg activator molecule. To experimentally check this hypothesis, chimeric proteins were constructed in which SK β and βγ domains were fused with SAK.

6.3.1 Cloning, expression and purification of SAKβγ and SAKβ fusion proteins

Overlap PCR was used for in-frame fusion of SK β and βγ encoding region of sk gene with sak gene as described in materials and methods and chimeric DNA was cloned in E. coli. SAKβ and SAKβγ fusion proteins were expressed in E. coli BL21DE3 under IPTG inducible T7 promoter and purified using column chromatography. SAKβγ was partially purified using DEAE-sepharose column. Column was equilibrated with 20 mM Tris.HCl, pH 7.5 and protein was eluted in 100-125 mM NaCl in 20 mM Tris.HCl, pH 7.5. Partially pure SAKβγ was loaded on Phenyl-sepharose column equilibrated with 0.5 M NaCl in 20 mM sodium phosphate buffer, pH 7.2 and protein was eluted in distilled water (Fig. 6.9). SAKβ fusion was subjected to single step purification using DEAE-sepharose column. Protein was eluted in 50-70 mM NaCl in 20 mM Tris.HCl, pH 7.5 (Fig. 6.9). The Western blot analysis of SAKβγ using polyclonal antibodies against SAK and SK confirmed the presence of both SAK and SK epitopes (Fig. 6.10a, b).

6.3.2 Pg activation by SAKβγ and SAKβ

The specific Pg activator activities of SAKβ and SAKβγ fusion proteins were determined by using Pg-coupled chromogenic substrate assay. Briefly, purified SAK or fusion protein (5 nM) was added to Pg (1.5 μM) in a quartz cuvette carrying 1.0 mM Chromozym PL in 0.1 M phosphate buffer, pH 7.5 containing 0.1% BSA and 0.01% Tween 80. Generation of Pm was measured at 25°C from change in absorbance at 405 nm. Both
Fig. 6.9 Expression and purification of SAK fusion proteins.
Lane 1: Protein molecular weight marker; Lane 2, 9: *E. coli* BL21 pET1; Lane 3: *E. coli* BL21 pETSAK; Lane 4: purified SAK; Lane 5: *E. coli* BL21 pETSAKβγ; Lane 6: purified SAKβγ; Lane 7: *E. coli* BL21 pETSAKβ; Lane 8: purified pETSAKβ. The arrows indicate the purified proteins.

Fig. 6.10 Western blot analysis of SAKβγ protein using antiSAK (a) and antiSK (b) antibodies. Lane 1: *E. coli* BL21 pETSAK; Lane 2: *E. coli* BL21DE3 pETSAKβγ; Lane 3: *E. coli* BL21DE3 pETSK. The arrows indicate the position of SAK, SK and SAKβγ.
fusion proteins exhibited more than 90% decrease (SAKβγ 11 IU/μg, SAKβ 12.5 IU/μg) in specific activation activity as compared with SAK (160 IU/μg). The catalytic amount of SAK (5 nM) induced rapid activation of Pg to Pm resulting in 50% of Pg activation within 7 min. In contrast, SAKβγ and SAKβ activated Pg to Pm slowly and reached to the 50% level in about 11 min (Fig. 6.11). Initial lag (about 4 min) in Pg activation by fusion proteins indicate that like SAK, SAKβγ and SAKβ form the activator complex with Pm. High initial velocity of Pg activation is observed for SAKβ in comparison to SAKβγ (Fig. 6.11).

6.3.3 Catalytic efficiency of pre-formed SAK fusion–Pm complex

Although SAK fusion proteins exhibit SAK like ability to generate activator complex with its partner Pm, its Pg activation rate is approximately 10-fold slower in comparison to SAK. These results prompted us to explore whether this deficiency in the Pg activation is due to alteration in the functionalities of Pg activator complex or the interaction of SAK fusion complex with the substrate Pg. For this purpose, the pattern of Pg activation by pre-formed complexes of Pm with SAK fusion proteins or SAK was compared. Equimolar mixtures of SAK (1.5 μM), SAKβ (1.5 μM) or SAKβγ (1.5 μM) and Pg (1.5 μM) were pre-incubated in 0.1 M phosphate buffer, pH 7.5 containing 0.1% BSA and 0.01% Tween 80 at 37°C for 5 min to generate the activator complex. These pre-formed activator complexes (5 nM) were mixed with Pg (1.5 μM) and 1.0 mM Chromozym PL. The change in absorbance at 405 nm was measured as a function of time in Shimadzu (UV–1601) spectrophotometer at 25°C. Activation of Pg by pre-formed SAK fusion-Pm complex occurs progressively with a brief lag phase followed by an exponential phase (Fig. 6.12). In contrast to direct activation where SAKβ shows better activity, SAKβγ-Pg pre-complex activates substrate Pm faster than SAKβ-Pg. SAK fusion proteins were further checked for any alteration in active-site exposure in substrate Pg using NPGB. A small increase in lag time is observed upon integration of SK domains with SAK (Fig. 6.13).
Fig. 6.11 Pg activation by SAK fusion proteins.

Fig. 6.12 Substrate Pg activation by equimolar complexes of Pm and SAK fusion proteins.
Fig. 6.13 Time-course acylation of Pm active site in equimolar mixtures of Pm with SAK fusion proteins.
6.3.4 Determination of steady state kinetic parameters of Pg activation by SAK fusion proteins

To calculate the steady state kinetic constants of Pg activation by SAKβ and SAKβγ, equimolar mixtures of SAK fusions (1.5 μM) and Pg (1.5 μM) were pre-incubated in 0.1 M phosphate buffer, pH 7.5 containing 0.1% BSA and 0.01% Tween 80 at 37°C for 5 min to generate the activator complex. These pre-formed activator complexes (5 nM) were mixed with different concentrations of Pg (1-10 μM) and 1.0 mM Chromozym PL. The change in absorbance at 405 nm was measured as a function of time in Shimadzu (UV-1601) spectrophotometer. The overall activation pattern obeys Michaelis-Menten kinetics and Km of SAKβ-Pg and SAKβγ-Pg are 1.52 and 1.5 μM respectively (Table 6.2). Activator complex of Pg with SAK fusion proteins exhibit more than 50% reduction in kcat values (Table 6.2) suggesting that although SAK fusion protein-Pm complex are interacting efficiently with substrate Pg but their catalytic activity is markedly reduced. Cleavage of substrate Pg to Pm by SAK fusion proteins was monitored by SDS-PAGE to further compare the catalytic efficiency of SAKβ and SAKβγ proteins. For this experiment, equimolar amounts of SAKβ or SAKβγ were incubated with Pg (1.5 μM each) at 25°C in 50 mM Tris.HCl, pH 7.5. At each time point, a 7.5 μl aliquot was removed and analyzed for the conversion of Pg into Pm by 12% SDS-PAGE. Densitometric analysis of the protein profile indicates that almost 100% Pg was converted to Pm by SAK within 5 min whereas SAKβ and SAKβγ takes more than 15 min for 100% processing of Pg (Fig. 6.14).

6.3.5 Sensitivity of chimeric SAK proteins to inhibition by α2-antiplasmin

When βγ domain of SK was provided in purified form to SAK-Pg complex, it showed partial resistance to α2-antiplasmin inhibition. To check whether this property was retained by SK domains after fusion with SAK, Pg activation by SAK fusion proteins was studied in the presence of α2-antiplasmin. SAKβ and SAKβγ strictly follows SAK pattern (Fig. 6.15) in the presence of α2-antiplasmin indicating that like SAK, SAK fusion proteins
Fig. 6.14 Cleavage of Pg by complexes of Pg with SAK fusion proteins.
Lane M: Protein molecular weight marker; Lane 2: Pg; Lane 3: purified SAK; Lane 4-7: SAK-Pg reaction mixtures incubated for 0, 1, 2, 5 min respectively; Lane 8: purified SAKβ; Lane 9-13: SAKβ-Pg reaction mixtures incubated for 0, 5, 10, 15, 30 min respectively; Lane 14: purified SAKγ; Lane 15-19: SAKγ-Pg reaction mixtures incubated for 0, 5, 10, 15, 30 min respectively. The arrows indicate the Pg cleavage products.
are dependent on Pm for the generation of activator complex and βγ domains fail to provide any resistance against α2-antiplasmin upon integration with SAK.

<table>
<thead>
<tr>
<th>Bimolecular complex</th>
<th>Km (μM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (s⁻¹ μM⁻¹)</th>
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<td>SAK-Pm</td>
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Table 6.2. Kinetic constants for the Pg activation properties of equimolar complexes of SAK fusion proteins and Pg. The results are mean±S.E.M. of three determinations.

6.3.6 CD analysis of SAK fusion proteins

Unexpected reduction of SAK activity upon fusion of SK domains prompted us to check the protein for any major structural alteration. Far UV CD spectra of the fusion proteins in the wavelength range of 197–250 nm were recorded on Jasco J710 CD spectrophotometer using 0.2 cm path length cell at a protein concentration of 0.1 mg/ml in PBS, pH 7.4. Appropriate buffer baseline was subtracted in all cases and a mean residue weight of 114 for SK was used (Radek and Castellino, 1989). Final spectrum was an average of 20 scans. Far-UV spectra were recorded for SAK, SK, SAKβγ and SAKβ. Interestingly, CD spectra for SAKβγ protein overlap perfectly with that of full length SK (Fig. 6.16), ruling out the possibility of major secondary structure alteration in SAK or βγ domain after fusion.
Fig. 6.15 Residual Pg activation activity of SAK fusion proteins in the presence of various concentrations of α2-antiplasmin.

Fig. 6.16 CD spectral analysis of various purified proteins.
6.4 Shuffling of SAK loops with corresponding SK α domain loops

In native SK, integration of βγ domains to α domain, which has negligible Pg activation activity results in a three-domain protein having very high Pg activation activity. In contrast, when βγ domains of SK are fused with the single domain molecule SAK, which is structurally homologous to SK α domain and possesses very high Pg activation activity, results in a chimeric protein with drastically reduced Pg activation capacity. These observations indicate that instead of increasing the functional activity of SAK upon fusion of SK βγ domains, SAK fail to fully retain even its native activity. In order to understand the reason for reduced activity of SAK fusion, SK α domain and SAK were structurally compared. Computer based modeling studies indicates that the replacement of two SAK loops i.e. SAKLys35-Tyr44 and SAKThr71-Phe76 with SK α domain loops i.e. SKGly28-Phe37 and SKAla89-Phe76 respectively could facilitate the interaction of SAK with substrate Pg.

Based on these studies, following loop-replaced mutants of SAK were generated:

a) SAKLys35-Tyr44 replaced with SKGly28-Phe37, construct was named as SAK 35/44.

b) SAKThr71-Phe76 replaced with SKAla89-Phe76, construct was named as SAK 71/76.

c) SAKLys35-Tyr44 and SAKThr71-Phe76 replaced with SKGly28-Phe37 and SKAla89-Phe76 and construct was named as SAK 71/35.

d) SAK 35/44 fused with SK βγ domains, construct named as SAKβγ 35/44.

e) SAK 71/76 fused with SK βγ domains, construct named as SAKβγ 71/76.

f) SAK 71/35 fused with SK βγ domains, construct named as SAKβγ 71/35.

All above mentioned loop-replaced mutants were generated by overlap PCR and cloned in pBS KS+, sequenced and expressed in E. coli Bl21DE3 cells under T7 promoter.
6.4.1 Purification and primary characterization of the loop-replaced mutants

SAK 35/44, SAK 71/76 and SAK 71/35 were purified using single step purification protocol, as was followed for native SAK. Loop-replaced mutants of SAK were purified using DEAE-sepharose and Phenyl-sepharose column chromatography and the elution profile followed the pattern similar to SAKβγ protein (Fig. 6.17a, b). Purified proteins were checked for their Pg activation property. All the loop-replaced mutants showed further reduction in specific activity of Pg activation as indicated in Table 6.3.

<table>
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<th>Construct</th>
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<td>SAK 71/35</td>
<td>0.15</td>
</tr>
<tr>
<td>SAKβγ 35/44</td>
<td>0.1</td>
</tr>
<tr>
<td>SAKβγ 71/76</td>
<td>5.7</td>
</tr>
<tr>
<td>SAKβγ 71/35</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 6.3. Pg activation activity of SAK and SAK fusion loop replaced mutants.

6.5 Pg activation by SAK fusion proteins with increased linker length

SAKβγ and SAKβ fusion proteins were generated based on close structural homology between SAK and SK α domain. While fusing sak gene with the DNA encoding SK βγ or β domains, DNA fragment encoding five residues was introduced at the site of fusion. Although structure-modeling studies indicated that five-residue long linker is
**Fig. 6.17a)** Expression and purification of SAK loop replaced mutants.
Lane 1: Protein molecular weight marker; Lane 2: *E. coli* BL21DE3 pET; Lane 3: *E. coli* BL21DE3 pETS\(\text{AK35/44}\); Lane 4: purified SAK\(\text{35/44}\); Lane 5: *E. coli* BL21DE3 pETS\(\text{AK71/76}\); Lane 6: purified SAK\(\text{71/76}\); Lane 7: *E. coli* BL21DE3 pETS\(\text{AK71/35}\); Lane 8: purified SAK\(\text{71/35}\). The arrow indicates the purified proteins.

**Fig. 6.17b)** Expression and purification of SAK fusion loop replaced mutants. Lane 1: Protein molecular weight marker; Lane 2: *E. coli* BL21DE3 pETS\(\text{AKβγ35/44}\); Lane 3: purified SAK\(\text{βγ35/44}\); Lane 4: purified SAK\(\text{βγ71/76}\); Lane 5: purified SAK\(\text{βγ71/35}\). The arrow indicates the purified proteins.
sufficient for independent folding of fusion protein domains, SAK fusion showed reduced activity. Therefore, an attempt was made to alter the linker region. A stretch of twenty residues, (STSGG)$_5$ was introduced at the site of fusion by a series of PCR to minimize inter-domain interference during protein folding. SAK fusion proteins with increased linker length (SAKL$\beta\gamma$ and SAKL$\beta$) were purified (Fig. 6.18) and characterized for their Pg activation properties. Specific activity of SAKL$\beta\gamma$ and SAKL$\beta$ are 28 IU/$\mu$L and 30 IU/$\mu$L, respectively. Although specific activity increases nearly 3-folds in comparison to SAK$\beta\gamma$ and SAK$\beta$ but fusion proteins are not able to fully regain the Pg activation activity of native SAK.

6.6 Effect of kringle domains on the activation of Pg by SAK in the presence of SK $\beta/\beta\gamma$ domains

Instead of showing increased Pg activation activity upon fusion with SK domains, SAK fail to display even its native activity indicating that fused and isolated SK domains are interacting with Pg in different modes than isolated SK domains. Along with the catalytic domain, SK $\beta$ domain has affinity for kringle domains of Pg. Pg activation by SAK in the presence of SK domains was studied to check for any interference by kringle domains. Presence of kringle domains (K1+K2+K3) reduce Pg activation by SAK upto 45% the presence of SK $\beta/\beta\gamma$ domains (Fig. 6.19). Since SAK also carries the site for interaction with kringle domains (Rajamohan et al., 2002), a concentration dependent inhibition was also observed in SAK but to a lesser extent (up to 20%) as shown in figure 6.19. Unlike in the presence of isolated domains, inhibitory effect of isolated kringle on SAK$\beta$ and SAK$\beta\gamma$ is comparable to SAK (Fig. 6.19), indicating that free kringles may be competing with Pg for the binding of isolated SK domains.
Fig. 6.18 Expression and purification of SAK fusion proteins with increased linker region. Lane 1: protein molecular weight marker, Lane 2: *E. coli* BL21DE3 pETSAKLβγ; Lane 3: purified SAKLβγ; Lane 4: *E. coli* BL21DE3 pETSAKLβ; Lane 5: purified SAKLβ. The arrows indicate the purified proteins.
Fig. 6.19 Percentage residual Pg activation activity of SAK in the presence of SK domains with various amounts of kringle domains of Pg.
6.7 μPg activation by SAK in the presence of isolated SK β/βγ domains

The inhibitory effect of free kringle domains of Pg is more pronounced on the Pg activation by SAK in the presence of isolated SK domains indicating that isolated SK domains might be interacting with Pg via kringle domains. To check this possibility, instead of Pg, μPg was used to study the functional activity of SAK in the presence of isolated SK domains. For this experiment, 1.5 μM of μPg was incubated for 5 min at 37°C with 3-fold molar excess of β or βγ domains and after incubation, 5 nM of SAK was added and amidolytic activity of μPg was monitored using Chromozym PL. As compared to Pg, where cofactor activity of SAK increases up to 75%, when μPg was used, isolated SK domains largely fail to alter the cofactor activity of SAK (Fig. 6.20). These results further support the idea that isolated SK domains are increasing the Pg activation activity of SAK by interacting with Pg via kringle domains.

Discussion

SAK is the smallest known, single domain Pg activator that exhibits close similarity with the α domain of the 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 (Parry et al., 1998; Wang et al., 1998). 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 (Loy et al., 2000; Sundram et al., 2003). Structural elements required for SAK versus SK mechanism of Pg activation and contribution of domain interactions in bringing distinct functional differences between these two Pg activators are not clearly understood at present. In the present work, ability of different domains of SK to act synergistically with SAK during Pg activation was examined. Isolated β domain or its two domains
Fig. 6.20 Percentage increase in cofactor activity of staphylokinase in the presence of isolated streptokinase domains with different molecular forms of plasminogen.
combination with γ domain of SK brought dramatic increase (up to 60%) in Pg activation potentiality of SAK, whereas, γ domain of SK alone had 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. This is supported by the fact that the time course exposure of active site in substrate Pg by SAK-Pm activator complex formed along with β or βγ domains of SK reduced the lag period for the active site generation. It has been contended that in SK, α domain provides the activator complex with the function of substrate Pg recognition whereas, SKγ and βγ are capable of inducing Pg catalytic activity in the activator complex (Esmon and Mather, 1998). Although presence of isolated SK γ did not have any additive enhancement effect on catalytic activity of SAK-Pm activator complex, two domain structure of β and γ domain combination amplified the catalytic efficiency of the activator complex better than SK β alone.

Surprisingly, in contrast to isolated domains, fusion of β and βγ domains with SAK resulted in drastic reduction in Pg activation potential of SAK suggesting that integration of these SK domains generates some constrain in the fusion protein due to which SAK is not able to display its cofactor activity efficiently. Although Km of SAK-Pm complex for substrate Pg remained same when SK domains were either fused or provided in-trans (Table 6.1, 6.2), catalytic activity of SAK fusion-Pm complex was drastically attenuated. This was also reflected by increase in lag period for the exposure of active site in substrate Pg by SAK fusion-Pm activator complex suggesting that SAK fusion-Pm complex is a poorer enzyme.

α2-antiplasmin interacts with Pg via lysine binding site (Mimuro et al., 1987) and inhibits the Pg activation activity of SAK in free plasma (Sakharov et al., 1996). Pg activation activity of SAK in the presence of isolated β domain was rapidly inhibited by α2-antiplasmin but isolated βγ domain provided SAK with partial resistance to inhibition by α2-antiplasmin. SAK fusion proteins also failed to activate Pg in the presence α2-
antiplasmin. These results suggest that isolated βγ domains are partially blocking the α2-antiplasmin binding sites on Pg. However, free β domain alone failed to impart any resistance in SAK against α2-antiplasmin, indicating that either β domain is not interacting with kringles or binding of β alone is not strong enough to compete with α2-antiplasmin.

No secondary structure alterations were observed upon in-frame fusion of SK domains with SAK as CD spectrum of SAKβγ fully overlapped with that of SK.

Overall results from biochemical characterization of SAK fusion proteins showed that the substrate Pg processing by SAKβ or SAKβγ complex with Pm is significantly slow after fusion. Substitution of loop region in SAK with SK α domain loops further reduced the functional activity of SAK. The SAK 35/44 and SAK 71/76 loop regions were found to be indispensable for the functional activity of SAK. Upon increasing the linker length, SAK fusion proteins did show nearly 30% increase in activity but full activity was not regained, suggesting that there might be some constrain in the molecule after fusion, due to which intermolecular interactions are not optimum.

Drastic differences in the behavior of free SK domains and their fusion with SAK with respect to 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 showed 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 (Dhar et al., 2002). It is well established that fibrin amplifies the Pg activation activity of SAK by changing the conformation of Pg via kringle domain mediated intermolecular interactions. Addition of purified kringle domains of Pg, inhibited 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 (Fig. 6.19). This implies that instead of interacting with Pg kringle domains, SK β and βγ domains interacted largely with free kringle domains and therefore, failed to display their positive effect on Pg activation activity of SAK. Since, free kringles can interfere with SAK as well as with isolated SK β domain, percentage inhibition of SAK cofactor activity was more pronounced when isolated SK domains were used. Low level of inhibition in case of SAK fusion proteins in the presence of isolated kringle domains suggest that after fusion SAK and/or SK domains are not interacting efficiently with kringle domains of Pg. Studies on Pg activation by SAK in the presence of SK domains and purified kringle domains indicated that isolated SK domains may be amplifying the cofactor activity of SAK mainly by kringle mediated interaction with Pg. Interaction of isolated SK domains with kringle domains may restructure Pg making it a better substrate for SAK mediated activation process. This hypothesis was supported by studies on μPg activation by SAK in the presence of isolated SK β and βγ domains. Isolated SK domains failed to alter the cofactor activity of SAK for μPg (Fig. 6.20), the catalytic domains of Pg lacking all kringle domains.

The present study, thus demonstrates that isolated β and βγ domains of SK interact with Pg and increase the cofactor activity of SAK. The enhanced Pg activation activity of SAK in the presence of isolated domains of SK involves their interactions with Pg that at least, in part are mediated via the kringle structures. Extended intermolecular interaction between Pg and isolated SK domains presumably reconfigures Pg and enhance the substrate Pg activation by the 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 the catalytic domain of Pg but β domain possesses affinity for the kringle domains also. Binding affinity of isolated SK α domain for Pg is much higher than SAK (Loy et al., 2000), 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
impacted by the presence of isolated SK domains to SAK fusion proteins.