IN Volvement of a nine-residue loop of streptokinase in the generation of macromolecular substrate-specificity by the activator complex through interaction with substrate kringle domains

Jayeeta Dhar#, Abhay H. Pande#, Vasudha Sundram, Jagpreet S. Nanda, Shekhar C. Mande and Girish Sahni

Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, India

Address correspondence to:
Dr. Girish Sahni
Institute of Microbial Technology
Sector 39-A, Chandigarh-160036
India.

Telephone: +91 (172) 695215
Fax: 0091-172-690585
e-mail: sahni@imtech.res.in

Running title: Kringle mediated streptokinase-plasminogen interaction
SUMMARY

Computer modeling studies of the three structurally homologous domains of streptokinase (SK) superimposed on each other revealed a discrete surface-exposed epitope (residues 254-262; 250-loop) in the β domain that is absent in the other two otherwise conformationally similar domains. The deletion of this nine-residue loop in SK led to significant decrease in the rates of substrate HPG activation by the mutant (SK_{del254-262}). A kinetic analysis of SK_{del254-262} revealed that its low HPG activator activity arose primarily from a five-six fold increase in $K_m$ for HPG as substrate, with little alteration in $k_{cat}$ rates. Under these conditions, the $k_{cat}$ for the small peptide substrate tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate was essentially unchanged. Remarkably, the observed increase in the $K_m$ for the macromolecular substrate was proportional to a similar decrease in the binding affinity for substrate HPG as observed in a new Resonant Mirror based assay for the real-time kinetic analysis of the docking of substrate HPG onto pre-formed binary complex, although under those conditions the 1:1 affinity of SK_{del254-262} for ‘partner’ HPG was minimally changed. In contrast, kinetic studies on the interaction of the two proteins with microplasminogen (having just the catalytic domain of HPG without any of the five kringles) showed no difference between formation of ternary complex with µPG by preformed equimolar complexes of HPG with either SK or SK_{del254-262}, nor between the rates of activation of microplasminogen to microplasmin under conditions where native HPG was activated differentially by nSK and SK_{del254-262}, clearly indicating a kringle-mediated mechanism for the role of the 250-loop of SK in substrate docking/recognition. The involvement of kringles was further indicated by a hyper-susceptibility of the SK_{del254-262}-plasmin activator complex to epsilon-aminocaproic acid (EACA) inhibition of substrate HPG activation in comparison to that of the native
SK-plasmin activator complex. Further, ‘ternary’ binding experiments on the Resonant Mirror showed that the binding affinity of kringles 1-5 of plasminogen to the preformed binary complex of the 250-loop deleted mutant and HPG was reduced by about three-fold in comparison to that of nSK-HPG. Overall, these observations identify the 250-loop of SK as an important structural determinant of the inordinately stringent substrate specificity of the streptokinase-plasmin(ogen) activator complex and demonstrate that this structural epitope in the β domain promotes the binding of substrate HPG to the activator via the kringle/s during the HPG activation process.
Streptokinase (SK)\textsuperscript{1}, a bacterial protein secreted by the Lancefield Group C \(\beta\)-hemolytic streptococci, is widely used as a thrombolytic agent in the treatment of various circulatory disorders, including myocardial infarction (1). Unlike other human plasminogen (HPG) activators, like tissue-plasminogen activator and urokinase (UK), SK does not possess any intrinsic enzymatic activity. Instead, SK forms an equimolar, stoichiometric complex with ‘partner’ HPG or plasmin (HPN), which then catalytically activates free ‘substrate’ molecules of HPG to HPN by selective cleavage of the Arg\textsubscript{561}-Val\textsubscript{562} peptide bond (2, 3). It is believed that consequent to the initial SK-HPG complexation, there is a structural rearrangement within the complex, and even before any proteolytic cleavage takes place, an active center within the HPG moiety capable of undergoing acylation is formed (3). This activated complex is rapidly transformed into an SK-HPN complex and develops a HPG activator activity. Unlike free HPN, however, which is essentially a trypsin-like protease with broad substrate-preference, SK-HPN displays a very narrow substrate specificity (4). Elucidating the structural basis of the conversion of the broadly-specific serine protease HPN to a highly substrate-specific protease, virtually with exclusive propensity for acting on the target scissile peptide bond in HPG once complexed with the ‘cofactor’ SK has been the subject of active investigations with both fundamental and applied implications (5-12).

SK has been shown to be composed of three structurally similar domains (termed \(\alpha\), \(\beta\) and \(\gamma\)), separated by random coils and small, flexible regions at the amino and carboxyl termini (5, 7, 8). The recently solved crystal structure of the catalytic domain of HPN complexed with SK strongly indicates how SK might modulate the substrate-specificity of HPN by providing a “valley” or cleft in which the macromolecular substrate can dock through protein-protein interactions, thus positioning the scissile
peptide bond optimally for cleavage by the HPN active site, thereby conferring a narrow substrate preference onto an otherwise ‘indiscriminate’ active center. In this structure, SK does not appear to induce any significant conformational changes in the active site residues directly but, along with ‘partner’ HPG, seems to provide a template on which the substrate molecule can dock through protein-protein interactions, resulting in the optimized presentation of the HPG activation loop at the active center of the complex (6, 8). However, the identity of these interactions, and their contributions to the formation of the enzyme-substrate intermediate/s remains a mystery so far.

Besides the well-recognized “switch” in substrate preference (4), the binding of SK to HPN results in a several-fold enhancement of the $K_m$ for diverse small molecular weight chromogenic peptide substrates but relatively little alteration in their $k_{cat}$ values as compared to free HPN indicating that the primary, covalent specificity characteristics of the active center of HPN upon SK binding are unchanged but result in steric hindrance/reduced accessibility for even the small molecular weight peptide substrates. Thus, the remarkable alteration of the macromolecular substrate specificity of HPN by SK is currently thought to be due to ‘exosites’ generated on the SK-HPN complex, as shown recently by the elegant use of active site-labeled fluorescent HPN derivatives (10). Peptide Walking studies in our laboratory had also indicated that short peptides based on the primary structure of SK, particularly those derived from selected regions in the $\alpha$ and $\beta$ domains, displayed competitive inhibition for HPG activation by the pre-formed SK-HPN complex under conditions where the 1:1 complexation of SK and HPN was essentially unaffected (11, 12) However, the crystal structure of SK complexed with microplasmin(ogen) (8), while providing a high degree of resolution of the residues involved in the SK-µPN complexation, yielded few unambiguous insights regarding the interactions engendered between the activator complex and ‘substrate’ HPG. This is
likely due to the binary nature of the complex i.e. an absence of a juxtaposed substrate molecule, large average thermal factors especially in the β domain, and a total absence, in both partner and substrate HPG, of the kringles which are known to be important in HPG activation (13). Thus, despite a detailed and high-resolution exposition of the overall nature of protein-protein interactions in the SK-μPN binary complex, discrete structures/epitopes of SK, if any, that are directly involved in the exosite formation process by the full-length activator complex have not yet been identified.

Previously, charged side-chains, both in HPG, particularly around the active center (14) as well as in SK in the beta domain (16, 17) have been shown to be important for HPG activation ability. However, a clear-cut identification of a structural epitope/element in conferring substrate HPG affinity onto the SK-PG activator complex has not been demonstrated till now. Of the three domains of SK, the central β domain displays maximal affinity for HPG (15), the N-terminal α domain displays relatively lesser affinity, and much less affinity of the isolated γ domain for HPG. 2 Solution and structural studies suggest that both α and β domains are involved in the substrate recognition phenomena (8, 12). Mutagenesis studies have also implicated positively charged residues in the β domain to be important, but failed to show that these residues are directly involved in substrate recognition by the binary complex (17). An examination of the crystal structure of the free β domain (18) and its comparison with the other two SK domains possessing closely similar (but not identical) structures revealed the presence of a distinct flexible loop in the β domain (the 250-loop) that protrudes into the solvent (Fig.1).

In the present study, we chose to delete this loop based on the premise that if this structural motif is involved in substrate recognition, discrete deletion of this loop would lead to selective increase in $K_m$ of the activator complex. The results obtained
provide clear-cut evidence of the role played by this nine-residue loop in substrate recognition, and thus identify a functionally important component of the macromolecular substrate-specific exosite operative in the SK-HPN complex which interacts via the kringle/s in HPG.
EXPERIMENTAL PROCEDURES

Reagents- Glu-plasminogen was either purchased from Roche Diagnostics Inc., Germany or purified from human plasma by affinity chromatography (19). The RNA polymerase promoter-based expression vector, pET23(d) and E. coli strain BL21 (DE3) were products of Novagen Inc. (Madison, WI). Thermostable DNA polymerase (pfu) was obtained from Stratagene Inc. (La Jolla, CA), and restriction endonucleases, T4 DNA ligase and other DNA modifying enzymes were acquired from New England Biolabs (Beverly, MA). Oligonucleotide primers were supplied by Integrated DNA Technologies Inc., (Indianapolis, IN). HPN was prepared by digesting Glu-HPG with urokinase covalently immobilized on agarose beads using a ratio of 300 Plough units/mg HPG in 50 mM Tris-Cl, pH 8.0, 25 % glycerol and 25 mM L-Lysine at 22 °C for 10 h (15, 16). All other reagents were of the highest analytical grade available.

Design and construction of SK_{del254-262} and β_{wild-type}\beta_{del254-262} - A set of mutagenic and flanking primers, carrying unique restriction sites, were used in polymerase chain reactions to generate DNA fragments having overlapping ends. Thereafter, splicing-overlap-extension PCR (SOE-PCR) reactions (20) were carried out, resulting in amplification products which were cloned in the pET23(d) expression vector (16, 21). Oligonucleotide primers for SOE-PCR for the construction of SK_{del254-262} -

(a) Sequences of the mutagenic primers:

upstream primer:

5’AACAGGCTTATAGGGAAATTAACCAACACTGACCTGATATCTGAGAAA 3’

downstream primer:

5’ TGTGTTATTTCCCTATAAGCCTGTTCCGATTTTTAA 3’
(b) Sequence of the flanking primers:
upstream primer: 5’ ATTTATGAACGTGACTCCTCAATCGTC 3’
downstream primer: 5’ ATAGGCTAAATGATAGCTAGCATTCTCTCC 3’

Oligonucleotide primers for SOE-PCR for the construction of $\beta_{\text{wild-type}}$ and $\beta_{\text{del254-262}}$ -

(a) Sequences of the mutagenic primers:
upstream primer:
5’ AACAGGCTTATAGGGAAATAAACACACTGACCTGATATCTGAGAAA 3’
downstream primer:
5’ TGTGTGTTATTTCCCTATAAGCCTGTCCCGATTTTTAA 3’

(b) Sequence of the flanking primers:
upstream primer: 5’ GTGGAATATATACTGTACAGTTTACTCC 3’
downstream primer:
5’ ATCGGGATCCTATTTCAAGTGACTGCGATCAAAGGG 3’

Expression and purification of nSK/SK$_{\text{del254-262}}$ - Both proteins were expressed intracellularly in E. coli BL21 (DE3) cells after induction with IPTG essentially according to the instructions of the supplier (Novagen Inc., WI). The host-vector system for the expression of the cDNA corresponding to mature SK from Streptococcus equisimilis H46A after in-frame juxtaposition of an initiator methionine codon (so as to express the protein as Met-SK) has been described earlier (16). However, protein sequence analysis of the purified SK expressed intracellularly in E. coli (referred to as nSK hereafter) was found to have its N-terminal Met removed at a 50 % level. The same case was seen in the mutant (SK$_{\text{del254-262}}$) prepared similarly from E. coli employing the same expression vector. The pelleted cells were sonicated and the proteins in the supernatants were precipitated with ammonium sulphate (16). This fraction, after dissolution in 20 mM Tris-Cl buffer, pH 7.5, was then chromatographed on a Poros-D
anion exchange column fitted onto a Bio-Cad Sprint liquid chromatographic work-station (Perceptive Biosystems Inc., Framingham, MA). nSK/SK$_{del254-262}$ were eluted using a linear gradient of NaCl (0 to 0.5 M) in 20 mM Tris-Cl buffer, pH 7.5. The eluted proteins were more than 95 % pure, as analyzed by SDS-PAGE.

**Expression and purification of $\beta_{\text{wild-type}}/\beta_{\text{del254-262}}$** - Both proteins were expressed intracellularly in *E. coli* BL21 (DE3) cells as inclusion bodies. The pellet obtained after sonication was taken up in 8 M urea and placed under gentle shaking conditions for 30 min to effect dissolution. After a high-speed centrifugation step, the protein in the supernatant was refolded by 20 fold dilution with 20 mM Tris-Cl buffer, pH 7.5. The $\beta$ domain was then purified to more than 95 % homogeneity by chromatography on DEAE-Sepharose Fast-flow (Pharmacia Biotech. Ltd., Uppsala, Sweden) at 4°C using a linear NaCl gradient (0-0.25 M NaCl in 20 mM Tris-Cl buffer, pH 7.5).

**Preparation of $\mu$PG and K1-5** - Microplasminogen, the catalytic domain of plasminogen (residues Lys530-Asn790) devoid of all kringles was prepared by cleavage of HPG by HPN under alkaline conditions (0.1 N glycine/ NaOH buffer, pH 10.5) at 25°C. Microplasminogen was purified from the reaction mixture by passing through a Lys-Sepharose column (Pharmacia), followed by a soybean-trypsin inhibitor-Sepharose 4B column to absorb HPN and $\mu$PN, as reported (22). The flow-through was then subjected to molecular sieve chromatography, after concentration by ultrafiltration, on a column (16 mm x 60 cm) of Superdex-75™ (Pharmacia). The purity of $\mu$PG formed was analyzed by SDS-PAGE which showed a single band moving at the position expected from its molecular size (22). The proteolytic fragment containing all of the HPG kringle domains (K1-5) was prepared by incubating HPG with urokinase-free HPN (5:1 ratio of HPG and HPN) under alkaline conditions (0.1 N glycine/ NaOH, pH 9.0) for 72 h at 25 °C. Under these conditions, the proteolytic conversion of native HPG to K1-5 was found to be
quantitative, with minimal residual HPG. HPN was removed from the reaction mixture by passing through a soyabean trypsin inhibitor-Sepharose column (1.6 x 3.6 cm). This was followed by gel filtration on Superdex-75, to obtain HPG- and μPG-free K1-5. The purity of this preparation was confirmed by SDS-PAGE analysis. (23). Digestion with urokinase, which is known to be a good activator of μPG irrespective of the presence of kringle domains (22), was used to establish that the activation of this preparation, when used as substrate, was comparable to that obtained when using SK-HPN as the activator species.

Characterization of SK_{del 254-262}

**Amidolytic activation of equimolar HPG-nSK/SK_{del254-262} complexes** - Aliquots (50 nM) were withdrawn from equimolar HPG-nSK/SK_{del254-262} complexes at regular periods and transferred to a 100-μl quartz micro-cuvette containing 2 mM Chromozym PL (Tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate) and 50 mM Tris-Cl, pH 7.5 at 22°C. The change in absorbance at 405 nm was monitored to compute the kinetics of amidolytic activation (12, 24).

**Esterolytic activation of equimolar HPG-nSK/SK_{del254-262} complexes** - Five μM HPG was added to an assay cuvette containing 5.5 μM nSK/SK_{del254-262}, 100 μM NPGB and 10 mM phosphate buffer, pH 7.5 and the ‘burst’ of p-nitrophenol release was monitored at 410 nm as a function of time at 22°C (3, 25).

**Determination of kinetic constants for HPG activator activity of nSK/SK_{del254-262}** - Varying amounts of HPG were added to the assay cuvette containing fixed amounts of nSK/SK_{del254-262} and chromogenic substrate (1 mM) and the change in absorbance was monitored at 405 nm as a function of time at 22°C. Also, the kinetics of HPG activation by HPN-nSK/SK_{del254-262} complexes were measured by transferring suitable aliquots of preformed HPN-nSK/SK_{del254-262} complexes to the assay cuvette containing different
concentrations of substrate HPG (24). To compute the $k_{cat}$, the number of HPN active sites was determined using the NPGB reaction (3, 25, 26).

**Assay for the determination of the steady-state kinetic constants for amidolytic activity of**

$nSK/ SK_{del254-262}$ - $nSK/ SK_{del254-262}$ and HPN were pre-complexed at 4 °C in equimolar ratios (100 nM each) for 1 min in 50 mM Tris-Cl, pH 7.5, containing 0.5 % BSA and an aliquot of the reaction mixture was transferred to a 100-µL assay cuvette containing 50 mM Tris-Cl buffer, pH 7.5, and varying concentrations of the chromogenic substrate (0.1-2 mM) to obtain a final concentration of 10 nM of the complex in the reaction. The reaction was monitored spectrophotometrically at 405 nm for 5 min at 22°C. The kinetic constants were calculated by standard methods (25).

**Kinetic analysis of protein-protein interactions using Resonant Mirror technology**

*a) Binary interaction analysis* - Association and dissociation between HPG and the $nSK/ SK_{del254-262}$, called hereafter as binary interaction, were followed in real time by resonant mirror based detection using the IAsys Plus™ system (Cambridge, UK) (27, 28). In these experiments, streptavidin was captured on biotin cuvette according to the manufactur’s protocols (IAsys protocol 1.1). This was followed by the attachment of (mildly) biotinylated HPG to the streptavidin captured on the cuvette. Non-specifically bound HPG was then removed by repeated washing with PBS followed by three washes with 10 mM HCl. The net response chosen for the immobilized biotinylated HPG onto the cuvette was 700-800 arc sec in all experiments. Experiments were performed at 25°C in 10 mM PBS, pH 7.4 containing 0.05% Tween 20 and $5 \times 10^{-3}$ M NPGB (binding buffer). The latter was included in order to prevent plasmin mediated proteolysis.

After equilibrating the cuvette with binding buffer, varying concentrations of either $nSK$ or $SK_{del254-262}$ were added and each binding response was monitored during the ‘association’ phase. Subsequently, the cuvette was washed with binding buffer and the
'dissociation' phase was recorded (29). Following each cycle of analysis, the cuvette was regenerated by washing with 10 mM HCl, and baseline was re-established with binding buffer. In parallel, in the control cell in the dual channel cuvette, immobilized streptavidin alone was taken as a negative control for the binding studies. In experiments where EACA was used to examine its effect on SK-HPG interaction, the binary complex was formed between ligate nSK/SK_{del254-262} and immobilized HPG, in binding buffer (as described above). The dissociation of the binary complexes was done by washing the cuvette with EACA, instead of buffer alone.

The data were analyzed after subtraction of the corresponding non-specific refractive index component/s, and the kinetic constants were calculated from the sensorgrams by nonlinear fitting of the association and dissociation curves according to 1:1 model A+B=AB using the software FASTfit\textsuperscript{TM}, supplied by the manufacturers. Briefly, the association curves at each concentration of ligate were fitted to the pseudo-first order equation to calculate the observed rate constant (k_{on}). Then the concentration dependence of k_{on} was fitted using linear regression to find the association rate constant (k_a) from the slope of the linear fit (30). The dissociation rate constant (k_d) was calculated from the average of four dissociation curves obtained at saturating concentration of ligate. The equilibrium dissociation constant (K_D) was then calculated as k_d/k_a. Values of K_D obtained using this relationship were in good approximation to those obtained by Scatchard analysis of the extent of association (data not shown).

b) Ternary interaction analysis - Resonant mirror technology based biosensor was also used to measure the rate and equilibrium dissociation constants describing interactions between soluble ligate (PG, µPG or K1-5) and nSK/SK_{del254-262} complexed with immobilized HPG, a situation simulating substrate binding to binary complex and hereafter called as ternary interaction. In binary interaction studies, it was evident that
when soluble nSK/SK_{del254-262} was added to immobilized HPG, a rapid and avid SK:HPG binary complex formation occurs. The dissociation of this complex is very slow due to the high stability of the SK-HPG complex, as has been observed by others also (15). After allowing the complex to dissociate maximally (~20 min), the dissociation baseline becomes stable which remains unaffected even after washing with 2.5 mM EACA. It has been reported that when SK was pre-incubated with immobilized HPG, EACA was > 100-fold less potent at dissociating the binary complex than it was at preventing binary complex formation when SK and EACA were added synchronously to immobilized HPG (13). Thus this comparative resistance to dissociation of the SK-HPG binary complex by EACA permitted us to study ternary substrate interaction, under conditions that did not adversely affect the stability of the binary interaction. In contrast, EACA was found to be strongly inhibitory to ternary complex formation (given below).

In a typical ternary interaction experiment, a stable binary complex was formed by adding saturating amount of either nSK or SK_{del254-262} onto HPG, immobilized on streptavidin captured on biotin cuvette. After maximally dissociating the binary complex with binding buffer and washing with 2.5 mM EACA, a stable dissociation baseline was obtained. Varying concentrations of either ‘ternary’ HPG (0.1 – 1.0 µM), µPG (1-6 µM) or K1-5 (1-6 µM) were then added to monitor the binding by recording the association phase. Subsequently, the cuvette was washed with binding buffer three times and the dissociation phase was recorded. After each cycle of analysis, the original baseline was re-established by stripping off the undissociated ‘ternary’ ligate with 2.5 mM EACA followed by three washes with binding buffer. It was established that EACA, at this concentration, completely abolishes the interaction of ternary HPG with the binary complex, while the binary complex remains stable. The latter attribute was considered to be a necessary precondition to obtain reliable and reproducible values of the kinetic
constants for ternary complex formation and dissociation. In order to test whether this was indeed so, control experiments were carried out in which the ternary complex formation and dissociation experiments were done at various time intervals after a stable baseline was attained subsequent to binary complex formation followed by buffer and EACA washes, as described above. It was observed that the rate constants so obtained did not differ significantly (within a margin of ±5%) as a function of time. In experiments where μPG was used as soluble ternary ligate, 1 mM EACA was found to be sufficient to strip off the undissociated μPG, while washing with binding buffer alone resulted in incomplete regeneration of baseline. Association and dissociation phases at varying ligate (HPG, μPG and K1-5) concentrations were fitted as described above and the equilibrium dissociation constant/s were calculated as $k_d/k_a$.

The effect of EACA on the binding of soluble ‘ternary’ HPG to nSK/SK$_{del254-262}$ complexed with immobilized HPG was assessed by measuring the binding extents, measured in arc second units, at equilibrium at a fixed concentration of substrate HPG (0.4 μM) in the presence of varying EACA concentrations (0-2 mM). For the analysis of these data, binding extent in buffer alone was taken as 100% and varying extents of formation of ternary complexes at equilibrium were plotted as a function of EACA concentration to obtain EACA-dependant binding isotherms for nSK and SK$_{del254-262}$. As controls, the effect of EACA was similarly examined for dissociation of preformed complexes between HPG and either nSK or SK$_{del254-262}$ in the absence of substrate HPG.

Circular dichroic analysis of nSK/SK$_{del254-262}$ – Far-UV CD spectra of proteins (concentration 0.15 mg/ml in PBS, pH 7.2) were recorded on a Jasco-720 spectropolarimeter. Measurements were carried out from 200 to 250 nm in a 0.1-cm path length cell and the appropriate buffer baseline was subtracted from the protein spectra. The final spectrum analyzed was an average of 10 scans (31).
Modeling studies - Cartesian coordinates of the kringle 5 domain of HPG (used as a prototypical representative structure of HPG kringle domains) and those of SK β domain, and SK-µPN complex were retrieved from the Protein Data Bank (codes - 5HPG, 1C4P and 1BML, respectively). The β domain in SK-µPN reveals several disordered loops, including the 250-loop (8). The coordinates in this complex were hence replaced with those of the isolated β domain (18). The isolated β domain was superimposed on the β domain of the complex, and the corresponding set of coordinates were simply replaced. The isolated β domain has the 250-loop clearly defined, and hence it is more suitable for docking analysis.

Molecular surface for a typical HPG kringle domain was generated using the kringle 5 co-ordinates (taken as a prototype) with the aid of GRASP (32) with standard atomic radii, and the probe radius of 1.4 Å. Electrostatic potential was then mapped onto the molecular surface. There are two distinct regions of negative charges on the surface. The negatively charged surface of kringles is likely to interact with the positively charged loop of the SK structure, and therefore these regions were of particular interest in generating the SK β-µPN-kringle docked complex. One of the negatively charged regions is at the interface of the dimeric kringle structure. This region was therefore not considered appropriate for docking. The other negatively charged region was then manually brought into close proximity of the ‘250 loop’ of SK β domain. The best docked complex is shown in Fig. 7. Remarkably, the surface of the kringle showed nearly perfect complementarity to the surface of the SK-µPN complex. The mode of docking also revealed that the C-terminal of the kringle domain is within connecting distance of the N-terminal of the µPN moiety, as would be expected in the physiological situation. Thus, the close complementarity of kringle and SK-µPN surfaces, the perfect electrostatic
match among the two structures, and the proximity between connecting peptide units suggests that indeed the kringle might dock onto SK-μPN in the mode shown in Fig. 7.
RESULTS AND DISCUSSION

In order to investigate the functional role of the 250-loop viz. its involvement in the modulation of substrate specificity of HPN by SK, we prepared a deletion mutant (SK$_{\text{del254-262}}$) employing the SOE-PCR method (20). The desired deletion mutation was confirmed by sequencing of both strands of the cloned DNA (see Experimental Procedures for other details). This was followed by sub-cloning of the PCR amplification product in a T7 RNA polymerase promoter-based expression vector (21). With the nine-residue deletion, we expected that a minimal perturbation of the underlying $\beta$ sheet would occur (Fig. 1), since the two residues flanking the loop (Tyr 252 and Glu 263) had a distance of 4 Å between the C$\alpha$ atoms (18). The E. coli BL21 (DE3) cells carrying the plasmid were grown in shake-flasks to mid log phase and the expression of the protein of interest was then induced by addition of IPTG. SK$_{\text{del254-262}}$ was then purified from the intracellular milieu to homogeneity using a rapid, two-step protocol (16). When purified SK$_{\text{del254-262}}$ was examined for its ability to activate substrate HPG, it showed a specific activity that was only about 20 % that of native-like SK (termed nSK) similarly expressed and purified using the same expression plasmid and host system.

We checked whether the deletion of the 250-loop resulted in changes in the overall folding characteristics of SK as judged by its secondary structure analyzed by CD. The far-UV CD spectra of nSK and the mutant (SK$_{\text{del254-262}}$), however, did not show any significant discernable differences (data not shown). However, since a native-like CD spectrum of the mutant could still result if relatively small, local conformational changes occurred in and around the site of the deletion in the $\beta$ domain that might get ‘averaged out’ in the presence of the CD contributions from rest of the two domains of SK in the full-length molecule, we carried out a deletion at the same site in a truncated gene
encoding for the isolated β domain. The cDNAs encoding for the β domain alone and β⇌del254-262 were then expressed in *E. coli*, purified (see Experimental Procedures for details), and their far-UV CD spectra were recorded. Interestingly, no noticeable changes were observed in the secondary structure of β⇌del254-262 with respect to β⇌wild-type similarly expressed in *E. coli* also (data not shown), indicating that neither the overall secondary structure of SK nor that of the β domain alone had been perturbed by the deletion of the nine-residue loop from the protein.

In order to explore whether the deletion of the 250-loop resulted in any significant alteration in the affinity of the mutant with partner HPG, the kinetics of interaction between immobilized HPG and nSK were compared with that of SK⇌del254-262 by the Resonant Mirror approach using a semi-automated instrument for measuring protein-protein interactions in real time (IAsys, Cambridge UK). To examine the effect of immobilization chemistry on the values of kinetic constants, preliminary experiments were performed by either immobilizing nSK or SK⇌del254-262 onto carboxymethylatedextran (CMD) cuvettes, using a amino-coupling protocol recommended by the manufacturers. The kinetics of association and dissociation of HPG with immobilized nSK/SK⇌del254-262 were then measured as described in Experimental Procedures. In another approach, biotinylated HPG was immobilized on streptavidin captured on biotin cuvette and the binding kinetics with SK were studied as described. For these assays, nSK/SK⇌del254-262 were added at concentrations ranging between 5-80 nM. Relatively fast association kinetics were observed for the binding of both proteins, which is consistent with a monophasic pattern of association (Fig. 2). When the association data for the interaction were fitted to a single exponential curve, a linear relationship was observed between $k_{on}$ and added ligate concentration, according to the equation $k_{on} = k_d + k_a [\text{ligate}]$. The results
show that the mutant exhibited an affinity which was not significantly different from that of nSK (Table 1).

Interestingly, experiments performed by immobilizing nSK/SK_{del254-262} on CMD cuvettes using standard amino-coupling procedures, as shown by other groups also (15, 33) yielded similar binding constants for nSK and SK_{del254-262} (data not shown), indicating that the coupling procedure per se does not interfere in the binding interaction between SK and HPG significantly.

To further explore the underlying reason for partial loss of activator activity of the loop-deletion mutant, we examined if, like the native protein, it could expose the active site in ‘partner’ HPG. For this purpose, we employed the active site acylating agent, NPGB, to test whether the characteristic ‘burst’ that occurs upon mixing equimolar SK and HPG (3, 25) was also observed with the mutant. Neither SK nor HPG alone give the burst, but only once the two are mixed in equimolar proportions is this burst, characteristically associated with a rapid NPGB hydrolysis, observed due to the formation of a ‘virgin’ SK-HPG complex and consequent acylation at the cryptic active site of HPG with NPGB (3). When the recombinant nSK (used as a control) was tested with this reagent in the presence of equimolar HPG, the characteristic colorimetrically detectable burst was indeed observed. A similar response was evident in the case of SK_{del254-262} as well (Fig. 3, inset). However, the maximal level of these bursts was approximately half of that observed with natural SK prepared from *S. equisimilis* (data not shown). It has been demonstrated recently that a free N-terminal (Ile) is required for the NPGB reaction of native SK due to the participation of the α-amino group of the N-terminal residue (34), as also some flanking residues (35), in the auto-activation of HPG by SK. However, the positive NPGB reaction of nSK, albeit less than that of natural SK, was easily explained when N-terminal sequence analysis of the purified nSK and SK_{del254-}
showed that approximately 50% of the purified protein fractions had (like natural SK) a free Ile at the N-terminus, probably due to partial processing by host methionyl aminopeptidase (36). The mutant (SK_{del254-262}) was then further verified for its ability to activate partner HPG by carrying out amidolytic assays of its equimolar mixtures with HPG. In this case too, it exhibited a similar, though slightly delayed time-course of generation of amidolytic activity with respect to nSK (Fig. 3) suggesting that it could open the active site in partner HPG. Thus, these results demonstrated that the observed lower HPG activator activity of SK_{del254-262} was not due to any major effect on its ability to activate partner HPG at the binary complex formation stage.

In order to obtain a glimpse of the functional characteristics of the active site, the kinetic constants associated with the substrate binding and processing of both macromolecular substrate (HPG) and the small MW amidolytic, peptide substrate tosyl-glycyl-prolyl-lysine-4-nitranilide acetate were measured by the equimolar complexes of HPN with either nSK or SK_{del254-262}. Remarkably, the results for the steady-state kinetic analysis of the activation of substrate HPG by the equimolar SK_{del254-262}-HPN complex revealed a 5-6 fold increase in the $K_m$ for HPG when compared to that of nSK-HPN, with relatively little alteration in the $k_{cat}$ values (Table 2). This clearly indicated that the loop-deleted mutant formed an activator complex with HPN that had an apparent decreased affinity for the macromolecular substrate as compared to that of the native SK. On the other hand, the $k_{cat}$ values exhibited either by nSK-HPN or SK_{del254-262}-HPN for the small MW peptide substrate were essentially unaltered as compared to that of ‘free’ HPN (suggesting that the primary covalent specificity characteristics of the active site in HPN remained unchanged), but, revealingly, the $K_m$ for this substrate was decreased as compared to that of nSK-HPN.
It is known that the complexation of SK with free HPN leads to an overall reduced accessibility of the HPN active site by small molecular weight substrates and inhibitors probably due to steric hindrance brought about by SK binding in the vicinity of the active site; this phenomenon is manifested, for example, in a significant increase in the $K_m$ for the amidolysis of chromogenic substrates by SK-HPN complex compared with that of HPN alone (8, 37, 38). The characteristic increase in $K_m$ of HPN for amidolytic substrates has also been observed with the isolated $\beta$ domain (11). The present results on the abolishment of the ‘$K_m$ shift’ (Table 3) in SK$_{del254-262}$ suggest that the 250-loop interacts with a region in partner plasmin(ogen) that is situated close to the active center, a surmise that is entirely consistent with its proposed role in sequestering substrate HPG to the activator complex and with peptide inhibition experiments reported earlier (11). Noticeably, the $k_{cat}$ for the hydrolysis of the low-molecular weight peptide substrate by the 1:1 HPN complex of the deletion mutant remained unchanged (Table 2). These results clearly demonstrate that the affinity for macromolecular substrate (HPG) was selectively decreased in the case of SK$_{del254-262}$-HPN, without a concomitant alteration in the processivity of the small-molecular weight peptide substrate.

The foregoing results on increased $K_m$ for HPG prompted us to explore the comparative affinity of substrate HPG with activator complexes between HPG and nSK, on the one hand, and HPG with SK$_{del254-262}$ on the other, using a more direct physicochemical approach. For this purpose, a new ‘docking’ assay for ternary complex formation between SK and two molecules of HPG, one in the binary mode, and the other docked as a substrate was devised based on a real-time approach utilizing Resonant Mirror based biosensor equipment. Earlier, a ‘static’ analysis of the formation of such a ternary complex with radioactively labeled substrate HPG bound onto the binary complex of immobilized HPG and nSK on plastic surfaces has been reported (39). In order to
establish the authenticity of ternary complex formation with substrate HPG onto the preformed binary SK-HPG complex on Resonant Mirror cuvettes, we have used two criteria: (a) sensitivity of the ternary complex, and comparative refractoriness of the preformed binary complex, to EACA, and (b) distinctive concentration range-dependence of binary (low nM) and ternary complex/es (high nM range) to HPG binding (see Experimental Procedures for detailed protocols). It has been well-established that the preformed SK-HPG complex is highly refractory to EACA whereas the action of the activator complex on the substrate is susceptible to inhibition in the low millimolar range (13), a fact which we also observed, while the same concentration of EACA potently inhibited the interaction of substrate HPG with preformed SK-HPG complex. This comparative resistance of the pre-formed binary complex, and susceptibility of the ternary complex to EACA allowed us to selectively examine the interaction of ‘ternary’ HPG with SK-HPG binary complex. Similarly, due to the comparatively low affinity of the substrate HPG towards the SK-HPG binary complex (24), higher concentration of substrate HPG was required, while the SK-HPG binary interaction, due to its intrinsically high affinity, could easily be monitored at the sub- and low-nanomolar ranges of concentration (Tables 1 and 4).

Ternary interaction studies of HPG with SK-HPG binary complex have been reported earlier using solid phase assay (39). Though such an assay can provide a clear estimate of affinity in terms of equilibrium dissociation constants ($K_D$), it fails to give insight into the dynamics of the interaction of the activator complex with the macromolecular substrate. The results obtained for the interaction of substrate HPG, μPG and K1-5 with either nSK or SK_{del254-262} complexed with immobilized HPG are given in Table 4. It is clear that substrate HPG interacts with SK_{del254-262}-HPG binary complex with a 5-fold lower affinity ($K_D \sim 0.75 \mu\text{M}$) as compared with nSK-HPG binary
complex ($K_D \sim 0.15 \mu M$). This decrease in affinity is remarkably proportionate to the increase in the $K_m$ for substrate HPG (5-6 fold) using enzymatic activity as the criterion for discrimination between nSK and mutant. These results clearly indicate the importance of the 250-loop in ‘capturing’ substrate HPG molecules by the activator complex.

Recent biochemical studies suggest that an ‘exosite’ mediated substrate HPG binding, independent of the primary covalent specificity of the HPN active site, represents the major mechanism of SK-induced changes in the macromolecular substrate specificity of HPN (10, 12). The structural basis whereby such an exosite contributes towards the change in substrate specificity of the HPN active site consequent to SK binding has, however, remained essentially unknown so far. The data presented in this paper clearly implicates the 250-loop of the $\beta$ domain as an important determinant of the macromolecular substrate specificity of the SK-HPG activator complex. The presence of two tandem lysine residues at the tip of the 250-loop suggests that interactions with the kringle domain/s in substrate HPG may be the operative mechanism behind this interaction. To understand the role of kringles in substrate affinity, the activities of nSK and the mutant were compared in the presence of varying concentrations of EACA, a lysine analogue that is well-known to inhibit HPG activation by SK as well as disrupt ternary complex formation through kringle-mediated mechanism (2, 13, 39). Kinetic studies to check the effect of varying concentrations of EACA on substrate HPG activation by nSK-HPN and SK$_{del254-262}$-HPN showed that the mutant exhibited a greater susceptibility to EACA-mediated inhibition of substrate HPG activation than nSK (Fig. 5A), in a concentration range that did not affect the activity of the two binary complexes or the activity of free HPN. It has been shown earlier (13, 39), using sandwich binding assay, that EACA completely abolishes the docking of substrate HPG onto SK-HPG binary complex formed on plastic surface. However, this does not allow the measurement
of the rates of binding and dissociation of substrate HPG with the high affinity binary complex. Experiments were carried out to see the effect of EACA on the ternary complexation of substrate HPG to nSK/SK$_{\text{del254-262}}$ pre-complexed with immobilized PG on Resonant Mirror cuvettes, as described earlier. With SK$_{\text{del254-262}}$-HPG binary complex, an IC$_{50}$ of 0.3 mM was obtained which is approximately 3-fold less than the IC$_{50}$ (1 mM) obtained with nSK-HPG binary complex (Fig. 5B). These data clearly support the conclusion that the binary complex of the mutant, with the 250-loop deleted, interacted with substrate in a manner that is more vulnerable, as compared to nSK-plasmin(ogen), to disruption with the lysine-binding site competitive ligand, EACA. However, it is worth noting that if all of the kringle mediated interactions between activator and substrate had been abolished by the deletion of the loop, the mutant should have been completely resistant to inhibition by EACA. The fact that the mutant is susceptible to lower concentrations of EACA suggests that other EACA sensitive kringle dependant interactions are still operative, but at least one of the critical interactions has been abolished by the selective deletion of the 250-loop.

If the observed difference in substrate affinities between nSK-HPG and SK$_{\text{del254-262}}$-HPG activator complexes is indeed kringle-mediated, it is reasonable to assume that the rates of activation of substrate µPG, which is devoid of all five HPG kringles, by nSK and SK$_{\text{del254-262}}$ should not substantially differ from each other. This, indeed, was found to be the case (Fig. 6). Microplasminogen is known to be a poor substrate for the preformed SK-PG activator complex (22). Interestingly, kinetic studies using µPG as the substrate showed no discernable difference between nSK and SK$_{\text{del254-262}}$ with respect to initial velocities of substrate µPG activation at sub-saturating concentrations (K$_{m}$ ~ 6 ± 2 µM), under conditions where the activator activities of the two complexes (nSK-HPN and SK$_{\text{del254-262}}$-HPN) showed a remarkable difference when the substrate (native HPG)
contained the kringle domains. These observations prove convincingly that interactions involving lysine binding site/s in the kringle domains are intimately involved in the mechanism of operation of the macromolecular substrate-specific exosite in the SK-plasmin(ogen) activator complex. To further establish that kringles are involved in activator-substrate interactions, ternary binding experiments on the Resonant Mirror where the binding of the substrates, µPG and kringles 1-5 of HPG, to the binary complex pre-formed between either nSK or the mutant with immobilized HPG were carried out (Table 4). Remarkably, in consonance with kinetic data, µPG showed the same affinities for both nSK and SK del254-262 complexed with HPG. When K1-5 was used as the docking ‘substrate’ during ternary complex formation, the nSK-HPG binary complex showed an affinity of 4.1 µM for K1-5, while that of SK del254-262-HPG complex was determined to be 12 µM, indicating approximately a 3-fold higher affinity for nSK (Table 4). Again, these results strongly argue that the 250-loop of the β domain of SK interacts with substrate HPG via the latter’s kringle domains.

Molecular modeling studies wherein the intermolecular surfaces between the β domain of SK and the isolated kringle 5 (which was used as a typical representative structure of the five HPG kringles, and does not necessarily indicate any preferred role in the substrate-activator complex interplay; however, see below) were explored for mutual complementarity (see Experimental Procedures for details) indicate that a kringle structure can indeed dock the 250-loop in a remarkably optimal fashion (Fig. 6). Although whether kringle 5 per se or any other kringle is involved in this interaction cannot be judged at this stage, during the course of revision of this manuscript, another report was published which demonstrates the involvement of kringle 5 in the 1:1 binding with the β domain of SK (33). Although speculative at this stage, this offers a tantalizing possibility that this kringle-mediated interaction at the levels of both binary and ternary
complex formation, operate through kringle 5. If this scenario is correct, kringle 5 might “switch” its binding specificity towards partner or substrate depending on the temporal stage in the catalytic cycle, a possibility that is strengthened by a previous observation (11) that a synthetic peptide encompassing the 250-loop exhibits bifunctional behaviour by competitively inhibiting both SK-HPG binding as well as HPG activation by preformed SK-HPN activator complex. On the other hand, the current evidence does not rule out that the different kringles in substrate and partner HPG are directed to different sites in SK. Whatever the exact mechanism, this study provides unmistakable evidence of the direct involvement of a discrete epitope in SK in substrate recognition and binding via the kringle/s of HPG. Undoubtedly, further studies are needed to identify the relative contributions of the kringle/s versus the catalytic domain of the substrate towards the latter’s recognition, docking and turnover in the “valley” formed by the activator complex. This would aid in a better understanding of this enigmatic interaction at the molecular level.

It needs to be realized, however, that the observation that even after the excision of the 250-loop, the HPG activation reaction still survived (albeit with increased $K_m$), indicates that this loop is not the sole determinant of the SK-HPN exosite property. Indeed, earlier studies had suggested that the $\alpha$ domain as well as the $\beta$ domain, together, contribute to the generation of HPG specificity in the activator complex (12, 16). The potential of the $\alpha$ domain to interact with substrate HPG is also evident from the SK-μPN crystal structure (8), although whether it does so by interacting with kringle/s of substrate is still unclear. It is also established from recent work that the $\beta$ domain contributes a major share towards SK’s affinity for partner HPG as well (12). However, the fact that mutagenesis of several residues in the $\beta$ domain that, without altering the overall folding of the protein or its affinity for partner HPG, resulted in appreciable diminution in the $k_{cat}$
for activator activity with little change in the $K_m$ for HPG per se (16), suggests that regions other than the 250-loop in this domain are also intimately involved in the modulation of the substrate specificity of the activator complex. We have observed\(^3\) that mutations of residues immediately flanking the 250-loop (e.g., SK\textsubscript{Y252A, E263G}) lead to further increase in the molecule’s $K_m$ for substrate HPG (to about 15-fold that of nSK) with only minimal alteration in the $k_{cat}$ of its complex with HPN.

It is quite intriguing that the $\beta$ domain provides a major portion of the intermolecular affinity of SK for HPG necessary for the formation of the tightly held equimolar activator complex (15), and at least some of the affinity required for the (transient) interaction of the latter with substrate HPG, as the present study indicates. This arouses curiosity whether this domain would, by itself, possess HPG activator activity, however compromised at a quantitative level, since two seemingly fundamental requirements for a single-domain HPG activator protein viz. ability to bind with partner plasmin(ogen) and to then interact with substrate, are present in this domain. This question assumes significance because staphylokinase (SAK), a single-domain bacterial HPG activator, is also known to work as a ‘protein co-factor’ in a fashion akin to that of SK (6), and has both these distinctive HPG interacting properties. The ternary structure of $\mu$PN-SAK-$\mu$PN suggests that SAK appears not to modify the active site conformation of the enzyme, but creates new exosites that indirectly alter the substrate specificity of $\mu$PN (40). Identification of such functional ‘hotspots’ in PG activators in general, and SK in particular, that help in exosite mediated modulation of substrate specificity may greatly aid the future de novo design of improved HPG activators.

**Acknowledgements**- The authors thank Dr. Amit Ghosh for the facilities provided and support, and Mrs. Paramjit Kaur for expert technical assistance. We express our gratitude...
to Mr. K. Rajagopal for providing the plasmid construct for the expression of $\beta_{\text{wild-type}}$ domain. Automated DNA sequencing was carried out with the unstinted help of Drs. Jagmohan Singh and K. Ganesan. We express our gratitude to Dr. Rajendra P. Roy, National Institute of Immunology, New Delhi for the use of CD facilities.
REFERENCES

1. ISIS-3. (1992) The Lancet 339, 753-781
2. Castellino, F. J. (1981) Chem. Rev. 81, 431-446
3. McClintock, D. K., and Bell, P. H. (1971) Biochem. Biophys. Res. Commun. 43, 694-702
4. Markus, G., and Werkheiser, W. C. (1964) J. Biol. Chem. 239, 2637-2643
5. Parrado, J., Conejero-Lara, F., Smith, R. A. G., Marshall, J. M., Ponting, C. P., and Dobson, C. M. (1996) Protein Sci. 5, 693-704
6. Esmon, C. T., and Mather, T. (1998) Nat. Struct. Biol. 5, 933-937
7. Conejero-Lara, F., Parrado, J., Azuaga, A. I., Smith, R. A. G., Ponting, C. P., and Dobson, C. M. (1996) Protein Sci. 5, 2583-2591
8. Wang, X., Lin, X., Loy, J.A., Tang, J., and Zhang, X. C. (1998) Science 281, 1662-1665
9. Parry, M. A., Zhang, X. C., and Bode, W. (2000) Trends Biochem. Sci. 25, 53-59
10. Boxrud, P. D., Fay, W. P., and Bock, P. E. (2000) J. Biol. Chem. 275, 14579-14589
11. Nihalani, D., Raghava, G. P. S., and Sahni, G. (1997) Protein Sci. 6, 1284-1292
12. Nihalani, D., Kumar, R., Rajagopal, K., and Sahni, G. (1998) Protein Sci. 7, 637-648
13. Lin, L-F., Houno, A., and Reed, G. L. (2000) Biochemistry 39, 4740-4745
14. Dawson, K. M., Marshall, J. M., Raper, R. H., Gilbert, R. J., and Ponting, C. P. (1994) *Biochemistry* **33**, 12042-12047
15. Conejero-Lara, F. C., Parrado, J., Azuaga, A. I., Dobson, C. M., and Ponting, C. P. (1998) *Protein Sci.* **7**, 2190-2199
16. Chaudhary, A., Vasudha, S., Rajagopal, K., Komath, S. S., Garg, N., Yadav, M., Mande, S. C., and Sahni, G. (1999) *Protein Sci.* **8**, 2791-2805
17. Lin, L.-F., Oeun, S., Houng, A., and Reed, G. L. (1996) *Biochemistry* **35**, 16879-16885
18. Wang, X., Tang, J., Hunter, B., and Zhang, X. C. (1999) *FEBS Lett.* **459**, 85-89
19. Deutsh, D., G., and Mertz, E., T. (1970) *Science* **170**, 1095-1096
20. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77**, 51-59
21. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60-89
22. Shi, G.-Y., and Wu, H.-L. (1988) *J. Biol. Chem.* **263**, 17071-17075
23. Wu, H.-L., Chang, B.-I., Wu, D.-H., Chang, L.-C., Gong, G.-C., Lou,, K.-L., and Shi, G.-Y. (1990) *J. Biol. Chem.* **265**, 19658-19664
24. Wohl, R. C., Summaria, L., and Robbins, K. C. (1980) *J. Biol. Chem.* **255**, 2005-2013
25. Chase, T., Jr., and Shaw, E. (1969) *Biochemistry* **8**, 2212-2224
26. Wohl, R. C., Arzadon, L., Summaria, L., and Robbins, K. C. (1977) *J. Biol. Chem.* **252**, 1141-1147
27. Cush, R., Cronin, J. M., Stewart, W. J., Maule, C. H., Molloy, J., and Goddard, N. J. (1993) *Biosensors Bioelectronics* **8**, 347-354
28. Buckle, P. E., Davies, R. J., Kinning, T., Yeung, D., Edwards, P. R.,
Pollard-Knight, D., and Lowe, C. R. (1993) Biosensors Bioelectronics 8,
355-363
29. Myszka, D. G. (1997) Curr. Opin. Biotech. 8, 50-57
30. Morton, T. A., Myszka, D. G., and Chaiken, I. M. (1995) Anal. Biochem.
227, 176-185
31. Radek, J. T., and Castellino, F. J. (1989) J. Biol. Chem. 264, 9915-9922
32. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins 11, 281-296
33. Loy, J. A., Lin, X., Schenone, M., Castellino, F. J., Zhang, X. C., and
Tang, J. (2001) Biochemistry 40, 14686-14695
34. Wang, S., Reed, G. L., and Hedstrom, L. (1999) Biochemistry 38, 5232-
5240
35. Boxrud, P. D., Verhamme, I. M. A., Fay, W. P., and Bock, P. E. (2001) J.
Biol. Chem. 276, 26084-26089
36. Hirel, P. H., Schmitter, J-M., Dessen, P., Fayat, G., and Blanquet, S.
(1989) Proc. Natl. Acad. Sci. USA 86, 8247-8251
37. Robbins, K. C., Summaria, L., and Wohl, R. C. (1981) Methods Enzymol.
80, 379-387
38. Wohl, R. C. (1984) Biochemistry 23, 3799-3804
39. Young, K-C., Shi, G-Y., Wu, D-H., Chang, L-C., Chang, B-I., Ou, C-P.,
and Wu, H-L. (1998) J. Biol. Chem. 273, 3110-3116
40. Parry, M. A. A., Fernandez-Catala, C., Bergner, A., Huber, R., Hopfner,
K-P., Schlott, B., Gührs, K-H., and Bode, W. (1998) Nature 5, 917-923
FOOTNOTES

* This work was supported by grants from the Department of Biotechnology (D.B.T.) and the Council of Scientific and Industrial Research (C.S.I.R), Government of India.

# Both authors have contributed equally in this work.

1 Abbreviations:

SK, streptokinase

PG, plasminogen, irrespective of source

HPG, human plasminogen

HPN, human plasmin

µPN, microplasmin

µPG, microplasminogen

K1-5, kringles 1-5 of plasminogen

NPGB, p-nitrophenyl p-guanidinobenzoate

IPTG, isopropyl-1-thio-β-D-galactopyranoside

CD, circular dichroism

SOE, splicing-overlap-extension

PCR, polymerase chain reaction

PBS, phosphate buffered saline

BSA, bovine serum albumin

SDS, sodium dodecyl sulphate

EACA, epsilon-amino caproic acid

Chromozym® PL, tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate

2 Vasudha Sundram, K. Rajagopal, A. Chaudhary, S. S. Komath and G. Sahni, unpublished observations.
3 J. Dhar and G. Sahni, unpublished observations.
FIGURE LEGENDS

Figure 1. Superposition of α and β domains of streptokinase.

The β domain is shown in green and the α domain in red. The 250-loop is a distinct feature of the β domain, while the rest of the structure between the two domains overlaps extensively. The superposition with the γ domain of SK showed similar results (not shown). The residues 253 and 263, where the loop was truncated, are indicated.

Figure 2. Optical biosensor determination of binding constants for the interaction of nSK(SKdel254-262) with immobilized HPG.

Overlay plots representing the binding and dissociation of nSK to the immobilized HPG are depicted. Human PG was biotinylated and immobilized on streptavidin surfaces of IAsys cuvettes, as described under Experimental Procedures. For each individual concentration of ligate (only data for nSK is shown), the association or binding to immobilized HPG was monitored. Subsequently, the cuvette was washed with binding buffer and the dissociation phase was monitored (see Experimental Procedures for details). For the sake of clarity, only dissociation at saturating ligate concentration is shown. The value of $k_{on}$ for the binding curves for each ligate concentration was determined using FASTfit™ program and each value was plotted against the corresponding concentration of the ligate. Inset: The plot of $k_{on}$ against ligate concentration for nSK (filled squares) or SKdel254-262 (open circles), which gives a straight line. The $k_a$ values were obtained from the slope of the straight line and $k_d$ values were calculated from the average of four dissociation curves obtained at saturating ligate concentrations, as described under Experimental Procedures.
Figure 3. Time-course of the generation of amidolytic activity of nSK and SK_{del254-262}.

Equimolar nSK-HPG and SK_{del254-262}-HPG complexes were made and aliquots were withdrawn at regular periods and transferred into a micro-cuvette containing 2 mM chromogenic substrate. The generation of amidolytic activity was monitored at 405 nm at 22°C, as described under Experimental Procedures. The percentage of maximum amidolytic activity as a function of pre-incubation time by nSK-HPG complex (filled squares) and SK_{del254-262}-HPG complex (filled circles) are plotted. **Inset:** Active site titration of HPG on complexing with nSK or SK_{del254-262} using the active site acylating agent, NPGB. The figure shows progress curves of NPGB hydrolysis by nSK-HPG (filled squares); SK_{del254-262}-HPG (filled inverted triangles) and control reaction (filled circles).

Figure 4. Tracings from the IAsys™ Resonant Mirror based system to quantitate the interactions between substrate HPG and the equimolar binary complex of nSK and immobilized HPG.

The experiment was carried out at 25°C in binding buffer as described under Experimental Procedures. Human PG was biotinylated and immobilized on streptavidin captured onto the biotin cuvette. A stable binary complex was formed by adding saturating concentration of nSK onto immobilized HPG. After washing with binding buffer (point of addition of binding buffer as depicted by a), a stable dissociation baseline (b) was obtained due to high affinity and stability of the SK-HPG binary complex, which remained unaffected even after washing with 2.5 mM EACA (data not shown). Thereafter, varying concentrations of substrate HPG (0.1-1 μM) were then added (point of addition of substrate as depicted by (c) to monitor the association phase (6 min) and subsequently, the cuvette was
washed with binding buffer thrice (point of addition as depicted by d), and the
dissociation phase was recorded for the next 6 min. After each cycle of analysis,
the undissociated substrate HPG was stripped off with 2.5 mM EACA (point of
addition of EACA as shown by e), followed by re-equilibrating the cuvette with
binding buffer (f) which re-established the original baseline (g). The immobilized
streptavidin alone was taken as the negative control and it was subjected to the
same kind of treatments as given to the test cell containing immobilized HPG. No
significant non-specific binding was observed (data not shown).

Figure 5. Differential susceptibility to EACA of the substrate with binary
complexes of nSK and SK<sub>del254-262</sub>-HPG.

A. Differential susceptibility to EACA of substrate activation by nSK and
SK<sub>del254-262</sub>. The effect of different concentrations of EACA (0-1 mM) on the
substrate HPG activation by nSK (filled squares) and SK<sub>del254-262</sub> (filled circles)
was examined. Controls containing 10 nM each of SK and HPN (filled triangles),
or HPN alone (filled inverted triangles) are also shown. The HPG activator
activity of constant, catalytic amounts of the pre-formed equimolar activator
complexes between HPN and either nSK or SK<sub>del254-262</sub> to obtain final
concentrations of 0.25 nM and 3 nM, respectively in presence of varying
concentrations of EACA, along with substrate HPG and chromogenic substrate in
50 mM Tris-Cl buffer, pH 7.5, was monitored at 405 nm at 22°C. The initial
velocities in different concentrations of EACA are expressed relative to the
controls not containing any EACA (taken as 100 %). Similar differential effects
between nSK and the mutant were observed at HPG concentrations of 0.5 µM, 2
µM and 4 µM, though the IC<sub>50</sub> values were different (data not shown). B. Effect
of EACA on the physical binding of substrate HPG to nSK-HPG or SK<sub>del254-262</sub>-
HPG binary complex examined by Resonant Mirror technique. Binding of substrate HPG (0.4 µM) to either nSK-HPG (filled triangles) or SK_{del254-262}-HPG (filled circles) binary complex was measured at various concentrations of EACA (0-2 mM) using IAsys™ system, as described under Experimental Procedures. The maximal extent of binding under varying concentrations of EACA was quantitated and the maximal binding in buffer alone was used to normalize the extent of binding under different experimental conditions. The extent of substrate HPG binding is plotted as a function of EACA concentration. The effect of respective EACA concentrations on the binary nSK/SK_{del254-262}-HPG complex has been represented by filled diamonds (control).

**Figure 6. Abolishment of the differential substrate activation phenomenon by nSK and SK_{del254-262} by using substrate µPG.**

Fixed, catalytic amounts of the respective pre-formed activator complexes of each protein with HPN were added to the cuvette containing sub-saturating concentrations of HPG or µPG as the substrate and Chromozym® PL in 50 mM Tris-Cl buffer, pH 7.5, and the reactions were monitored at 405 nm at 22°C. The figure shows progress curves of activation of (A) HPG by nSK (filled diamonds) and SK_{del254-262} (filled triangles) and of (B) µPG by nSK (filled circles) and SK_{del254-262} (filled squares).

**Figure 7. Docking of kringle 5 of HPG with the β domain of SK.**

Connolly surfaces of the β domain and kringle 5 of HPG were calculated by using the GRASP program. Kringle 5 has been used here as a typical representative structure of the five HPG kringles. In order to generate the molecular surface, the radius of a water molecule was considered to be 1.4 Å. Electrostatic potentials were mapped onto the surfaces with GRASP. The β domain showed a distinct
patch of positive charges in the 250-loop. Similarly, the kringle domain also showed a distinct patch of negative charges. The two patches were brought in close proximity by manual docking, which showed close surface and electrostatic complementarities (see Experimental Procedures for details).
Dhar et al.

Table 1. Association and dissociation rate constants and apparent equilibrium dissociation constants for the binding of immobilized HPG to the derivatives of SK

| Ligate                  | $k_a$ (x 10$^6$) | $k_d$ (x 10$^{-3}$) | $K_D$ (x 10$^{-9}$) |
|-------------------------|------------------|---------------------|--------------------|
|                         | (M$^{-1}$ s$^{-1}$) | (s$^{-1}$)          | (M)                |
| nSK                     | 1.98 ± 0.08      | 1.81 ± 0.21         | 0.91 ± 0.34        |
| SK$_{del254-262}$       | 1.43 ± 0.07      | 2.27 ± 0.15         | 1.58 ± 0.79        |

$^a$ Biotinylated HPG was immobilized on streptavidin captured onto biotin cuvette. Different concentrations of the nSK/SK$_{del254-262}$ were then titrated as outlined under the Experimental Procedures. The pseudo-first order rate constant ($k_{on}$) was determined using the FASTfit$^\text{TM}$ program. The concentration dependence of $k_{on}$ was fitted using linear regression to find the association rate constant ($k_a$) from the slope of linear fit. The dissociation rate constant ($k_d$) value was calculated from the average of four dissociation curves obtained at saturating concentration of ligate. The $K_D$ was estimated from the ratio of kinetic constants as $k_d/k_a$. 

40
Table 2. Steady-state kinetic parameters for amidase activity of equimolar complexes of HPN and nSK/\textit{SK}_{del254-262}\textsuperscript{a}

| Protein            | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$/mM) |
|--------------------|------------|------------------------|-------------------------------|
| HPN                | 0.17 ± 0.03| 310 ± 18               | 1823.53                       |
| nSK-HPN            | 0.6 ± 0.02 | 370 ± 15               | 616.67                        |
| \textit{SK}_{del254-262} -HPN | 0.2 ± 0.07 | 320 ± 14               | 1600                          |

\textsuperscript{a}For the determination of the amidolytic parameters, nSK/\textit{SK}_{del254-262} and HPN were pre-complexed in equimolar ratio and an aliquot of this mixture was assayed for amidolysis at varying concentrations of Chromozym\textsuperscript{®} PL as detailed under Experimental Procedures. The data represent the mean of three independent determinations.
Table 3. Steady-state kinetic parameters for HPG activation by equimolar complexes of HPN and nSK/SK_{del254-262} \(^a\)

| Activator protein | \(K_m\) (\(\mu\)M) | \(k_{cat}\) (min\(^{-1}\)) | \(k_{cat}/K_m\) (min\(^{-1}\)/\(\mu\)M) |
|------------------|----------------|----------------|----------------|
| nSK-HPN          | 0.5 ± 0.05     | 11 ± 0.5       | 22.0           |
| SK_{del254-262} -HPN | 2.5 ± 0.3     | 9.7 ± 0.52     | 3.9            |

\(^a\)The kinetic parameters for substrate HPG activation were determined at 22°C as described under Experimental Procedures. The data represent the mean of three independent determinations. Closely similar values were obtained for the direct activation of substrate HPG by SK_{del254-262} (data not shown).
Table 4. Association and dissociation rate constants and apparent equilibrium dissociation constants for the interaction of substrate HPG, µPG and K1-5 with nSK/SK_{del254-262} complexed with immobilized HPG.

| [Ligand] | [Ligate] | $k_a$ (x 10^5) (M^{-1}s^{-1}) | $k_d$ (x 10^{-1}) (s^{-1}) | $K_D$ (x 10^{-6}) (M) |
|----------|----------|-------------------------------|----------------------------|---------------------|
| nSK-HPG  | HPG      | 10.70 ± 1.30                  | 1.76 ± 0.23                | 0.16 ± 0.04         |
| -do-     | µPG      | 0.75 ± 0.02                   | 1.29 ± 0.74                | 1.72 ± 0.91         |
| -do-     | K1-5     | 0.29 ± 0.06                   | 1.19 ± 0.17                | 4.11 ± 0.62         |
| SK_{del254-262} –HPG | HPG      | 4.62 ± 0.51                   | 3.35 ± 0.13                | 0.72 ± 0.09         |
| -do-     | µPG      | 0.90 ± 0.03                   | 1.33 ± 0.12                | 1.48 ± 0.20         |
| -do-     | K1-5     | 0.28 ± 0.12                   | 3.41 ± 0.21                | 12.02 ± 2.08        |

Kinetic constants for the interactions of substrate HPG, µPG and K1-5 with nSK-HPG or SK_{del254-262} -HPG binary complex were determined by applying the FASTfit™ program to the binding data obtained using IAsys biosensor, as described under Experimental Procedures. A stable binary complex between nSK/SK_{del254-262} and HPG immobilized onto the cuvette was made and then the binding of varying concentrations of either substrate HPG (0.1-1 µM), µPG (1-6 µM) or K1-5 (1-6 µM) was monitored, as described in the legend of Fig. 5.
Involvement of a nine-residue loop in the generation of the macromolecular substrate-specificity by the activator complex through interaction with substrate kringle domains

Jayeeta Dhar, Abhay H. Pande, Vasudha Sundram, Jagpreet S. Nanda, Shekhar C. Mande and Girish Sahni

J. Biol. Chem. published online January 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M108422200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts