CHAPTER 5

Product release is the major contributor to catalytic turnover for the Streptokinase-Plasmin (SK.PN) catalyzed activation of substrate plasminogen as probed in real time by Stopped-flow FRET studies.
Summary

In the present study, we demonstrate real-time evidence for the notion that the presence of kringle domains in substrate human plasminogen (HPG) is necessary for its optimal processing by streptokinase-human plasmin (SK.HPN) activator complex. Steady-state kinetic parameters for activation of different substrate HPG derivatives viz. microPG (catalytic domain of HPG only, devoid of all kringles), miniPG (catalytic domain with kringle 5), midiPG (catalytic domain with kringles 4 and 5) when compared with full-length HPG revealed the significant contribution made by kringle(s) in catalytic turnover ($k_{cat}$). It was observed that catalytic turnover by the SK.HPN activator for the kringle-less derivative, microPG, was very less (only 1-2 %) as compared to that against native full-length substrate HPG; however, upon addition of a single kringle, as in case of miniPG, the activity shoots up by nearly 30-fold. Addition of one more kringle, as in case of midiPG, further lifts the activity to levels equivalent to nearly $3/4$th of the full length HPG. These observations suggest that for optimal activation of substrate HPG by the SK.HPN activator complex, the involvement of long-range protein-protein interactions between kringle domains of the substrate and the enzyme are vital. Indirect measurement of enzyme-substrate affinity ($K_m$) by steady-state kinetics revealed that despite the nearly two-thirds reduction in catalytic activity in case of substrate miniPG as compared to full-length native HPG, there exists minimal difference in affinity for the two-substrate derivatives for the SK.HPN enzymatic complex. Interestingly, enzyme’s affinity for the two-kringle derivative (midiPG) is undistinguishable from that for full-length HPG although, the $K_{cat}$ for midiPG still remains approximately one quarter less than the full-length HPG. This apparent non-correlation between the catalytic efficiency, on the one hand, and enzyme-substrate affinity, on the other, is seemingly in contrast with the established model of HPG activation by SK and other bacterial HPG activators whereby the docking of the catalytic domain of substrate onto the HPG activator complex is believed to impart both specificity and catalytic drive to the plasmin active center. To gain further insight into the catalytic mechanism, pre-steady state kinetics of SK.HPN mediated activation of different substrate derivatives of HPG. For transient kinetics studies, preformed SK L260C’.HPN (equimolar) catalytic complex was rapidly mixed with various acceptor fluorophore-labeled HPG substrate derivatives, and the change in FRET efficiency during single catalytic cycle was monitored with milliseconds time resolution. Rapid mixing of SK L260C’.HPN enzymatic complex with either of the fluorescently labeled substrates viz. microPG, miniPG and midiPG resulted in a distinct
fluorescent change due to alteration in FRET efficiency during the catalytic cycle. Kinetic fitting of fluorescent traces indicated an early rapid phase followed by a relatively slow phase for each rapid-mixing experiment. The rate constant for the fast phase ($k'_1$) was found to be multifold higher than the actual turnover rates for all the substrate derivatives studied, while the rate constant ($k'_2$) for the slower phase was in remarkable unison with the respective catalytic turnover rates as deduced from steady-state kinetic studies for various substrate derivatives of HPG. Therefore, from these data, it appeared that the product release step (attributed to the slower rate constant, $k'_2$) is the major contributor to catalytic turnover for the SK-HPN catalyzed activation of substrate HPG derivatives. It was also found that formation of the initial “Michaelis complex” for all the different substrate HPG derivatives were indifferent, suggesting that none of the substrate derivatives was kinetically deficient in formation of enzyme-substrate complex, thereby suggesting that any change in catalytic turnover observed originated from the events that were subsequent to substrate binding. These results have been interpreted to suggest a model of SK action in which the major amplification in the catalytic drive is obtained through substrate kringle-dependent conformational changes generated in the nascent product immediately after scissile peptide bond cleavage.
5.1 Introduction

Activation of human plasminogen (HPG) to human plasmin (HPN) is a key event in the fibrinolytic system that results in the dissolution of blood clots and restoration of normal blood circulation. This makes HPG a very attractive target for therapeutic interventions to treat clinical conditions resulting from arterial thrombosis and myocardial infarction. Clot dissolver protein drugs such as streptokinase (SK), staphylokinase (SAK), urokinase (UK) and tissue plasminogen activator (tPA) exert their beneficial influence through the activation of HPG to its proteolytically active form, HPN that dissolves the pathological blood clots within the circulatory system. Among these, SK and SAK are bacterial cofactor proteins that are responsible for conformational rather proteolytic activation of HPG. SK is a widely-used thrombolytic protein that is secreted by the Lancefield Group C β-hemolytic streptococci (Castellino, 1981; ISIS-3, 1992). Although HPN is a serine protease with trypsin-like side-chain specificity, it cannot efficiently activate substrate HPG to HPN by itself, but once complexed with SK, the otherwise "non-specific" active site of HPN gains an inordinately high substrate selectivity for the cleavage of the single peptide bond (Arg 561-Val 562) in substrate HPG (Markus and Werkheiser, 1964; Wang et al., 1998). This amide bond is part of a nonapeptide activation loop that is restrained by a disulfide linkage at the base (Wang et al., 2000a). The remarkable alteration of the macromolecular substrate specificity of HPN by SK as a result of the latter's "protein co-factor" property, which has been the subject of intense investigations, is currently thought to be due to exosites generated on the SK-HPN activator complex (Wang et al., 1998; Nihalani et al., 1998; Boxrud et al., 2000). The crystal structure of SK complexed with microPN (the catalytic, serine protease domain of HPN, devoid of its five kringle domains) has provided invaluable initial insights regarding the molecular mechanism whereby SK manages to "switch" the substrate specificity of the active site of HPN (Wang et al., 1998), indications of which had also been gleaned earlier through solution studies using Peptide Walking and fragment complementation.
approaches (Nihalani et al., 1997, 1998). These studies collectively established that SK acts as a protein co-factor of the HPN active site by creating a substrate-binding environment around the HPN active centre by virtue of its tri-domain structure. Thus, it has been postulated that the docking of the catalytic sub-unit brings an optimal presentation of the “target” scissile peptide bond Arg 561-Val 562 in HPG at the active centre facilitating the preferred cleavage of this peptide bond in the macromolecular substrate by the otherwise non-specific HPN active site (Wang et al., 1998; Parry et al., 2000). However, some recent studies have suggested that unlike the mode of action of most proteolytic enzymes in general (as also several other hydrolytic enzymes), long-range protein-protein interactions not only between the scissile peptide bond bond-bearing catalytic domain but also between kringle domain(s) of the substrate HPG and the SK.HPN activator enzyme play a contributory role in the catalytic functioning evolved by this system (Sundram et al., 2003; Verhamme and Bock, 2008). Specific large-substrate recognition through extended macromolecular interaction sites (exo-sites) has been documented in the cases of prothrombinase (Boskovic et al., 2004), factor VIIa (Shobe et al., 1999; Baugh et al., 2000), and factor X (Duffy et al., 1992). Each of the kringle domains, initially identified in prothrombin (Magnusson et al., 1975) but later identified in HPG as well (Sottrup-Jensen et al., 1978), contains approximately 80 amino acids that are cross-linked through three intrakringle disulfide bonds. Earlier it was believed that these domains largely participate in only effector functions such as promotion of fibrin binding of kringle-bearing proteins (Wiman and Wallen, 1977), with little or no direct involvement in active site function per se. However, some recent findings have indicated that the kringle domains of macromolecular substrate (HPG) are responsible for improved enzyme-substrate interactions in the SK.HPN system (Dhar et al., 2002; Sundram et al., 2003) probably through initiating long-range non-covalent interactions between some, or all of the kringle domain(s) of substrate HPG and the SK.HPN activator.
complex. However, exact mechanistic details regarding this have not yet been deciphered.

According to the generally accepted mechanism, serine proteases catalyze the hydrolysis of amide bonds of their protein and peptide substrates according to a three-step mechanism (See Fig. 5.1). In the first step, the substrate and enzyme combine to form the Michaelis complex. From within this complex, the hydroxyl of the active site serine attacks the carbonyl carbon of the amide bond of the substrate to generate an acyl-enzyme intermediate. Finally, hydrolysis of the acyl-enzyme intermediate produces the reaction’s product, and regenerates free enzyme after product release. This reaction

\[
E-OH + RNHCO-R' \xrightleftharpoons[k_1]{k_{-1}} E-OH : RNHCO-R' \\
\xrightarrow{k_2} E-OH : CO - R' + H_2N-R' \\
\xrightarrow{k_3} E-OH + R'COH \\
\xrightarrow[k_4]{K_4} E + P
\]

*Fig. 5.1 Generally accepted kinetic mechanism for serine protease catalysis.* It involves Michaelis complex (Enzyme.Substrate) formation \((k_1)\), covalent acyl intermediate \((E_{acyl})\) formation \((k_2)\), and intermediate hydrolysis \((k_3)\) steps. Also shown is a kinetically relevant product \((P)\) release step \((k_4)\).
scheme gives rise to microscopic constants describing binding \( (k_1) \), acylation \( (k_2) \), deacylation \( (k_3) \) and product release \( (k_4) \) steps. Each of these steps influences the catalytic efficiency an enzyme can achieve. In addition to chemistry taking place at the active site, there are many other factors that determine high substrate specificity and catalytic efficiency. Important among these are enzyme-substrate interactions that are distal to active-site and cofactor induced modulation of substrate specificity (Wang et al., 1998; Sundram et al., 2003; Boskovic et al., 2004). Dependence of \( k_{cat} \) upon substrate chain length has been documented for several serine proteases, including pancreatic elastase (Thompson and Blout, 1973; Stein et al., 1987) and chymotrypsin (Case and Stein, 2003). There is a strong body of evidence that suggests that many of the blood coagulation proteases utilize their ability to engage exosites and active site independent conformational changes to achieve maximum catalytic turnover (reviewed by Krishnaswamy, 2005 and Bock et al., 2007). It is conceivable that similar processes are involved in SK.HPN based activation of HPG, wherein, SK bound to HPN utilizes the substrate kringle-dependent interactions and substrate-driven conformational assistance to achieve a catalytic rate of HPG activation known to be highest among all the HPG activators studied till date. The exact manner by which this happens, or the specific “nature” of such “global” enzyme-substrate interactions that contribute towards the enhanced substrate HPG activation by the native SK.HPN activator complex, however, remains to be deciphered. A recent study (Sundram et al., 2003) clearly showed that although the presence of kringle domains generated a native-like docking potential for substrate HPG even in the isolated bi-domains of SK (i.e. \( \alpha \beta \) and \( \beta \gamma \)) which, like SK, formed strong binary complexes with plasmin(ogen), yet by themselves this docking could not generate native-like high catalytic rates, which were found to be very low indeed (1-2 %) as compared to that of the native SK.HPN activator complex. Interestingly, however, when the co-factor activity of native SK.HPN activator complex alongwith the activator complex(es) of bi-domain derivatives and HPN was explored with kringle-less
derivative of HPG (microPG), the catalytic advantage of even the native SK.HPN activator complex (observed in case of full-length HPG as substrate) was abolished, with both the bi-domain derivatives (αβ and βγ) as well as three-domain native SK, showing only a relatively small decrease in substrate affinity (~5-fold less as compared to SK.HPN versus HPG) but a drastically reduced catalytic turnover (~1%). This indicated that the kringle domain(s) of substrate HPG not only contributes towards the optimal stereochemical docking of macromolecular substrate onto the activator complex but also contribute substantially in terms of generation of the maximal catalytic potentiation seen in the SK.HPN system. However, this study did not provide the much-needed direct evidence of kringle dependent alteration in actual turn-over in real time. Accordingly, in the present study, we have sought to more deeply explore the mechanistic contributions of the kringle-mediated long-range interactions in substrate recognition and turnover by SK.HPN enzymatic complex using steady-state and rapid kinetics methods. This was attempted mainly by evaluating the steady-state and transient kinetic studies for SK.HPN based catalysis using partially truncated substrate HPG derivatives with varying “kringle numbers”. Steady-state kinetic parameters established the differential contribution of kringles in the catalytic potentiation. In order to examine the individual steps that control the catalytic efficiency, we developed an intermolecular FRET system specifically to probe the pre-steady state kinetics of catalytic conversion of HPG by SK.HPN activator complex. Stopped-flow FRET studies were conducted under pseudo first order conditions, where excess enzyme was used over substrate, so that, information from a single turn-over of the catalytic cycle could be obtained. To generate a suitable FRET pair, a unique cysteine was substituted in the central domain (SK L260C, in the 250 loop of the beta domain) of the SK molecule. The free thiol (-SH) in the SK was specifically reacted with sulphydryl reactive 5-(((2-iodoacetyl)amino)ethyl) aminonaphthalene-1-sulfonic acid (IAEDANS) that acted as donor fluorophore. To place acceptor fluorophore, free cysteine variants of microplasminogen (microPG, serine protease domain devoid of kringles),
miniplasminogen (miniPG, serine protease domain with kringles 5) and midiplasminogen (midiPG, serine protease domain with kringles 4 & 5) were made by cysteine substitution at Met 585 of the 37-loop, present in the catalytic domain of the substrate. These free cysteine variants were reacted with QSY® 35 iodoacetamide, a non-fluorescent acceptor probe for IAEDANS emission. Under the condition of these experiments (enzyme in slightly molar excess to substrate) acceptor labeled substrate HPG derivatives were rapidly mixed with preformed SK.HPN enzymatic complex and fluorescence changes due to change in FRET efficiency could then be related to catalytic events. The Stopped-flow FRET experiments enabled us to monitor the rate of product release in real time for different substrate derivatives used. Kinetic fitting of the fluorescence traces for change in FRET efficiency provided characteristic rate constants that were correlated with the two crucial post-docking events viz. scissile-peptide bond cleavage and the product release. The results, presented below, clearly demonstrate the important role played by the kringles of substrate HPG in generating maximal catalytic turnover by the SK.HPN activator complex. The results obtained clearly suggest that long-range or "global" protein-protein interactions between substrate HPG and the SK.HPN activator complex are indeed effectively utilized to drive the reaction to the very high turnover rates associated with this HPG activator. In summary, undistinguished substrate docking but differential product release for kringeless and kringlerich HPG derivatives were strongly indicative of the vital role of kringles domains in post-docking events consequent to substrate capture. The observed rate constants associated with the catalytic events further suggest that the product release step is a major contributor towards the high $k_{cat}$ of the SK.HPN enzymatic system as opposed to substrate-binding associated with the docking step.
5.2 Experimental Procedures

5.2.1 Reagents

The RNA polymerase promoter-based expression vector, pET23d 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 Inc. (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 was 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 QSY® 35 iodoacetamide were purchased from Molecular Probes-Invitrogen CA, USA. HPG 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 an Applied Biosystems 3130xl Genetic Analyzer 16 capillary DNA sequencer. The N-terminal amino acid sequencing was done with Applied Biosystems sequencer, Model 491A.

5.2.2 Genetic constructs

Molecular Cloning of nSK and preparation of SK L260C are described in the section 3.2.2.1. There are no natural cysteines present in SK polypeptide sequence. Hence, single
cysteine variant of SK where cysteine is substituted for leucine (See Fig. 5.2 A) at 260th amino-acid position (flank of the 250 loop of beta domain) was created for site-specific fluorophore conjugation. Molecular cloning of microPG, miniPG and midiPG are described in section 2.2.3.

![Figure 5.2 Crystal structure of SK (A) and microPG (B) showing sites of cysteine substitution. (A) Cysteine was substituted for the Leu 260 present in the surface exposed 250 loop of the beta domain of SK. Side chain of Leu 260 is shown in green. The three different domains of SK viz. α (red), β (cyan) and γ (blue) are also shown. (B) Free cysteine containing variants of microPG, miniPG and midiPG were prepared by substituting Met 585 (side chain shown in green), present in the 37-loop of the catalytic domain. Activation loop of the microPG is shown in red.]

Each of the HPG derivative used in this study contain multiple cysteine residues. However, all these cysteines are engaged in disulfide bridging. Since none of the natural cysteines remain free in the natively folded microPG, miniPG and midiPG, this provides
an opportunity to incorporate an additional cysteine in these derivatives at any chosen location that could be possibly available free for conjugation with sulfhydryl reactive agents, albeit under the "correct" redox state. We therefore prepared free-cysteine containing variants of microPG, miniPG and midiPG for covalent modification with thiol-reactive polyethylene glycol (PEG) reagents. For cysteine substitution, the catalytic domain was selected since it was common to all the three derivatives utilized for study. Cysteine was substituted for Met 585, a residue central to the surface-exposed loop of the catalytic domain (See Fig. 5.2 B). Cysteine substitutions were made by the commercially available QuikChange® Mutagenesis kit (Stratagene, La Jolla, CA) and involved the usage of two complementary primers having the desired mutation (Please refer Appendix I for primer details).

5.2.3 Fluorescent labeling of proteins

For donor fluorophore conjugation, SK L260C was reacted with IAEDANS as described in section 3.2.7. Acceptor labeling was performed by reacting free cysteine variants of substrate HPG derivatives with QSY® 35 iodoacetamide. Concentrated samples of purified proteins (200-250 μM) in a total volume of 1 ml were reacted with 10X molar excess of fluorescent dyes under dark at room temperature. Components of reaction buffer included 50 mM Tris-Cl pH 7.5, 1 mM EDTA and 100 mM NaCl. Reactions were stopped by adding 1 μl of 1M cysteine. Free dye was removed using desalt column packed with Sephadex G25 medium beads. Desalted proteins were aliquotted and stored at -70 °C. The stoichiometry of the labeling was determined by measuring absorption at 340 nm for IAEDANS and 490 nm for QSY® 35 iodoacetamide and using an extinction coefficient of 6100 M⁻¹ cm⁻¹ for IAEDANS (Hudson and Weber, 1973) and 25000 M⁻¹ cm⁻¹ for QSY® 35 iodoacetamide (Molecular probes-Invitrogen, USA). Protein concentration was determined spectrophotometrically at 280 nm using extinction coefficients ε (M⁻¹ cm⁻¹), 84,670 for midiPG, 67,821 for miniPG and 50,500 for microPG and 44,762 for SK.
The molar extinction coefficient at 280 nm for different proteins used were determined by Vector NTI® software from Invitrogen, USA.

5.2.4 Steady-state kinetic studies

Steady-state kinetic parameters for different substrate HPG derivatives were determined for their activation by using catalytic amounts of preformed SK.HPN enzyme complex (Wohl et al., 1980; Dhar et al., 2002). Fixed concentrations of SK.HPN was added to assay cuvettes containing various amounts of refolded and purified HPG variants (0.2 to 2 μM) plus chromogenic substrate (0.5 mM) in assay buffer (50 mM Tris-Cl, pH 7.4), in a total volume of 100 μL. Change in absorbance was then monitored continuously at 405 nm as a function of time at 25 °C in a Versa-Max tunable microplate reader from Molecular Devices, USA. The kinetic parameters for midiPG, miniPG, and microPG and their fluorescent adducts' activation were then calculated from inverse Lineweaver Burk plots (Wohl et al., 1980). To compute the $k_{cat}$, the number of HPN active sites was determined using the p-nitrophenyl p-guanidinobenzoate (NPGB) reaction (McClintock and Bell, 1971; Chase and Shaw, 1969; Wohl et al., 1977).

5.2.5 Transient kinetic studies

Rapid reaction kinetic experiments were performed on a Biologic SFM-4 Stopped-flow instrument. The dead time of measurement was ~2 ms. Excitation was carried out at 340 nm with excitation slit set at 8 nm, and the emission data was collected using a 410 nm cut-off filter (Bio-Logic SAS, France) that restricted any potential fluorescence contribution from proteins intrinsic fluorophores. Since acceptor (QSY® 35 iodoacetamide) was nonfluorescent, therefore, we monitored the change in donor fluorescence intensity ($E_{max}$ = 490 nm) due to change in FRET efficiency after rapid-mixing of enzyme and substrate. The reaction volume was 226 μL, and cuvette used was FC 15 with path length of 1.5 mm. All experiments were performed at 25 °C. Preformed SK.HPN
and each of the HPG derivatives were filled in separate syringes (10 ml capacity) immediately before the rapid mixing experiments. Contents of syringes containing enzyme and substrate were mixed rapidly in a ratio that always resulted in at least two fold excess of enzyme over the substrate in the reaction. In a typical Stopped-flow rapid mixing experiment, enzyme and substrate were mixed in a ratio to achieve final enzyme and substrate concentrations of 10 \( \mu \text{M} \) and 5 \( \mu \text{M} \) respectively. Alternatively, SK complexed with pre inhibited (with 10 molar excess NPGB) HPN was also used for rapid mixing with either of the HPG substrate derivatives to follow the substrate docking steps but without actual turnover to product. Time-traces (8000 data points) of near-saturation exponential fluorescence increases were collected for each experiment. Control reactions containing labeled SK L260C only were performed to quantitate the initial fluorescence, and also to see the photo stability of the conjugated fluorophore. Fitting of the data was done with the Biokine software provided by the manufacturer with the instrument, and for each experiment average of several kinetic traces was used for fitting.

5.3 Results

5.3.1 Protein purification and site-specific fluorescent labeling

Purification, site-specific labeling and functional characterization of SK L260C are discussed in sections 3.3.1, 3.3.2 and 3.3.3 respectively. All the substrate HPG derivatives used in this study were purified to near homogeneity (>95% pure) as evidenced by SDS-PAGE (See Fig. 5.3). Incorporation of QSY\(^\text{\textregistered} 35\) iodoacetamide in microPG, miniPG and midiPG was found to be 0.82, 0.75 and 0.62 mole per mole of protein on an average.
Fig. 5.3 SDS-PAGE showing purified HPG derivatives. 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 as indicated in KDa; lane 2, midiPG, lane 3, miniPG and lane 4, microPG.

5.3.2 Steady-state kinetic parameters for the activation of substrate microPG, miniPG, midiPG and their fluorescently labeled cysteine variants with SK.HPN activator complex

In order to explore the contribution of the kringle domains in substrate HPG activation by SK.HPN activator complex, if any, HPG derivatives having either one or two kringles attached to the catalytic domain (miniPG and midiPG, respectively) were used as substrate for the SK.HPN activator complex. The steady-state kinetic analysis (Please refer to Table 5A) of this activation reveals that substrate miniPG and midiPG have nearly the same apparent affinity ($K_m$ -0.5 μM) as that of full-length substrate HPG for the SK.HPN activator enzyme. Like full-length HPG, the affinity of miniPG and midiPG for
SK.HPN was observed to be 4-5 fold higher than that of the kringle-less derivative, microPG. However, the catalytic turnover ($k_{cat}$) of substrate miniPG by SK.HPN was observed to be 25-30-fold higher than that of microPG. The kinetic parameters for fluorescent derivatives of substrates, namely microPG', miniPG' and midiPG' remained indistinguishable from their respective unmodified variants.

<table>
<thead>
<tr>
<th>Protein purified from E. Coli inclusion bodies</th>
<th>Steady-State Kinetic Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ ($\mu$M)</td>
<td>$k_{cat}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>microPG</td>
<td>2.0±0.3</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>microPG'</td>
<td>1.6±0.25</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>miniPG</td>
<td>0.5±0.25</td>
<td>3.75±0.05</td>
</tr>
<tr>
<td>miniPG'</td>
<td>0.55±0.3</td>
<td>3.4±0.02</td>
</tr>
<tr>
<td>midiPG</td>
<td>0.5±0.15</td>
<td>9±0.1</td>
</tr>
<tr>
<td>midiPG'</td>
<td>0.5±0.20</td>
<td>8.8±0.2</td>
</tr>
</tbody>
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See "Experimental Procedures" for labeling method and assay conditions

Clearly, with the addition of only one kringle (in this case, K5), which is closest (at least in term of covalent proximity) to the catalytic domain, a nearly 150-fold increase in catalytic efficiency ($k_{cat}/K_m$) of SK.HPN over that of substrate microPG was generated. Interestingly, with the HPG derivative where both kringle 4 and kringle 5 are attached to the catalytic domain (midiPG), the catalytic turnover increased significantly and became equivalent to almost 3/4$^{th}$ of the $k_{cat}$ value obtained with the full length HPG. Consequently, the catalytic potentiation upon addition of two kringles resulted in overall...
200-fold increase in the catalytic efficiency as compared to the kringle-less derivative, microPG.

These results highlight the importance of kringle domain(s) particularly the 4\textsuperscript{th} and 5\textsuperscript{th} kringles, in substrate HPG activation by SK.HPN activator complex. It is clear that the addition of only one kringle domain to the catalytic domain of substrate HPG brings the affinity of substrate to near-native levels and also significantly increases the catalytic turnover in comparison to the kringle-less derivative, microPG. The addition of another kringle, as in case of midiPG, further augments the activatibility of the substrate by SK.HPN complex. Overall, these results suggest that the presence of kringle 5 alone adjacent to the catalytic domain is able to restore the affinity of substrate to near native levels as compared to the kringle-less derivative, microPG, yet the catalytic turnover achieved is still only one-third of that observed when native full-length HPG is employed as substrate. Furthermore, the observation that addition of one more kringle (kringle 4) to the miniPG brings up the catalytic turnover at the level of 3/4\textsuperscript{th} of that obtained with HPG suggests that although kringle 5 alone is able to “lift” the affinity to near-native, the catalytic turnover has dependency on the other kringles as well. However, the most important revelation that emerges from these kinetic observations is that the gain in substrate affinity does not necessarily exhibit a linear relation with catalytic potentiation seen upon addition of kringles. Fig. 5.4 presents this in a figurative manner and shows that the major gains in both affinity and activity to near-native levels could be achieved with addition of only one or two kringles into the catalytic domain of the macromolecular substrate. It also suggests that kringle 5 is sufficient by itself to reinstate the substrate affinity, however; this inclusion is not able to scale up the activity to native-like levels. Increasing the length of substrate by addition of kringle 4 further adds to catalytic turnover and brings it close to that seen with native HPG containing all the 5 kringles (K1-K5). Also, the fluorescent labeled single-cysteine variant of SK, i.e. SK L260C' showed similar kinetics as observed for nSK (described in Table 3C., Chapter 3).
Fig. 5.4 Effect of sequential kringle additions to the catalytic domain of HPG on substrate affinity and catalytic turnover by SK.HPN enzymatic complex. Curves were generated from steady-state kinetic parameters (Table 5A) for the four different substrate forms of HPG, viz. microPG (catalytic domain, CD), miniPG (K5+CD), midiPG (K4+K5+CD) and full length HPG (K1+K2+K3+K4+K5+CD). All derivatives were expressed in E. coli except full length HPG which was purified from blood plasma. Non-correlation between enzyme-substrate affinity and catalytic activity is apparent in the figure. (See "Experimental Procedures" and Discussion section for details).

Table 5A lists the kinetic parameters for the different HPG derivatives and their fluorescently labeled cysteine variants determined from their activation by SK.HPN activator complex (See "Experimental Procedures" for details).
5.3.3 Pre-steady state kinetics of SK L260C'.HPN mediated catalysis of different HPG derivatives

Rapid kinetics of catalysis employing the different truncated HPG derivatives as substrate by the SK L260C'.HPN activator complex was monitored by Stopped-flow FRET experiments. Rapid complexation of preformed SK L260C'.HPN with either of the fluorescently labeled substrate HPG derivatives under conditions wherein the enzyme is at least two molar excess over the substrate so as to allow only one catalytic cycle, resulted in rapid fluorescence increase due to change in FRET efficiency during catalysis. Fig. 5.5 shows stopped flow traces observed after mixing SK L260C'.HPN activator complex with either of the substrate HPG derivatives. Near saturation, fluorescence increase consequent to enzyme-substrate complexation was observed in ~5 s for the substrate midiPG, whereas, in case of miniPG it took more than 20 s to reach near saturation. Fluorescent traces for microPG under similar conditions saturated in more than 80 s.

In order to establish whether the change in FRET efficiency is consequence of catalytic process, we inhibited the HPN with active site acylating agent, NPGB. The NPGB has a very slow rate of deacylation, hence, it traps the enzyme in acyl-enzyme intermediate (Chase and Shaw, 1969) which cannot further act upon other substrates. However, NPGB inhibited SK.HPN enzyme complex can still combine with the oncoming substrate to make a ternary complex. This allowed us to carry out rapid kinetic studies under conditions that did not turnover the substrate. The NPGB inhibited HPN was complexed with SK L260C' to make an inactive “cofactor.enzyme” complex. Rapid mixing of this complex with substrate midiPG did not show any change in FRET efficiency (See Fig 5.5) hence, it appeared that change in FRET efficiency observed under our experimental condition was related with catalysis. Fluorescent traces for change in FRET efficiency were interpreted according to scheme presented in next section.
Fig. 5.5 Stopped-flow fluorescence traces obtained after rapid complexation of pre-formed SK L260C'.HPN activator complex with different substrate HPG derivatives. Reactions were carried out under pseudo first order conditions with excess of enzyme over substrate (as described in “Experimental Procedures”). Time traces for near-saturation fluorescence increase are shown for each of the three different substrate derivatives used. In a typical Stopped-flow rapid mixing reaction the final concentrations of enzyme (SK.HPN) and substrate (different PG derivatives) were 10 μM and 5 μM respectively. This ensured that upon mixing majority of the substrates reacted rapidly with the preformed SK L260C'.HPN activator complex and hence approached a single turn-over condition. Fluorescent traces for midiPG (a), miniPG (b) and microPG (c) are shown with fitted curves. Curve (d) represents the fluorescent trace obtained with rapid mixing of NPG8 inhibited SK L260C'.HPN complex and substrate midiPG.
5.3.4 Interpretation of Stopped-Flow FRET traces

Stopped flow FRET experiments could be explained according to scheme presented in Fig. 5.6. In a typical catalytic cycle the preformed SK.HPN complex when rapidly mixed with substrate HPG derivatives makes an initial "encounter" complex. Once this initial ternary complex is in place, the post-docking events can be expected to take place, which includes formation of the acyl-enzyme intermediate followed by deacylation and product formation and, finally, product release from the surface of the enzyme. The fluorescence of donor fluorophore present in the 250-loop of β-domain of SK gets rapidly

![Diagram](image-url)

**Fig. 5.6** Schematic representation of fluorescence changes in stopped flow FRET experiments. Donor (IAEDANS) labeled SK and acceptor (QSY® 35 iodoacetamide) labeled substrate HPG derivatives constitute an intermolecular FRET pair. Rapid mixing of preformed SK L260C'.HPN complex with the substrate HPG derivative results in instant quenching of donor fluorophore (i.e. increase in FRET efficiency), however traces of which could not be recorded due to its completion before the instruments' dead time (-2 ms). Rapid rise in fluorescence (decrease in FRET efficiency) which is recordable originates from a post docking event and could be kinetically linked to a particular step of catalysis.
quenched upon substrate binding due to transfer of a part of its energy to the acceptor fluorophore present in the 37-loop of different truncated substrate derivatives. Therefore, substrate docking event probably leads to high FRET efficiency. Any subsequent recovery of fluorescence i.e. decrease in FRET efficiency is likely due to conformational changes in the process of catalysis and finally due to product release. It means that as the substrate reorients itself during catalysis which leads to increase in inter-probe distances, resulting in a decrease in FRET efficiency. FRET is completely annihilated once the product leaves the enzyme’s surface. Under our experimental conditions, phase of donor fluorescence quenching upon rapid mixing of SK L260C’.HPN with either of the substrate was not recordable, thus indicating that initial encounter or the formation of “Michaelis’ complex” completes within the dead time of the instrument (-2 ms) and any subsequent fluorescence changes can therefore be attributed to post-docking events, such as scissile-peptide bond cleavage and product release. Since the acceptor probe (QSY® 35 iodoacetamide) is a non fluorescent species, this allows one to collect purely the emission from the donor fluorophore (IAEDANS).

The time course of fluorescence change during catalysis of all the three substrates showed a rapid fluorescence increase which saturated in different time scale (5 s to 80 s) for the various substrate HPG derivatives used. Fluorescent traces for all the reactions were fitted to biexponential which yielded characteristic rate constants consistent with an early rapid fluorescence change (k’1) followed by a relatively slow increase in fluorescence (k’2) that corresponds to the rate of catalytic turnover (k_cat) as estimated experimentally from equilibrium (steady-state) kinetic studies (Please refer to Table 5A for steady-state kinetic parameters). Table 5B lists the rate constants characterizing two distinct structural transitions observed under rapid kinetics experiments with three truncated substrate HPG derivatives viz. microPG, miniPG and midiPG. It also lists the corresponding turn-over rates (k_cat) for each of them derived from steady-state
experiments. It can be seen that the two different rate constants observed in the fluorescence burst revealed two distinct, kinetically separable events during the catalysis of HPG substrates by SK.HPN activator complex.

**TABLE SB**

<table>
<thead>
<tr>
<th>Substrate derivative</th>
<th>$K'_1$ (min$^{-1}$)</th>
<th>$K'_2$ (min$^{-1}$)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>microPG'</td>
<td>12±2</td>
<td>0.36±0.1</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>miniPG'</td>
<td>22±3</td>
<td>4.0±0.2</td>
<td>3.4±0.02</td>
</tr>
<tr>
<td>midiPG'</td>
<td>84±6</td>
<td>10.0±1.2</td>
<td>8.8±0.2</td>
</tr>
</tbody>
</table>

Fluorescent traces for midiPG' under biexponential fit gave rate constants of 84 min$^{-1}$ ($K'_1$) and 10 min$^{-1}$ ($K'_2$) respectively. The slower rate constant of 10 min$^{-1}$ ($K'_2$) was comparable to the $k_{cat}$ value of -9 min$^{-1}$, observed under equilibrium studies. Rapid reaction kinetics where miniPG' was used as the substrate showed relatively slow rate constants as compared to midiPG' with values 22 min$^{-1}$ ($K'_1$) and 4.0 min$^{-1}$ ($K'_2$) for fast and slow steps, respectively. The rate constant characterizing the slower step in this case also corresponded to the turn-over rate (3.4 min$^{-1}$) of miniPG by SK.HPN activator complex obtained by steady-state kinetics. Rapid reaction traces for microPG were also fitted to biexponential with observed rate constants of 12 min$^{-1}$ ($K'_1$) and 0.36 min$^{-1}$ ($K'_2$) for fast and slower steps respectively. Remarkably, the slower rate constant obtained with microPG' was also corresponding well with its respective $k_{cat}$ value deduced from steady-state kinetic studies.
state kinetics. The slower rate constant observed for microPG was found to be nearly 30 fold and 10 fold less than that observed with midiPG' and miniPG' respectively. Since the rate constants for the slower steps are in consonance with the experimentally determined (steady-state) turn-over rates obtained from Steady-State kinetics, it can be concluded that the slower step results from the release of the product from the enzyme's surface.

As compared to slower phase, similar fold differences were not observed when the rate constants for faster phase were compared. It was observed that fast phase for microPG' was nearly two fold and four fold less rapid in comparison to miniPG' and midiPG', respectively. Since the slower rate constants observed with all the three substrates are in consonance with the \( k_{cat} \), the relatively faster rate constant could be safely assigned to an event that precedes the product release. However, with the present information it is not possible to correlate this conformational change to a particular post-docking event in a definitive manner. It is probable that this could arise most likely due to cleavage of the scissile peptide bond, since large structural transitions in the catalytic domain during this event are likely to occur, as elegantly documented with, X-ray evidence upon zymogen activation (Wang et al., 2000a). Additional support for this conclusion was obtained in the present study when the pre-inhibited (with the irreversible active site inhibitor, NPGB) SK.HPN activator complex was used for the rapid kinetics experiment (See Fig. 5.5). We could not observe any fluorescence increase when the transient kinetics experiment was repeated with active-site inhibited enzyme. Since the NPGB rapidly arrests the enzyme (HPN) in the acylated form, therefore, it cannot further act upon the scissile peptide bond of the non-covalently bound substrate HPG. This suggests that any fluorescence changes observed under uninhibited conditions are dependent on cleavage of the scissile peptide bond. Therefore, it seems probable that the relatively fast rate constant observed upon rapid mixing of SK.HPN enzyme and the substrate is very likely related to an event that obligatorily follows the amidolysis step.
The experimentally observed rate constants (Please refer to Table 5B) further suggest that the slowest and the rate limiting step in the catalytic cycle is probably product release from the enzyme surface. This observation is further supported by the finding that formation of non-covalent enzyme-substrate complex for all the three substrates viz. microPG, miniPG and midiPG completes well within the dead time (-2 milliseconds) of the instrument, therefore, it appears to be a diffusion limited process. This suggests that none of the three HPG derivatives used as substrate are compromised in formation of initial enzyme-substrate complex. Therefore, the differential rate of the catalysis for the three different truncated substrates observed, originated most likely due to differences in the post-docking events that lead to product formation and its release from the enzyme surface.

Furthermore, since the experimentally observed rate constants for the faster phase is multifold higher than the respective turn-over rates \( (K_{cat}) \) obtained for the three truncated substrate derivatives, hence, it appears that the product release step (attributed to the slower rate constant, \( K'_{2} \)) is the major contributor to catalytic turnover for the SK.HPN catalyzed activation of substrate HPG derivatives.

5.4 Discussion

The X-ray crystal structures of the catalytic domain of HPN (microPN) in complexation with either SK or SAK (Wang et al., 1998; Parry et al., 1998) have provided useful insights as to how the binding of ‘co-factor’ SK or SAK to ‘partner’ microPN modulates the change in specificity of HPN towards the activation bond in substrate HPG. Extensive potential contacts between the three domains of SK and substrate microPG were observed by modeling the docking of substrate microPG, with its activation bond Arg 561-Val 562 positioned in the active site of SK-microPN enzyme (Wang et al., 1998). The comparison of this modeled structure with the X-ray crystal structure of the ternary complex of microPN.SAK bound to substrate microPN (Parry et al., 1998; 2000) has
indicated a high degree of similarity between the interaction of substrate microPN with the two activator enzymes viz. SK.microPN and SAK.microPN. The information generated from these structural and modeling studies provides us evidence as to how the interaction of ‘co-factors’ with the catalytic domain of HPN might be able to convert an active site with a generalized trypsin-like specificity into a highly substrate-specific one. Yet, the mechanistic insights regarding the overall catalytic phenomenon i.e. dynamic events such as substrate binding, cleavage of the scissile peptide bond of the substrate HPG and, finally, product release from the enzyme surface have remained a mystery so far. Moreover, the possible conformational transitions occurring during the conversion of substrate HPG to product, HPN, especially ones that may potentially influence the release of the product from the enzyme active site, need to be discerned and studied by solution based approaches.

The possibility that “post-docking” events could also be as vitally important in protein cofactor-mediated catalysis as the initial substrate docking is strengthened by comparing the crystal structure of the catalytic domain of HPG (Wang et al., 2000a) with the structure of the catalytic domain of HPN (Wang et al., 1998). Modeling studies based on the crystal structure of SK.microPN and SAK.microPN (Parry et al., 1998) further indicate that when the proteolytic cleavage is made at the Arg 561-Val 562 bond located in the nonapeptide activation loop of substrate HPG, the structural strain (disulfide bond linking residues Cys 558 and Cys 566 which spans the scissile peptide bond) imposed by this loop is likely to be released, leading to a widespread conformational change in the catalytic domain of substrate microPG. These conformational changes, in fact, have been postulated to cause a decrease in contact surface area between the substrate (microPG) and the SK/SAK.microPN activator enzyme, which likely assists in product dissociation. However, until now these studies were limited only to the use of either microPG, i.e. kringle-less derivative of HPG or the full length HPG containing all the five kringles to draw surmises about possible allostery and protein-protein interactions in the
mechanism of SK.HPN based activation of substrate HPG (Loy et al., 2001; Dhar et al., 2002; Sundram et al., 2003). Moreover, no previous study has attempted to characterize the contribution of individual kringles attached to the catalytic domain for their possible involvement in the catalytic process. In the present study, we recombinantly expressed three different variants of HPG viz. microPG, miniPG and midiPG to explore the role of kringle-mediated protein-protein interactions and their quantitative contributions in the catalytic processivity by SK.HPN activator complex. The results presented in this study clearly established that interactions between substrate HPG and SK.HPN were not ‘local’ i.e. limited only to the serine protease domain, but are likely spread over other relatively remote regions of substrate HPG as well.

It was observed that catalytic turnover by the SK.HPN activator for the kringle-less derivative, microPG, was very less (only 1-2 % ) as compared to that against native full-length substrate HPG, demonstrating that for an optimal activation of substrate HPG by the SK.HPN activator complex, the involvement of long-range protein-protein interactions between kringle domains of the substrate and the enzyme are vital.

The mechanism whereby the kringle domain(s) of substrate HPG in SK mediated activation play a role was further revealed when the steady-state kinetic parameters with one (miniPG) and two kringle derivatives (midiPG) were explored. The results showed that with mere presence of only one kringle domain adjacent to the catalytic domain, a significant rise in catalytic efficiency (nearly 150-fold) over that of the completely kringle-less derivative, microPG, was observed. Interestingly, when midiPG was used as substrate, the catalytic turnover increased further by nearly 200 fold of full-length HPG. Besides this, the results also showed that although the juxtaposition of K5, next to the catalytic domain was able to generate a near-native affinity between substrate miniPG and the SK.HPN activator complex, yet the resultant increment in catalytic potentiation in terms of \( k_{cat} \) rates generated with substrate microPG were still only one-third compared to that observed in case of full-length HPG. However, the presence of two kringles i.e. K4
and K5 in proximity of the serine protease domain further lifted the $k_{cat}$ to nearly $3/4$th of that observed with full length HPG. This suggests that there is significant enhancement in catalytic turnover upon kringle additions to the serine protease domain. These results strongly suggest that in case of the SK.HPN system, merely a rise in substrate enzyme affinity does not automatically correlate with a proportionate increase in catalytic turnover, even though the substrate specificity of the reaction (a key property) may have been substantially enhanced. In other words, despite an apparently native-like docking with a given macromolecular substrate, a native-like catalytic power is not generated automatically. This discrepancy in enzyme-substrate affinity and turnover, observed in case of substrate miniPG and midiPG, strongly suggested that at least in case of the SK.HPN system, substrate specificity and catalytic turnover are likely to be mutually exclusive events. These results thus point towards the dual nature of the kringle-mediated long-range interactions, in that the presence or absence of critical kringle-mediated substrate-enzyme interactions very likely, independently, dictates docking and turnover events. Although our findings with steady-state kinetics revealed a definitive role of kringle mediated interactions in catalytic potentiation, yet, the vital question, as to exactly at which kinetic step the different substrate derivatives differ, still remained elusive. In other words, do the kringles have contribution in substrate binding events or they contribute in the stages that are subsequent to formation of initial enzyme-substrate complex was needed to be explored further. In order to do this, the role of kringles in different steps of catalysis was examined by using the pre-steady state kinetics of SK.HPN mediated activation of substrate HPG derivatives. This was made possible by development of an intermolecular FRET pair in which the donor fluorophore resides in the SK molecule and the nonfluorescent acceptor probe was placed in one of the surface exposed loop (37-loop) of serine protease domain of the different miniature substrate derivatives successfully shown to be good 'models' for the full-length substrate. The observation of significant restoration of affinity and activity with the one- and two-
kringle substrates allowed us to use these as miniaturized models for the full-length substrate for studies requiring site-specific labeling which is not easily feasible with HPG and its variants due to their size and complex domain structure. We reasoned that design of such a system would allow us to actually examine the events taking place within a single catalytic cycle of substrate-to-product conversion. Since the energy transfer efficiency is extremely sensitive to interprobe distances, we reasoned that it is quite possible to detect the conformational transitions taking place during the various catalytic events, and correlate these to particular kinetic steps. Indeed the use of transient kinetic techniques successfully allowed a more detailed temporal dissection of the catalytic process and revealed an early rapid phase, followed by a relatively slow phase. The rate constant for the fast phase \( (K'_4) \) was found to be multifold higher than the actual turnover rates for all the substrate derivatives studied, but the fluorescence changes associated with the slower rate constant \( (K'_2) \) was in close unison with the respective catalytic turnover rates as deduced from steady-state kinetic studies for different substrate derivatives of HPG. Under our experimental conditions, formation of initial enzyme-substrate complex for all the different substrates used was complete within the dead time of the instrument, suggesting that, all the miniature HPG variants under study were indistinguishable at least in terms of the rate of the formation of initial enzyme-substrate complex. If this were not true, we would likely have observed an initial phase of fluorescence decrease due to rapid quenching of donor fluorophore upon substrate binding. The minimalistic conclusion that could be drawn from this observation is that the rate of substrate docking is not actually affected by presence or absence of kringle(s) in the substrate. In other words, the catalytic process of SK.HPN activator complex for different HPG derivatives, whether *kringle-rich* or *kringle-less*, is indifferent at least in the first step i.e. substrate binding. Even though the enzyme-substrate complexation was not rate limiting for different substrate derivatives used, interestingly we observed drastically different rates for the slower phases \( (K'_2) \) for all the truncated derivatives used for study.
Steady-state kinetic studies (Please refer to Table 5A for kinetic parameters) established that the presence of a single kringle i.e. K5 increased the activity to 30 fold as compared to kringle-less derivative. Furthermore, addition of two kringles i.e. both K4 and K5 fostered the activity to a level equal to $3/4^{th}$ of that observed with full length HPG. Remarkably, similar trends were also observed under fast kinetics reaction when the kringle-less and kringle-rich HPG derivatives were used as substrates for SK L260C'.HPN activator complex. Kinetic dissection of the observed rate constants suggested that a relatively fast conformational change that could be attributed to cleavage of scissile peptide bond was much faster than the rate of actual product release for all the three substrates used. This indicated that the rate limitation might not be in the hydrolysis of the enzyme-acyl intermediate, but rather, in the dissociation of the product from the enzyme surface. Furthermore, it appears that the rate of product release is extremely compromised when kringle-less HPG derivative (microPG) is used as substrate for SK L260C'.HPN activator complex. It was also found that the addition of a single kringle, K5, to the catalytic domain increased the $K_{cat}$ significantly, and the contribution of substrate kringle domains became even more prominent when two kringles (K4 and K5) were attached to the catalytic domain. These observations strengthen the conclusion that once the substrate is docked onto the SK.HPN enzyme, the scissile peptide bond hydrolysis occurs in a facile and non-rate limiting manner but a major amplification in the catalytic turn-over is achieved through the relatively fast rate of product release in case of kringle-rich substrates. However, there appears no quantitative linear relationship between sequential kringle addition and the gain in turn-over, since major amplification in $k_{cat}$ (-200 fold) is achieved merely by addition of two out of the 5 kringles present in full length HPG. It is also clear from our results that K4 and K5 are the major contributors towards the catalytic potentiation, while other kringles viz. K1, K2 and K3, together add, very little to the turn-over rate. The present study, thus, establishes the important role of events that are ‘independent’ of the initial
macromolecular substrate docking \textit{per se}, in amplifying the catalytic potentiation of substrate HPG from the basal levels generated by SK.HPN with microPG as substrate, purely through long-range enzyme-substrate interactions centered on the kringle domains of the macromolecular substrate. Therefore, in essence, it appears that kringle mediated long range protein-protein interactions are the molecular epitopes key for the fast decoupling of nascent product from the enzyme surface that this system has exploited during its evolution.

We believe that this study provides the first tantalizing glimpse into the kinetic events subsequent to the post-docking phase of the catalytic reaction in SK-mediated HPG activation. However, more detailed analysis of the individual microscopic events in the catalytic cycle along with an identification of all the important protein-protein contacts between enzyme and substrate would require further, more involved, studies.

In conclusion, our results are consistent with a "self-sustained" model of SK action in which the activator enzyme captures the substrate not only through the catalytic domain (which is indeed a 'primary' requisite, since it is this region of the macromolecule substrate that contains the target of enzyme action, namely, the scissile peptide bond) but, also, through the kringle domains of the substrate as well. Once the substrate is positioned optimally, and the scissile peptide bond is cleaved, the enzyme is probably so "designed" that the product formed rapidly diffuses away from the enzyme surface. In other words, the SK.HPN enzyme is probably evolutionary "programmed" to sense a conformational change in the substrate when it is converted to product, thus decoupling the latter from the enzyme's surface. Since the SK.HPN enzyme is not dependent on an external energy source e.g. ATP/GTP hydrolysis, the molecular motions in substrate consequent to conversion into product, quite likely act as the driving force for the completion of the enzymatic reaction that involves not only the active site \textit{per se} but structural units in both the enzyme and the substrate that are located far away from the active center. There is unmistakable evidence in literature that several discrete regions in
the catalytic domain (microPG) undergo conformational alterations upon conversion to microPN (Lamba et al., 1996; Parry et al., 2000; Wang et al., 2000a). However, by themselves, these cannot be enough to generate the catalytic power associated with the native SK.HPN complex, as evidenced by the fact that the SK.HPN complex acts upon the kringle-less substrate very slowly. From our study, it seems probable that the unique kringle mediated changes observed during the activation of miniPG and midiPG signify the key molecular components of the post-docking events that act as a trigger for the highly efficient release of the product and consequent increase in overall catalytic rates.