Multi-site FRET studies reveal a substrate kringle binding "exosite" on the 250-loop of the β-domain of Streptokinase (SK) that bestows increased binding affinity and substrate specificity to the SK-Plasmin (SK.PN) activator complex for the macromolecular substrate.
Summary

Streptokinase (SK) acts as a protein cofactor of human plasmin (a non-specific protease) and modulates the latter’s substrate specificity for the cleavage of the single scissile peptide bond (Arg 561-Val 562) in the macromolecular substrate, human plasminogen (HPG). This well-recognized “switch” in substrate preference consequent to binding of SK to human plasmin (HPN) is believed to be due to conferment of high affinity for substrate plasminogen onto the PN by the complexed SK. The increase in the macromolecular substrate specificity of the HPN by SK is currently thought to be due to new “exosites” generated on the SK-HPN complex. Previous kinetics and fluorescent studies on SK-mediated PG activation indicate that the interactions with extended macromolecular recognition sites (exosites) rather than the active site of HPN are the principal determinants of binding affinity for the substrate. Our earlier solution studies indicated that the 250-loop of the beta domain of SK can potentially promote the initial low affinity binding to more avid binding (Dhar et al., 2002) likely due to interaction with kringle domains. However, the nature of this interaction and the exact role and identity of participating kringle(s) remained unelucidated. In the present study, we have attempted to address this issue through a multi-site FRET based approach utilizing the measurements of various prospective enzyme-substrate interactions by placing a site-specific donor probe (IAEDANS) in the 250-loop, and the multiple-acceptor fluorophore (Fluorescein 5-maleimide) in different loci of the substrate miniPG (PG derivative containing kringle 5 and catalytic domain). The leucine at 260th position in the SK 250-loop was substituted with cysteine to generate a single-cysteine variant of SK (SK L260C). For site-specific fluorophore conjugation in miniPG, additional cysteine were substituted for residues contributing the lysine binding sites (N53C and L71C) of kringle 5, as also three other distinct locations in the catalytic domain of the substrate, namely the 37-loop (F583C), the Ca-binding loop (N625C) and the autolysis loop (G690C). Stable ternary complex of SK(cofactor).HPN(enzyme).miniPG(substrate) was made possible by covalent modification of the active site of HPN with the highly specific peptide inhibitor (Glu-Gly-Arg-Chloromethyl ketone) which inhibited turnover of the substrate. The steady-state FRET between one donor location situated in the SK 250-loop and five different acceptor sites in the individual miniPG derivatives, used as substrates, was then measured to obtain five distinct intermolecular distances. Among all, the least spatial separation was observed with the Kringle 5 derivatives, namely N53C (27 Å) and L71C (29 Å). According to FRET-measured distances autolysis loop was found to be located at the farthest (44 Å) from the 250-loop, while the 37-loop (32 Å) and Ca-binding loop (33 Å) derivatives were at intermediate distances between activator enzyme and the bound substrate. The
distance geometry obtained through these FRET measurements were then utilized to select a most suitable computer modeled structure wherein substrate miniplasminogen (miniPG) was optimally docked onto the X-ray diffraction structure of the SK.µPN enzyme complex, whose X-ray coordinates (PDB ID 1BML) are available, albeit without docked substrate. The comparison of the chosen ternary interaction model on the basis of experimentally determined distances from FRET experiments suggested proximal placing of Kringle 5 to the 250-loop of beta domain of SK. This provides the first physical evidence that it is indeed Kringle 5 that specifically interacts with the 250-loop of the SK β domain.
3.1 Introduction

Streptokinase (SK), a bacterial protein secreted by several species of β-hemolytic streptococci, is widely used as thrombolytic agent for treatment of diverse circulatory disorders, including myocardial infarction (ISIS-3, 1992). SK has ability to activate human plasminogen (HPG), a 92 KDa protein of blood coagulation system. However, unlike other physiological activators such as urokinase (UK) and tissue plasminogen activator (tPA), which are proteases, SK is catalytically inert. 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 561-Val 562 peptide bond (McClintock and Bell, 1971; Castellino, 1981). 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 (Markus and Werkheiser, 1964; Castellino, 1981; Boxrud et al., 2004; Boxrud and Bock, 2004). This activated complex further combines with free HPG to activate the latter into HPN. The SK.HPG activator complex then rapidly exchanges conformationally activated HPG with HPN (the latter having three -3 orders higher affinity to SK) to form a SK.HPN complex and develops a HPG activator activity (Boxrud et al., 2004). The SK.HPN enzymatic complex then rapidly converts the free HPG to HPN. An understanding of the structural processes involved in, first, the exposure of the active site in the “virgin” SK-HPG complex, followed by its transformation to a highly specific protease either “activated” SK.HPG, or SK.HPN that, unlike free HPN, displays a very high substrate preference for HPG (Markus & Werkheiser, 1964), is crucial to the design of improved SK-based thrombolytic agents (Marder, 1993).

SK has been shown to be composed of three structurally similar domains (termed α, β, and γ), separated by random coils and small, flexible regions at the amino and carboxyl termini (Parrado et al., 1996; Conejero-Lara et al., 1996; Wang et al., 1998). The
X-ray diffraction structure of the catalytic domain of HPN (μPN) 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 it appears that SK along with partner μPN provides a template onto which substrate molecule can dock with optimal presentation of activation loop (Arg 561-Val 562) at the active centre (Wang et al., 1998). However, the identity of protein-protein interactions cardinal to formation of the enzyme-substrate intermediate(s) remains mystery so far.

It is currently thought that well recognized "switch" in substrate preference (McClintock and Bell, 1971) after binding of SK to HPN is facilitated due to generation of "exosites" on the SK.HPN complex (Boxrud et al., 2000). Peptide walking studies in our laboratory have also indicated that short peptides based on the primary structure of SK, particularly those derived from selected regions in the α- and β-domains, displayed competitive inhibition for HPG activation by the preformed SK.HPN complex under conditions where the 1:1 complexation of SK and HPN was essentially unaffected (Nihalani et al., 1997; 1998). However, the binary nature of SK,μPN crystal structure (i.e. absence of a juxtaposed substrate molecule) limits our understanding about the potential regions that could be involved in interaction with the macromolecular substrate. Thus, despite a detailed and high resolution exposition of the overall nature of protein-protein interactions in the SK,μPN binary complex, there exists little information, how the discrete structural epitopes of SK in SK,μPN binary complex communicate with oncoming substrate. However, according to crystal structure, among all the three domains of SK, β-domain has the least contact (950 Å² out of total 4100 Å² contact area with SK) with the partner μPN and most of the regions of this domain have high thermal factors as well. This strongly favors the belief that β-domain is key among all the three
domains of SK which could have maximum contact with substrate. Evidences in this regard were also obtained when we examined a relatively small locus in the β-domain (residues in and around 230–290), termed the “core” region through “Peptide walking” approach and implicated its role in the interaction with substrate HPG (Nihalani et al., 1997). We further explored this locus through selective deletion of a surface exposed loop (residues 254–262; 250-loop) that resulted in drastic reduction of substrate HPG activation ability (Dhar et al., 2002). However, deletion of this loop did not alter its capability to activate microplasminogen (μPG, catalytic protease domain of PG devoid of all kringles) thus, behaving native like SK for kringle-less substrate derivatives. In addition to this, 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) when used to explore for mutual complementarity of binding surfaces, indicated that a kringle structure can indeed dock the 250-loop in a remarkably optimal fashion (Dhar et al., 2002). These observations provided convincing evidences that the interactions involving kringle domains are intimately involved in the mechanism of operation of the macromolecular substrate-specific exosite in the SK-plasmin(ogen) activator complex. However, it was still unclear whether kringle 5 or any other kringle of the substrate HPG specifically interacted with the 250-loop. Moreover, unavailability of a decisive picture of enzyme-substrate interaction limits our understanding of how the extreme covalent specificity is generated, once SK combines with HPG or HPN. Any characterization of such interactions requires a stabilized ternary complex which may allow us to map the way substrate docks on the enzyme and engage in exosite-mediated binding with SK.HPN complex. In this study, we aimed to establish that the origin of inordinately high substrate processivity does not only depend on selected nodal interactions that are localized around the scissile peptide bond of the incoming substrate, but utilize a global enzyme substrate contact for recognition and binding in which distantly placed kringles have participation as well.
The present study describes the design of multisite fluorescence resonance energy transfer (FRET) system to map intermolecular interactions between SK and the substrate HPG under a condition where enzyme-substrate are stabilized in a ternary state without actual turn-over of the substrate. This was achieved by deactivation of enzyme's active site using highly specific peptide inhibitor (Glu-Gly-Arg-Chloromethyl ketone). Attenuated HPN took part in formation of binary activator complex with SK, and this was subsequently complexed with substrate miniplasminogen (miniPG) to derive a stable cofactor (SK).enzyme (HPN).substrate (miniPG) ternary complex. Steady-state FRET enabled us to physically map distances of different loci present in both serine protease domain and kringle 5 of substrate from a single reference point present in SK β-domain (250-loop). For site-specific fluorophore conjugation, cysteine variants of both SK and miniPG were generated. Single cysteine variant, where 260th leucine of SK 250-loop is substituted with cysteine was derivatized with IAEDANS. Generation of free cysteine variants of miniPG was possible with a highly efficient in house developed in vitro refolding strategy for cysteine rich proteins (Please refer section 2.2.7 for details). Disulfide bridging of all 18 natural cysteines present in miniPG under natively refolded condition allows incorporation of one more cysteine that remains free for covalent modification with sulfhydryl reactive reagents. Altogether, we selected five different regions, one each in 37-loop, Ca-binding loop, autolysis loop of serine protease domain and two in Lysine binding sites (LBS) of kringle 5 for site-specific conjugation of acceptor fluorophore, fluorescein 5-maleimide. The IAEDANS placed in SK 250-loop acted as donor fluorophore to transfer energy to the acceptor fluorophore present in chosen locations of substrate miniPG. The distance geometry obtained through FRET measurements were used to select a most suitable computer modeled ternary structure wherein substrate miniPG was docked onto X-ray diffraction structure of SK.μPN enzyme complex. The chosen ternary interaction model that satisfied the experimentally determined distances was used to gain insight about of the substrate miniPG binding to the SK.μPN enzymatic

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The selected ternary interaction model generated by computer modeling showed an optimal placing of the substrate scissile-peptide bond for proteolytic cleavage by partner μPN complexed with SK. The experimentally measured distances further suggested proximal placing of kringle 5 to the 250-loop of β-domain of SK thus, providing first physical evidence that it is indeed kringle 5 that specifically interacts with the 250-loop of the SK β-domain. Our finding established that at least one of the determinants of binding affinity in SK.HPN enzyme and miniPG substrate complex is principally governed by “exosite” provided by the SK 250-loop for substrate kringle 5 interactions.

3.2 Experimental Procedures

3.2.1 Reagents

The RNA polymerase promoter-based expression vector, pET-23d) and Escherichia coli strain BL21(DE3) were products of Novagen Inc. (Madison, WI). Thermostable DNA polymerase (Pfu) and the QuikChange™ Site-Directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA). Restriction endonucleases, T4 DNA ligase, and other DNA-modifying enzymes were acquired from New England Biolabs (Beverly, MA). Oligonucleotide primers were supplied by Biobasic, Inc., Canada. Purifications of DNA and extraction of PCR amplified products from agarose gels were performed using kits available from Qiagen GmbH (Germany). Urokinase, N-Acetyl L-tryptophanamide and dithio-bis-2-nitobenzoate (DTNB) were purchased from Sigma Chemical Co., St. Louis, USA. The chromogenic substrate, Chromozyme®PL (tosyl-Gly-Pro-Lys-p-nitroanilide) used for HPG activation assays was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Phenyl Agarose 6XL, DEAE Sepharose (Fast Flow) and SP Sepharose (Fast Flow) were procured from GE-Amersham Biosciences. 5-(((2-Iodoacetyl)amino)ethyl) aminonaphthalene-1-sulfonic acid (IAEDANS) and Fluorescein 5-maleimide (F-150) were purchased from Molecular Probes (Invitrogen, USA). Peptide inhibitor (Glu Gly Arg-Chloromethyl ketone) was procured from Calbiochem (San Diego, USA).
USA). Glu-plasminogen was either purchased from Roche Diagnostics Inc. or purified from human plasma by affinity chromatography (Deutsch and Mertz, 1970). All other reagents were of the highest analytical grade available. Automated DNA sequencing using fluorescent dyes was done on Applied Biosystems 3130xI Genetic Analyzer 16 capillary DNA sequencer. The N-terminal amino acid sequencing was done with Applied Biosystems sequencer, Model 491A.

3.2.2 Genetic constructs

3.2.2.1 Molecular Cloning of native streptokinase (nSK) and preparation of SK L260C

The SK gene from *Streptococcus equisimilis* H46A in pBR 322 (Pratap et al., 1996), was subcloned into pET-23d (See Fig. 3.1), an expression vector containing a highly efficient ribosome binding site from the phage T7 major capsid protein (Studier and Moffatt, 1986) and described in details in Nihalani et al., 1998. There are no natural cysteines present in SK polypeptide sequence. Hence, single cysteine variant of SK where cysteine is substituted for leucine (See Fig. 3.2) at 260th amino-acid position (flank of 250-loop of β-domain) was created using QuikChange® Mutagenesis kit and involved usage of two complementary primers having the desired mutation (Please refer to Appendix 1 for details of primers). The primers were extended during temperature cycling by the PfuTurbo DNA polymerase. This enzyme replicates both plasmid strands with high fidelity and does not displace the mutant oligonucleotide primers. This generates PCR product having both the mutated plasmid having staggered nicks and the parental plasmid. The template strand/ parental plasmid was digested by the DpnI enzyme that cleaves specifically the methylated and hemi-methylated DNA and the newly synthesized mutated nicked plasmid was then transformed in XL1-Blue chemical competent cells (prepared by method described by Nishimura et al., 1990) and positive clones with desired mutation were confirmed by DNA sequencing.
Fig. 3.1 Circular map of pET-23d-SK. The circular map highlights a few selected, unique RE sites on the pET-23d vector, a T7-RNA polymerase promoter-based expression vector (Studier et al., 1990) and the incorporated gene encoding for SK that was used for the construction and expression of mutants of full-length SK.

Fig. 3.2 Crystal structure of SK showing site of cysteine substitution. The three different domains of SK viz. α (red), β (cyan) and γ (blue) are shown. Cysteine was substituted for the Leu 260 present in the surface exposed 250-loop of the β-domain of SK. Side-chain of Leu 260 is shown in green. The 250-loop of the β-domain of SK is missing in SK μPN crystal structure (PDB ID, 1BML; Wang et al., 1998) hence; coordinates for this loop were taken from isolated β-domain (Wang et al., 1999b) structure where the 250-loop is intact. See “Experimental Procedures” for details.
3.2.2.2 Molecular cloning of miniPG and preparation of free cysteine containing variants

Details of molecular cloning of miniPG are described in detail in section 2.2.4. In brief, full length HPG cDNA in pCMV6 vector was custom synthesized from OriGene Technologies Inc, USA. Nucleotide sequence coding for a full length HPG was referred from NCBI. Overhang primers were used to fish out desired coding sequences of miniPG (Ala 440-Asn 791, HPG numbering) along with generation of unique enzymatic sites for cloning in pET-11a. PCR products were digested to generate unique Nde I/Hind III site and the PCR amplified fragment coding for miniPG was subcloned in pET-11a to generate pET-11a-miniPG.

Native miniPG polypeptide contains 18 cysteines that make 9 disulfide bridges under refolded conditions. Since none of the natural cysteines are free in the natively folded molecule, this provides an opportunity to incorporate an additional cysteine in the miniPG that could be possibly available free for fluorescent labeling, albeit under the "correct" redox state. We selected four different regions in miniPG for strategic placing of additional cysteine residues. Three of these cysteines were located in surface exposed loops of serine proteases domain namely, 37-loop, Ca-binding loop, autolysis loop and two domicile in the LBS (Please refer to section 1.5.1 chapter one for details of LBS) of kringle 5. Figure 3.3 represents the polypeptide sequence for recombinantly expressed miniPG. Residues substituted with cysteine are marked by an asterisk. Substitution in kringles and catalytic domain are mentioned with most common numbering system such as 1-80 for kringle 5 and 543-791 for catalytic domain. LBS is defined by His 33-Thr 37, Pro 54-Val 58, Pro61-Tyr64 and Leu 71-Tyr 74, which together form an elongated depression on the kringle surface approximately 9 Å wide and 12 Å long (Chang et al., 1998). To engender acceptor sites (Please refer to Table 3A and Fig. 3.4) in LBS, Asn 53 and Leu 71 were substituted with cysteine to generate miniPG N53C and miniPG L71C constructs. Phe 583 which is centrally placed to the 37-loop (See Fig. 3.4) was substituted
Fig. 3.3 Amino-acid sequence of miniPG (1-352) used for recombinant protein expression. Region 1-103-represents kringle 5 amino-acids, 104-352-represents catalytic domain (µPG). Sequence for different structural epitopes such as, kringle 5, 37-loop, Ca-binding loop and autolysis loop are underlined. Residues substituted with cysteines are marked with an asterisk and numbered according to the most commonly used convention existing in literature for kringle 5 and the catalytic domain.
TABLE 3A

Free cysteine containing variants of miniPG are listed along-with sites of cysteine substitution. Nomenclatures of variants are based on most common numbering system frequent in literature. Respective positions in the cloned polypeptide (See Fig. 3.3) are also shown.

<table>
<thead>
<tr>
<th>Variants</th>
<th>Respective position in cloned polypeptide (See Fig. 3.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>kringle 5 mutants (kringle numbering)</td>
<td>kringle 5</td>
</tr>
<tr>
<td>miniPG N53C (LBS)</td>
<td>N75C (LBS)</td>
</tr>
<tr>
<td>miniPG L71C (LBS)</td>
<td>L93C (LBS)</td>
</tr>
<tr>
<td>catalytic domain mutants (HPG numbering)</td>
<td>catalytic domain</td>
</tr>
<tr>
<td>miniPG F585C (37-loop)</td>
<td>F144C (37-loop)</td>
</tr>
<tr>
<td>miniPG N625C (Ca-binding loop)</td>
<td>N186C (Ca-binding loop)</td>
</tr>
<tr>
<td>miniPG G690C (autolysis loop)</td>
<td>G251C (autolysis loop)</td>
</tr>
</tbody>
</table>

a each kringle of HPG are individually numbered (1-80)
b Lysine binding sites present in kringle 5 of miniPG
c HPG numbering starts at Glu1 and ends at Asn 791 (Please refer Fig. 1.6 for details)
d 37-loop, Thr 581-Met 585 (HPG numbering)
e Ca-binding loop, Gly 619-Val 630 (HPG numbering)
f autolysis loop, Gln 689-Leu 697 (HPG numbering)

with cysteine to generate miniPG F583C free cysteine variant. Asn 625 and Gly 690 were substituted with cysteine in Ca-binding loop and autolysis loop respectively (See Fig. 3.4) to generate miniPG N625C and miniPG G690C free cysteine variants. Table 3A lists the free cysteine variants constructed in different epitopes and their corresponding amino-acid position in the cloned polypeptide (1-352).
Fig. 3.4 Modeled structure of the miniPG showing sites of cysteine substitutions. Structure of the miniPG was generated by using coordinates from microPG (PDB ID, 1QRZ) and isolated kringle 5 (PDB ID, 5HPG) crystal structures (See “Experimental Procedures” for details). Residues substituted for cysteines are shown in spheres (red). To engender acceptor sites in LBS of kringle 5 (shown in cyan), Asn 53 and Leu 71 were substituted with cysteine to generate miniPG N53C and miniPG L71C constructs. Phe 583 which is centrally placed to the 37-loop of the catalytic domain (shown in green) was substituted with cysteine to generate miniPG F583C free cysteine variant. Asn 625 and Gly 690 were substituted with cysteine in Ca-binding loop and autolysis loop respectively to generate miniPG N625C and miniPG G690C free cysteine containing variants.

3.2.4 Expression and Purification of nSK and SK L260C

The nSK protein and its cysteinyln mutant to be purified were grown from single colony, streaked on LB-Amp plate from their BL21(DE3) glycerol stocks. The primary culture was developed by inoculating pET-23d-SK or SK variant into 10 ml of LB medium containing 100 microgram/mL ampicillin (LB-Amp medium) and incubated for 16 h at 37 °C, under shaking conditions (220 rpm). This pre-inoculum was used to seed 500 ml of LB-
Amp medium at 5 % v/v and allowed to grow at 37 °C., at 220 rpm to an OD\textsubscript{600} nm (optical density measured at 600 nano-meter) of 0.5-0.6. At this stage, it was induced with IPTG (final concentration of 1.0 millimol) and further grown at 40 °C, for 6-8 h under shaking condition (Chaudhary et al., 1999; Dhar et al., 2002). Cells were then harvested by centrifugation at 6000 g for 10 min. The pellet was then washed twice with ice-cold buffer (final concentrations-100 mM NaCl, 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA) and subjected to sonication (Heat System, New York) at 4 °C, under conditions of 30 sec sonic-pulses interspersed with equal periods of rest. The cell lysate was then centrifuged at high rpm (14000 g) for 15 min. The SDS-PAGE analysis showed that more than 90 % desired protein had gone to the Inclusion Bodies (IBs). The IBs were then solubilised in 8 M urea at room temperature for 45 min under constant gentle shaking condition. The protein in supernatant was refolded after 10-fold dilution (Sundram et al., 2003) in the loading buffer (0.4 M NaCl in 20 mM PB). The sample was then chromatographed on Phenyl Agarose 6XL beads (Hydrophobic Interaction Chromatography, HIC) and protein was eluted in water. Protein fractions after HIC were pooled and Tris-Cl pH 7.5 was added to a final concentration of 20 mM Tris-Cl, after which it was loaded onto a column packed with DEAE-Sepharose (Fast Flow) pre-equilibrated with 20 mM Tris-Cl (pH 7.5). After washes with buffer containing 20 mM Tris-Cl (pH 7.5), the bound protein was eluted using a linear gradient of salt (0-0.5 M NaCl) in 25 mM Tris-Cl. SK proteins eluted were generally more than 95 % pure, as analyzed by SDS-PAGE.

3.2.5 Expression and Purification of miniPG and free cysteine variants of miniPG

Method of expression for miniPG and its muteins was essentially same as that of SK with minor variations that include, pre-induction and post induction temperatures set at 30 and 37 °C respectively. Additionally, IBs of miniPG were washed with extended incubation period in wash buffer (10 mM Tris-Cl pH 8.0, 5 mM EDTA, 2M Urea and 1 % Triton X-100) to remove the impurities. IBs solubilized in 8 M urea and 10 mM DTT were
subjected to in vitro refolding according to procedure detailed in section 2.3.2. In brief, the solubilized IBs were slowly diluted in refolding buffer (50 mM Tris-Cl pH 8.0, 5 mM EDTA, 1.6 M Urea, 20 % Glycerol, 1.25 mM GSH and 0.5 mM GSSG) and kept on stirring at 4 °C for 24 h. After 24 h, refolding mix was dialysed thoroughly for additional 24 h against a buffer containing 50 mM Tris-Cl pH 8.0, 5 mM EDTA and 0.4 M urea. Refolded miniPG was purified in one step by anion exchange chromatography on a SP-Sepharose® column (GE-Amersham Biosciences). Refolded protein was equilibrated to 20 mM sodium phosphate buffer pH 6.0, 5 mM EDTA and 0.4 M urea and loaded onto a column packed with SP-Sepharose matrix. After washes with equilibration buffer, the bound protein was eluted using a linear gradient of salt (0-0.5 M NaCl) in equilibration buffer. Protein purity was checked on SDS-PAGE and it was found to more than 95 % pure. The refolded and purified miniPG was successfully activated to its functional form miniPN by activation with UK or SK, hence, establishing the native refolded state of purified products.

3.2.6 Preparation of HPN and attenuated HPN

Method of HPN preparation was essentially same as described in section 2.2.9. In brief, catalytically active HPN was prepared by digesting Glu-HPG with UK covalently immobilized on agarose beads (Stults et al., 1989) 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 25 °C for 8 hrs. Inactivated HPN was prepared by reacting HPN with 20 molar excess of peptide inhibitor (Glu-Gly-Arg-Chloromethyl ketone) at 25 °C for an hour. Complete attenuation of HPN activity was monitored spectrophotometrically by assaying its ability to cleave small chromogenic substrate (tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate, Chromozyme®). Excess of inhibitor was removed by passing the samples through desalt column packed with Sephadex G 25 fine beads.
3.2.7 Site-Specific labeling of cysteine variants of SK and miniPG

Concentrated samples of purified SK L260C or miniPG mutants (200 µM) in a total volume of 1 ml or less were reacted with 10 molar excess of IAEDANS or Fluorescein 5-maleimide respectively under dark at room temperature. Components of reaction buffer included 50 mM Tris-Cl pH 7.5, 1 mM EDTA and 100 mM NaCl. Reaction was stopped by adding 1 µl of 1 M cysteine. Free dye was removed using desalt column packed with Sephadex G 25 fine beads. Desalted protein was aliquoted and stored at -70 °C. The stoichiometry of the labeling was determined by measuring absorption at 340 nm for IAEDANS and at 490 nm for Fluorescein 5-maleimide. An extinction coefficient of 6100 M⁻¹ cm⁻¹ for IAEDANS (Lakowicz, 1999) and 83000 M⁻¹ cm⁻¹ for Fluorescein 5-maleimide was used to calculate molarities of dye. Protein concentration was determined by measuring absorption at 280 nm and using an extinction coefficient of 44,762 M⁻¹ cm⁻¹ for SK and 67,821 M⁻¹ cm⁻¹ for miniPG (Loy et al., 2001).

3.2.8 Functional characterization of proteins

For 'cofactor' activity determination nSK/SK L260C/SK L260C' were premixed with the partner molecule having preformed active site i.e. with HPN, and catalytic amounts were withdrawn and added to a range of HPG concentration (0.1-2.0 µM) and 0.5 mM Chromozyme® PL in the assay buffer containing 50 mM Tris-Cl, pH 7.5. The change in absorbance at 405 nm was then measured as a function of time in a Versa-Max tunable microplate reader from Molecular Devices USA, set at 25 °C. The activator activities were obtained from the slopes of the progress curves, which were plotted as change in absorbance/time² (Wohl et al., 1980). Functional characterization of free cysteine containing variants of miniPG and their fluorescently labeled derivatives were essentially carried out as described in section 2.2.10. In brief, fixed concentrations of SK.HPN was added to assay cuvettes containing various amounts of miniPG derivatives (0.1 to 2 µM) and chromogenic substrate (0.5 mM) in presence of assay buffer (50 mM Tris-Cl, pH 7.4),
total volume of reaction being 100 μL. Change in absorbance was then monitored continuously on a spectrophotometer at 405 nm as a function of time at 25 °C. The kinetic parameters for midIPG, miniPG, and μPG activation were then calculated from inverse Lineweaver-Burk plots (Wohl et al., 1980).

3.2.9 Fluorescence spectroscopy and steady-state FRET measurements

Fluorescence measurements were performed on a Perkin-Elmer LS 50B spectrofluorimeter in a 1-cm cuvette. Samples were excited at 340 nm with an excitation slit of 5 nm and emission spectra were collected from 400 to 600 nm with the emission slit-width set at 6 nm. Steady-state energy transfer measurements were performed by mixing preformed SK.HPN complex (0.5 μM) with saturating concentrations (1 μM) of acceptor labeled miniPG variants. For distance measurement, \( E_{max} \) (the maximum energy transfer) was taken into consideration for each of the donor-acceptor pair. All samples were equilibrated before subjecting for fluorescence emission scan and measurements were performed in 50 mM Tris-Cl pH 7.5 and 100 mM NaCl at 25 °C. For each sample multiple scans were taken and the spectra were corrected by subtracting the values obtained for acceptor only spectrum taken under similar conditions.

3.2.10 Theory of energy transfer for a donor-acceptor pair

The fluorescence energy transfer occurs whenever the emission spectrum of a fluorophore, called the donor, overlaps with the absorption spectrum of another molecule, called the acceptor (Stryer, 1978; Clegg, 1995; Lakowitz, 1999). The acceptor does not need to be fluorescent. It is important to note that there are no intermediated photons in resonance energy transfer and the donor and acceptor are coupled by a dipole-dipole interaction. The extent of energy transfer \( E \) is determined by the distance between the donor and acceptor and the extent of spectral overlap \( J \).
3.2.11 Analysis of FRET data

The FRET efficiency (E) was calculated using the equation

\[ E = 1 - \left( \frac{F_{DA}}{F_D} \right) \]  \hspace{1cm} (Equation 1)

Where \( F_{DA} \) and \( F_D \) are fluorescence intensity of the donor in presence and absence of acceptor respectively.

The efficiency is related to the distance (\( R \)) between probes and Förster’s critical distance (\( R_0 \)) at which the transfer efficiency is equal to 50% by Eqn (2)

\[ E = \frac{R_0^6}{(R_0^6 + R^6)} \]  \hspace{1cm} (Equation 2)

The value of \( R_0 \) was calculated using Förster’s equation (Eqn 3)

\[ R_0 = 9.79 \times 10^3 (\kappa^2 Q_D J n^{-4})^{1/6} \]  \hspace{1cm} (Equation 3)

Where \( \kappa \) is the dipole-dipole orientation factor, \( Q_D \) is the quantum yield of the energy donor in the absence of transfer, \( J \) is the spectral overlap integral that signifies the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and \( n \) is the refractive index of the medium.

A value of 2/3 for \( \kappa \) was used for all calculations and it assumes free rotation of the donor and acceptor fluorophores (Stryer, 1978). For refractive index, a value of 1.36 was used for distance calculations. The value of \( Q_D \) was determined by taking N-acetyl-tryptophan amide (NATA) fluorescence as a reference (Lakowicz, 1999). The value of \( J \) was obtained using:
\[ J = \int F(\lambda) \varepsilon(\lambda) \lambda^4 \, d\lambda / \int F(\lambda) \, d\lambda \]  \hspace{1cm} \text{(Equation 4)}

Where \( F(\lambda) \) and \( \varepsilon(\lambda) \) represents the fluorescence intensity of the donor and the molar extinction coefficient of the acceptor, respectively, at the wavelength expressed in centimeters.

### 3.2.12 Molecular Modeling studies

Cartesian coordinates of the kringle 5 domain of HPG, \( \mu \text{PG} \) and those of SK \( \beta \)-domain and the SK.\( \mu \)PN complex were retrieved from the Protein Data Bank (codes 5HPG, 1QRZ, 1C4P, and 1BML, respectively). The co-ordinates for the 250-loop of the \( \beta \)-domain of SK are missing in SK.\( \mu \)PN crystal structure due to high flexibility of this disordered loop (Wang et al., 1998). However, co-ordinates for this loop are intact in isolated \( \beta \)-domain crystal structure (Wang et al., 1999b). The coordinates for the \( \beta \)-domain in the SK.\( \mu \)PN complex were hence replaced with those of the isolated \( \beta \)-domain and the structure was energy minimized. Crystal co-ordinates of kringle 5 and \( \mu \text{PG} \) were used to generate various miniPG structures that differed mainly in spatial orientation of kringle 5 due to the presence of the flexible loop that connects \( \mu \text{PG} \) with kringle 5. Rigid body docking of SK.\( \mu \)PN structure (with intact 250-loop) and miniPG was performed using ZDOCK (Accelrys Inc, USA). Default setting was used for docking and 100 docked conformations were generated. Docking of substrate miniPG onto the SK.\( \mu \)PN generated various ternary interaction models out of which those having activation bond (Arg 561-Val 562) optimally positioned in the partner \( \mu \)PN were further evaluated to satisfy the experimentally measured distances from FRET.
3.3 Results

3.3.1 Preparation of mutants for Fluorescence resonance energy transfer

For intermolecular FRET measurements, variants of SK and miniPG were constructed containing additional cysteine residues (for free -SH groups for fluorescent labeling). SK does not contain any cysteine residue; however miniPG contains “18” cysteines and all are intramolecularly linked to form 9 disulfide bridges in a natively folded molecule, therefore either molecule does not contain any naturally existing free -SH group. Thus, the substitution of cysteine at the flank (260th amino acid) of 250-loop generated a single cysteine variant of SK. Additional cysteine residues were substituted in miniPG to generate multiple free cysteine containing variants of miniPG. Table 3A lists the free cysteine variants of miniPG and their respective location in the primary structure. The respective locations of substituted cysteines in crystal structure are shown in Fig. 3.4. All proteins used in this study (see experimental procedure for details) were purified to homogeneity as evidenced by SDS-PAGE (See Fig. 3.5).

![Fig. 3.5 SDS-PAGE showing purified SK L260C and miniPG variants. 10 µg of purified proteins were electrophoresed on 10% SDS-PAGE gels and stained with Coomassie blue R-250 dye. Lane 1, molecular mass markers; lane 2, SK L260C; lane 3, miniPG N53C; lane 4, miniPG L71C; lane 5, miniPG F583C; lane 5 miniPG N625C; lane 7, miniPG G690C.](image)
3.3.2 Protein labeling

In the present study fluorescence donor and acceptor groups were chosen that were suitable to act as FRET pairs. For donor, we used IAEDANS-derivatized SK L260C, and for acceptors, we utilized Fluorescein 5-maleimide-labeled miniPG constructs. The labeling procedure resulted in nearly stoichiometric incorporation of dye for different cysteine variants. Table 3B lists the dye to protein ratio for each of the molecule used in this study.

**TABLE 3B**

Extent of fluorophore conjugation (labeling efficiency) to various proteins. Molarity of dye and proteins were calculated from their respective molar extinction coefficients. Ratio of dye to Protein concentration yielded extent of labeling and it is expressed in percentage.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>SK L260C</th>
<th>miniPG N53C</th>
<th>miniPG L71C</th>
<th>miniPG F583C</th>
<th>miniPG N625C</th>
<th>miniPG G690C</th>
</tr>
</thead>
<tbody>
<tr>
<td>% labeling</td>
<td>130</td>
<td>101</td>
<td>80</td>
<td>85</td>
<td>84</td>
<td>92</td>
</tr>
</tbody>
</table>

See Experimental Procedures for determination of extent of labeling.

3.3.3 Determination of kinetic parameters for fluorescently labeled SK and miniPG variants

Fluorescently labeled SK and miniPG variants were characterized for their functional activity by determination of steady-state kinetic parameters and were found to be nearly equivalent to their native counterparts in terms of affinity ($K_m$) and catalytic turnover ($k_{cat}$) rates. The steady-state kinetic parameters for HPG activation by preformed complexes of either nSK, SK L260C or SK L260C' (fluorescent labeled SK L260C) with equal concentrations of HPN are listed in Table 3C.
The steady-state kinetic parameters obtained for free cysteine containing variants of miniPG and their fluorescently labeled derivatives with preformed complex of SK.HPN are listed in Table 3D.

### TABLE 3C
Steady-state kinetic parameters for HPG activation by equimolar complexes of either nSK, SK L260C or SK L260C' with HPN

<table>
<thead>
<tr>
<th>Activator species</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$/µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nSK</td>
<td>0.5 ± 0.1</td>
<td>11 ± 0.5</td>
<td>22</td>
</tr>
<tr>
<td>SK L260C</td>
<td>0.5±0.03</td>
<td>11 ± 0.6</td>
<td>22</td>
</tr>
<tr>
<td>SK L260C'</td>
<td>0.52 ± 0.1</td>
<td>11 ± 0.5</td>
<td>21</td>
</tr>
</tbody>
</table>

See “Experimental Procedures” for details of assay conditions

### TABLE 3D
Steady-state kinetic parameters for activation of fluorescent derivatives of miniPG by preformed SK.HPN activator complex are listed along with values for unmodified recombinant

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>miniPG</td>
<td>0.5±0.25</td>
<td>3.75±0.05</td>
<td>6.25±1.5</td>
</tr>
<tr>
<td>miniPG L71C'</td>
<td>0.52±0.3</td>
<td>4.10±0.02</td>
<td>7.8±1.1</td>
</tr>
<tr>
<td>miniPG N53C'</td>
<td>0.62±0.25</td>
<td>3.0±0.02</td>
<td>4.83±0.5</td>
</tr>
<tr>
<td>miniPG F583C'</td>
<td>0.5±0.25</td>
<td>3.9±0.05</td>
<td>7.8±1.5</td>
</tr>
<tr>
<td>miniPG N625C'</td>
<td>0.6±0.3</td>
<td>3.15±0.02</td>
<td>5.25±1.2</td>
</tr>
<tr>
<td>miniPG G690C'</td>
<td>0.61±0.15</td>
<td>4.0±0.3</td>
<td>6.55±1.1</td>
</tr>
</tbody>
</table>

See “Experimental Procedures” for details of assay conditions
3.3.4 Determination of spectral overlap \( (J) \) and Calculation of \( R_0 \)

Forster's critical distance \( (R_0) \) for a given donor-acceptor pair is dependent on spectral overlap \( (J) \), Quantum yield of the donor \( (Q_0) \), dipole-dipole orientation factor \( (\kappa) \) and the refractive index of the medium (Stryer, 1978) as shown by Eqn. 3 in section 3.2.11. Spectral overlap integrals were calculated using Eqn. 4 (refer to section 3.2.11). For determination of \( J \), emission spectrum of the donor alone sample and the absorption spectrum of the acceptor alone, each at 1 nm interval over a range of wavelengths, were acquired. Molar extinction coefficient of the acceptor at each wavelength was determined by simply dividing the absorption spectra by the used concentration of the acceptor. The spectral overlap was then obtained by numerical integration of a product of an area-normalized emission spectrum of donor only sample and the absorption spectrum of the acceptor (Stryer, 1978, Lakowicz, 1999). The other simple method to determine \( J \) and \( R_0 \) is to write a Matlab® (The MathWorks, Inc. MA, USA) routine (see Appendix 2 for Matlab® algorithm for calculation of spectral overlap \( J \) and \( R_0 \)). Spectral overlap values obtained for different donor-acceptor pair are listed in Table 3E along with experimentally determined \( R_0 \) distances. The donor quantum yield was determined to be 0.14 and the refractive index of the medium was taken 1.36 for calculation of \( J \).

**TABLE 3E**

Spectral overlap \( J \), and measured \( R_0 \) distances for IAEDANS-labeled SK L260C and different acceptor sites of miniPG labeled with Fluorescein S-maleimide

<table>
<thead>
<tr>
<th>Acceptor species</th>
<th>miniPG N53C'</th>
<th>miniPG L71C'</th>
<th>miniPG F583C'</th>
<th>miniPG N625C'</th>
<th>miniPG G690C'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spectral overlap ( J ) ( (cm^3 M^{-1}) )</strong></td>
<td>3.2x10^{-14}</td>
<td>3.4x10^{-14}</td>
<td>3.6x10^{-14}</td>
<td>3.8x10^{-14}</td>
<td>4.3x10^{-14}</td>
</tr>
<tr>
<td><strong>( R_0 ) ( (\AA) )</strong></td>
<td>31.2</td>
<td>31.5</td>
<td>31.8</td>
<td>32.1</td>
<td>32.8</td>
</tr>
</tbody>
</table>

Please refer to section 3.3.3 for the methodology used
3.3.5 Resonance energy transfer between SK L260C' and sites on substrate miniPG

The FRET approach exploited the ability to successfully develop a donor-acceptor system in the SK-HPG system, more specifically the interaction of the SK.HPN enzyme with miniPG as its substrate. Strategic placing of donor fluorophore (IAEDANS) in the SK 250-loop and acceptor fluorophore (Fluorescein 5-maleimide) in different loci of miniPG was used to measure the intermolecular distances between the 250-loop and the different regions of the partially truncated substrate. Complexation of fluorescein labeled miniPG constructs with attenuated SK L260C'.HPN binary complex where SK 250-loop is specifically labeled with IAEDANS resulted in transfer of a part of energy from IAEDANS to the Fluorescein (See Fig. 3.6).

Fig. 3.6 Emission spectra of SK L260C' (conjugated with donor fluorophore, IAEDANS) alone or in presence of different acceptors (labeled with fluorophore, fluorescein 5-maleimide) present in miniPG variants. Donor spectra in presence and absence of different acceptors are shown. Acceptor alone spectra for miniPG L71C' is also shown. Different extent of resonance energy transfer to acceptor results in corresponding quenching of donor fluorescence as is evidenced by lowered fluorescence intensity of SK L260C' emission.
Different extents of dipole-dipole energy transfer were detected when preformed SK.HPN was complexed with acceptor labeled miniPG variants under non-turnover conditions. The non-radiative transfer of donor (IAEDANS) energy to the acceptor (Fluorescein-5 maleimide) resulted in donor fluorescence quenching (See Fig. 3.6). Relative distances of donor fluorophore in SK and acceptor fluorophores in miniPG mutants were approximated using extent of energy transfer. For measurement of energy transfer, efficiency values of donor quenching were taken in consideration after subtracting the contributions from acceptor fluorophore (See “Experimental Procedure” for details). The extent of energy transfer was calculated from the Eqn 1 and used to measure the distances according to Förster’s equation (Eqn. 3, refer to section 3.2.11). R₀ values characteristic for each donor-acceptor pair were used for approximation of distances from energy transfer data. Table 3F lists the extent of energy transfer (E) and the calculated inter-probe distances for multisite FRET pairs in a stable enzyme-substrate complex. Maximum donor quenching was observed for those miniPG constructs where the acceptor probe was attached to the kringle 5 regions. The extent of energy transfer obtained for miniPG L71C’ acceptor was 0.57, indicated an apparent distance of -29 Å form the 250-loop of SK β-domain. Also, a proximally placed acceptor probe in miniPG N53C’ construct resulted in relatively high non-radiative transfer with an E value of 0.62, that approximated a computed distance of 27 Å from the reference probe placed in SK (250 loop of the β-domain). All other acceptor probes residing in the serine protease domain resulted in relatively low energy transfer which is indicative of their relatively distal positioning from SK 250-loop of SK β-domain. MiniPG F583C’ construct where acceptor was placed in the 37-loop of catalytic domain showed an apparent spatial separation of 32 Å with an E value of 0.46. Acceptor construct where probe was placed in the Ca-binding loop resulted in nearly 43% (E value, 0.43) energy transfer, indicative of an apparent distance of 33 Å. Non-radiative energy transfer was found to be least (E value, 0.16) with miniPG G690C’ construct, where acceptor probe was placed in autolysis loop,
suggesting that among the selected FRET pairs, this could be the most distantly placed locus relative to the 250-loop of the SK β-domain. The spatial separation of G690 from the 250-loop was determined as ~44 Å, which is clearly the farthest among all the intermolecular separations obtained through the present FRET measurements.

**TABLE 3F**

Intermolecular FRET parameters and measured distances between various acceptor loci present in substrate miniPG and a fixed donor probe placed in the SK 250-loop.

<table>
<thead>
<tr>
<th>Acceptor construct</th>
<th>Acceptor location</th>
<th>$F_{DA}/F_0$</th>
<th>Energy transfer ($E$)</th>
<th>$R_{FRET}$(Å)</th>
<th>$R_{modeled}$(Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miniPG N53C'</td>
<td>LBS of K5</td>
<td>0.38</td>
<td>0.62</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>miniPG L71C'</td>
<td>LBS of K5</td>
<td>0.42</td>
<td>0.58</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>miniPG F583C'</td>
<td>37-loop in catalytic domain</td>
<td>0.54</td>
<td>0.46</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>miniPG N625C'</td>
<td>Ca-binding loop in catalytic domain</td>
<td>0.57</td>
<td>0.43</td>
<td>33</td>
<td>28</td>
</tr>
<tr>
<td>miniPG G690C'</td>
<td>autolysis loop in catalytic domain</td>
<td>0.84</td>
<td>0.16</td>
<td>44</td>
<td>46</td>
</tr>
</tbody>
</table>

$F_{DA}/F_0$ ratio of donor fluorescence values in presence ($F_{DA}$) and absence ($F_0$) of acceptor. 
$E$ extent of energy transfer ($E$) was calculated by using Eqn 1 (See section 3.2.11). 
$R_{FRET}$(Å) experimentally measured distances from steady-state FRET experiments in Å. 
$R_{modeled}$(Å) distances in modeled ternary structure (Please refer to section 3.3.6) in Å.
Although FRET is generally considered to be a low-resolution structural technique, when complemented with computer modeling, it may result in a high definition picture of distance distribution in the complex under study. The crystal structure of the SK.µPN binary complex (Wang et al., 1998) provides critical insight into how the binding of SK with µPN results in generation of a possible concavity for avid binding of the catalytic domain of the substrate. This crystal structure also indicates how SK might modulate the substrate specificity of HPN by providing a “valley” or cleft in which the catalytic domain of the macromolecular substrate can dock through protein-protein interactions, thus positioning the scissile peptide bond optimally for cleavage by the HPN active site. This is crucial to confer the characteristically narrow substrate preference onto an otherwise “indiscriminate” active center of HPN. 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 (Conejero-Lara et al., 1996; Esmon and Mather, 1998). However, the structural positioning of kringle(s) into this “valley” or on the SK.HPN enzyme surface is neither self-evident from the X-ray crystal structure nor easily discernible by simple stereochemical docking of the macromolecular substrate. This situation is further compounded by the fact that full-length HPG is a multi-domain protein in which the kringles fold as separate flexible domains and can have a larger degree of “conformational freedom” with respect to catalytic domain. Moreover, since the activity of SK.HPN varies almost 100-fold between µPG and full length HPG as substrate, the structural disposition of the substrate kringles is very important in mechanistic terms. Further, even if one were to examine a ‘simplified’ substrate i.e. miniPG, wherein one of the kringle is intact, the rate enhancement seen over µPG is sufficiently significant (20-25 fold) to warrant a serious investigation into the structural
alignment of the 5th kringle with the SK.HPN activator complex to explain the mechanistic role of this domain kringle in catalytic potentiation.

3.3.6 Use of FRET-obtained distances to select most optimal computer modeled ternary structure

In order to examine the possible mode of substrate binding, we docked various conformations of miniPG (differing mainly in the orientation of the kringle domain due to the flexible linker connecting the kringle with the catalytic domain) that were generated by using individual crystal co-ordinates for μPG and kringle 5 onto the SK.μPN crystal structure. Docking of substrate miniPG onto the SK.μPN generated various ternary interaction models out of which those having activation bond (Arg 561-Val 562) optimally positioned in the partner μPN were further evaluated to satisfy the experimentally measured distances from the FRET. In order to choose from various available ternary interaction models, the FRET measured distances were used and the most suitable model satisfying closely the FRET mapped values was selected to gain insight about the probable mode of substrate miniPG binding to the SK.μPN activator complex. The catalytic domain of the docked substrate miniPG in the selected ternary model (that satisfied the FRET distances) when compared with the ternary crystal structure of SAK.μPN.μPN, where a substrate μPN is complexed with the SAK.μPN enzyme complex, showed complete superimposition for the corresponding catalytic domain bound as a substrate in the crystal structure. This comparison further validated our computer generated ternary structure of SK.μPN.miniPG and allowed us to examine the legitimate contact points between the cofactor SK and the docked substrate. The most optimal ternary interaction model is shown in Fig. 3.7 that shows multiple interactions between the substrate miniPG and the three domains of SK. Fig. 3.8 shows a section of the ternary interaction model highlighting specifically the substrate miniPG and the β-domain of SK.
Fig. 3.7 Ternary interaction model showing substrate interaction sites on SK. Docked substrate miniPG onto the SK.µPN crystal structure is shown (partner µPN is not shown for clarity). Three domains of SK, viz. α (red), β (cyan) and γ (blue) are shown interacting with the substrate miniPG catalytic domain (magenta) and kringle 5 (green). Coordinates of 250-loop from isolated β-domain structure were used to reconstruct the missing loop in the SK.µPN structure. Model shows that substrate miniPG interacts mostly with α- and β-domains of SK. Catalytic domain of substrate has extensive contacts with both α- and β-domains of SK while kringle 5 is positioned optimally towards 250-loop of SK β-domain. Sites of cysteine substitution are shown with spheres. See "Experimental Procedures" for details of model building and the PDB ID used.
Fig. 3.8 Ternary interaction model. Only a section is taken, showing, substrate miniPG (magenta for catalytic domain and green for kringle 5) and the β-domain of SK (cyan). Sites of cysteine substitution are shown with spheres. Interprobe distances (in Å) as obtained from FRET experiments for each of the substrate epitopes from one fixed location (in SK 250-loop) in SK are shown with connecting lines. Kringle 5 of substrate miniPG is positioned in a manner so as to have most favorable contacts with the 250-loop of β-domain, which extends out in the binary SK,μPN structure. Measured distances from FRET are in agreement with those obtained from modeled structure. See “Experimental Procedures” for details of model building and the PDB ID used.
Distance distribution in the modeled structure was in consonance with the experimentally calculated values (See table 3F). The distances present in the selected modeled structure matched closely with the experimentally measured values from FRET studies. For example, the spatial separation of N53C (present in lysine binding site of kringle 5) in modeled structure is 25 Å, while we measured the distance to be 27 Å. Another locus present in kringle 5 (L71C) was spaced at 28 Å in the modeled structure which was close to FRET based determination of 29 Å. Spatial separation value measured by FRET for miniPG N164C (residue present in Ca-binding loop) also matched very closely with the distance discerned from the selected computer generated ternary interaction model. The F583C (residue present in 37-loop of catalytic domain) was found to be placed at a distance of 25 Å in the modeled structure, while we measured it to be 32 Å. We reasoned that such a minor disagreement could possibly arise from a different dipole orientation of fluorophore that does not fit to assumption of free dye movement (Stryer, 1978). Another possibility could be the segmental flexibility of the 37-loop that could possibly shift it slightly more towards the core of the active site as soon as the substrate binds to the SK.HPN activator complex, therefore appearing to be distantly placed from the 250-loop as measured by FRET experiments. This argument is strengthened by the fact that the 37-loop immediately follows the activation loop bearing scissile-peptide bond, Arg 561-Val 562; hence, it is probable that structural changes which orient the activation loop of the substrate towards the active site, once the substrate is docked onto the SK.HPN, may lead to rearrangement of the 37-loop, causing the observed distance disagreement. The most distantly placed acceptor location was found to be G690C (residue present in the autolysis loop) for which the modeled distance of 44 Å was close to the experimentally measured distance of 46 Å (Please refer to Table 3F).
3.3.7 Mechanistic Insights from the ternary interaction model

The modeled ternary structure shows multiple interactions between the substrate miniPG and the three domains of SK (See Fig. 3.7). The structure shows that the serine protease domain of substrate miniPG interacts most extensively with the enzyme’s catalytic domain as well as the alpha domain of SK, while the kringle domain has close proximity with the SK β-domain. The 250-loop optimally positions itself towards the LBS of kringle 5 (See Fig. 3.8 which shows only substrate miniPG and the β-domain for clarity). The γ-domain has least contacts with the docked substrate miniPG (See Fig. 3.7). Kringle positioning on the β-domain is suggestive of presence of substrate binding exosites on the β-domain, which is also consistent with the fact that the β-domain has the least contact with the enzyme μPN in the SK-μPN crystal structure (Wang et al., 1998).

Quantitative determination of distances through steady-state FRET and selection of a suitable computer modeled structure based on FRET distances yields a convincing ternary model in which substrate miniPG is docked onto the SK-μPN enzyme complex. However, since FRET is sensitive only to distances that range from 20-100 Å, it is difficult to ascertain distances that are either too close or too far from this range. Additionally, the finite size of fluorophores (-10 Å, IAEDAND, and -15 Å Fluorescein-5-maleimide) allows them to sweep through an area proportionate to their sizes. Hence, fine mapping of interacting faces that could fall within the possible distance for ionic interactions to take place requires relatively high resolution techniques such as X-ray crystallographic or high resolution NMR pictures of the ternary complex. Nevertheless, at present juncture the results show proximal placing of 250-loop of the β-domain of SK and the kringle 5 of substrate miniPG. This indicates that a relatively low affinity binding of catalytic domain alone to the SK-μPN binary activator complex switches to a high avidity binding through kringle mediated interactions to the “exosites” present on the SK β-domain and perhaps, other regions of SK as well. This assumption is strengthened by the steady-state kinetic
studies where miniPG is observed to possess nearly four times higher affinity as compared to the kringle-less derivative, μPG, for the SK.HPN activator enzyme (This aspect is dealt in detail in Chapter 5).

3.4 Discussion

Besides the well recognized “switch” in substrate preference, the binding of SK to HPN results in a severalfold enhancement of the $K_m$ for diverse small molecular weight chromogenic peptide substrates but relatively little alteration in their $k_{cat}$ values as compared with free HPN, indicating that the change in preference for larger substrate is likely driven by global contacts with the macromolecular substrate. Multiple lines of evidence now suggest a predominant role of exosite interactions rather than mere engagement of the active site microenvironment in driving high substrate affinity and covalent specificity for macromolecular HPG activation by the SK.HPN enzyme complex.

Some of the early evidences in this regard were provided by “peptide walking” and fragment complementation approaches to map possible binding sites for HPG in the SK.HPN activator enzyme. (Nihalani et al., 1997; 1998). In these studies, peptides that mimic short stretches of α- or β-domains strongly inhibited the macromolecular substrate activation by SK.HPN enzyme complex, suggesting that enzyme-substrate interactions emanate from a larger surface-to-surface contact. The belief that exosite-mediated interactions are key to the inordinately high substrate specificity of SK.HPN were further supported by studies that elegantly used active site fluorescently-labeled HPN to examine the exosites generated on the enzyme-cofactor complex (Boxrud et al., 2000). Later on, we obtained more direct evidence for substrate kringle interactions in case of one of the surface-exposed loops (SK 254-262) of SK (Dhar et al., 2002). Solution studies that utilized both loop deletion and truncated substrate derivative (μPG) provided clear-cut evidence of the role played by this nine-residue loop in substrate recognition, and thus identified a functionally important component of the
macromolecular substrate-specific exosite operative in the SK.HPN complex, which interacts via some, or all, of the five kringle(s) in substrate HPG. However, the exact identity of a particular kringle involved in this interaction remained unclear. Moreover, any further information in this regard was severely limited by the unavailability of any direct structural information about the possible mode of substrate interactions with the SK.HPN enzyme complex. We therefore resorted to a simple biochemical strategy, using truncated substrate derivatives generated by the sequential removal of kringles from the full-length HPG, for the kinetic characterization of their activation by the SK.HPN complex. Another approach utilized the development of an intermolecular FRET based system where one could map the substrate binding in a stabilized ternary complex by using biophysical methods. However, this study, in principle, is complicated by the involvement of multiple proteins in the ternary assembly. Nevertheless, we have standardized a prototype study that utilizes a single kringle containing substrate derivative (miniPG) for making a stable enzyme.cofactor.substrate ternary complex, and successfully measured the various intermolecular distances. This approach was feasible because we had successfully developed an in vitro refolding strategy that could refold recombinant miniPG and its free cysteine variants in high yields. The basic design of study was to inactivate the catalytic site of HPN by using a peptide inhibitor (Glu-Gly-Arg-Chloromethyl ketone) and use it to make the SK.HPN complex which can no longer activate the substrate, but still binds to it favorably. The attenuated SK.HPN complex so generated was subsequently complexed with substrate miniPG to get a stable ternary complex. Hence, the enzyme-substrate complex was “frozen” in a non-turnover condition, wherein, presumably, the substrate retained all interactions with the binary activator complex except for few inhibitor binding regions. Strategic placing of additional cysteine residues in selected loci of miniPG enabled us to carry out a site-specific conjugation of extrinsic fluorophore, Fluorescein-5-maleimide, in order to carry out the planned FRET studies. The generation of single-cysteine mutants of SK was made more
facile due to absence of natural cysteine/s in the polypeptide, and the ease of refolding these mutants under \textit{in vitro} conditions. This allowed us an opportunity to successfully place a single cysteine in the 250-loop through substitution mutagenesis. The cysteine-substituted mutant at Leucine-260 position was then specifically derivatized with a sulfhydryl-reactive fluorophore, IAEDANS. Emission spectrum of IAEDANS has significant overlap with the absorption spectrum of fluorescein, hence they became a donor-acceptor pair of choice for many energy transfer studies (Bigelow \textit{et al.}, 1992; Rietveld and Ferreira, 1996; Garzon-Rodriguez \textit{et al.}, 1997). The extent of energy transfer between the donor and acceptor sites in the SK and substrate miniPG, respectively, enabled us to map some of the proximal and distal loci of the substrate from one reference point of SK in a stabilized ternary complex. The steady-state FRET between one donor location situated in the SK 250-loop, and five different acceptor sites allowed us to measure five intermolecular distances from this approach. Among all, the least spatial separation of SK 250-loop was observed with kringle 5 derivatives, namely N53C and L71C. The autolysis loop mutant was found to be located farthest from the 250-loop, while 37-loop and Ca-binding loop derivatives were at relatively intermediate distances. The most vital information emanating from these measurements is that the 250-loop has close proximity with the lysine binding sites of kringle 5. However, despite this an overall picture of substrate binding remained elusive. To obtain a broader understanding of possible substrate orientation, we generated multiple computer modeled ternary structures of SK.\mu N.miniPG (see “Experimental Procedures” for detail) and used the experimentally measured distances from FRET experiments to select the most suitable ternary model. Remarkably, the values obtained from the FRET measurement were in agreement with the distances present in the best selected modeled structure. The model detailed extensive contacts between the docked substrate and the domains of SK, wherein the 250-loop which extends outwards in the SK.\mu PN crystal structure for possible interaction with the substrate was found to be closely placed with kringle 5 of
the docked miniPG. Hence, the ternary modeling both complemented and validated our FRET based distance measurements, and also helped in interpreting the exact spatial separations between donor and acceptor sites. The new information obtained from this study lends further support to the earlier solution studies that it is exosite binding rather than active site docking per se of the substrate that predominantly determines the substrate affinity and substrate kringle mediated potentiation of catalytic activity. These results support our previous solution studies where we examined the role of SK 250-loop and showed its responsiveness for kringle-containing substrate (Dhar et al., 2002). The present findings provide yet another instance of exosite-mediated catalysis in action. For example, previous studies with thrombin and prothrombinase have established the significant role of exosite-substrate interactions in generation of high substrate affinity and binding specificity (Stubbs and Bode, 1995; Di Cera, 2003; Boskovic et al., 2004). Thematic features associated with exosite-dependent substrate recognition are also discernible in the action of the VIIa-TF complex on factor X (Shobe et al., 1999; Baugh et al., 2000), factor X binding to IXa-VIIIa (Duffy et al., 1992), and Va cleavage by activated protein C (Gale et al., 2002), and IXa formation catalyzed by Xla (Aktimur et al., 2003). Thus, exosite-mediated function increasingly appears to represent a generalized mechanism underlying the inordinately high substrate specificity in the coagulation enzymes. However, extension of this mechanism to a bacterial cofactor protein is new, wherein a heterologous protein of bacterial origin non-covalently associates with a eukaryotic serine protease, and then modulates its substrate specificity to favor a particular substrate. The finding supports a mechanism where domains of SK in SK.HPN enzymatic complex provide complementary surfaces for avid substrate binding and generation of extreme covalent specificity for scissile-peptide bond of the macromolecular substrate HPG. However, it must be realized that the substrate utilized in this study is a truncated HPG derivative with only one of the five kringles that are present in the full length molecule. Hence, a more comprehensive examination of
additional exosites on SK and their cognate binding sites in the substrate requires multiple full length recombinant HPG derivatives with additional cysteine residues placed strategically in different locations. Intermolecular FRET measurements with all such derivatives will provide us a distance geometry map, which could be used to construct a three dimensional, holistic view of enzyme-substrate interaction in this system.