Kringles of substrate plasminogen provide a “catalytic switch” in plasminogen to plasmin turnover by Streptokinase

Vandna Sharma1*, Shekhar Kumar1#, Girish Sahni1*

From the 1Division of Protein Science & Engineering, CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh 160036, India.

*Present address: Skaggs School of Pharmacy and Pharmaceutical Sciences, Center for Discovery and Innovation in Parasitic Diseases, University of California San Diego, La Jolla, CA 92093, USA.

#Present address: Research Institute, Children’s Hospital of Philadelphia, Philadelphia, Department of Pediatrics, University of Pennsylvania, Philadelphia, PA 19104.

Running title: Kringle dependent catalytic switch in plasminogen activation

*To whom correspondence should be addressed: Girish Sahni, CSIR Bhatnagar Fellow, 1Division of Protein Science & Engineering, CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh 160036, India; E-mail: girishsahni@gmail.com, +91-9888371548.

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Abstract

To understand the role of substrate plasminogen kringles in its differential catalytic processing by the streptokinase - human plasmin (SK-HPN) activator enzyme, Fluorescence Resonance Energy Transfer (FRET) model was generated between the donor labeled activator enzyme and the acceptor labeled substrate plasminogen (for both kringle rich Lys plasminogen – LysPG, and kringle less microplasminogen - μPG as substrates). Different steps of plasminogen to plasmin catalysis i.e. substrate plasminogen docking to scissile peptide bond cleavage, chemical transformation into proteolytically active product, and the decoupling of the nascent product from the SK-HPN activator enzyme were segregated selectively using (1) FRET signal as a proximity sensor to score the interactions between the substrate and the activator during the cycle of catalysis, (2) active site titration studies and (3) kinetics of peptide bond cleavage in the substrate. Remarkably, active site titration studies and the kinetics of peptide bond cleavage have shown that post docking chemical transformation of the substrate into the product is independent of kringles adjacent to the catalytic domain. Stopped-flow based rapid mixing experiments for kringle rich and kringle less substrate plasminogen derivatives under substrate saturating and single-cycle turn-over conditions have shown that the presence of kringle domains adjacent to the catalytic domain in the macromolecular substrate contributes by selectively speeding up the final step, namely the product release/expulsion step of catalysis by the streptokinase-plasmin(ogen) activator enzyme.

Introduction

Human plasmin (HPG), a single chain multi domain glycoprotein of molecular weight ~92 kDa, is an inactive zymogen form of the serine protease plasmin (PN). It consists of five triple loop structures called kringles linked in a series to the serine protease catalytic domain (CD) (1). Conversion of zymogen form of the plasminogen to its active form plasmin is a key event in physiological fibrinolysis and is broadly employed therapeutic means to alleviate circulatory disorders arising from the obstruction of blood flow by pathological thrombi (2). This zymogen to active protein conversion is mediated by various plasminogen activator proteases that can be categorized as either physiological (intrinsic) and non-physiological activators. Streptokinase (SK), which is of bacterial origin, is one of the non-physiological plasminogen activators and has been a widely used thrombolytic protein especially due to its comparatively
highly favorable economics against tissue Plasminogen Activator (tPA), a physiological activator, produced from animal cell culture (3, 4).

Streptokinase is a single-chain (~47 kDa) polypeptide comprising of three domains (α, β and γ) connected by short, flexible linkers (5). It initiates fibrinolysis by forming a high affinity (in the low nanomolar range) stoichiometric complex with ‘partner’ plasminogen. In this 1:1 equimolar stoichiometric complex, SK acts as a ‘cofactor’. After complexation with SK, plasminogen undergoes a conformationally driven activation step transforming into an amidolytically active enzyme complex (SK-HPG*) which acquires capability to selectively cleave the Arg<sup>561</sup>-Val<sup>562</sup> scissile peptide bond in ‘substrate’ plasminogen and thereby converting the latter into its proteolytically active form plasmin - HPN. These series of events are known as Pathway I, or as the conformational activation pathway (6-8). Besides Pathway I there is an alternative direct proteolytic activation pathway or Pathway II, where SK complexes with HPN (activated form of HPG) to form fully mature SK-HPN activator complex (9, 10). Elegantly designed kinetic studies and domain truncation studies in the past have shown that SK-HPN activator complex utilizes long range protein-protein interactions with the molecular substrate to attain higher order rate of catalysis and these interactions are mediated through the kringles of the substrate or the exosites generated on the cofactor SK or both (11-15). Steady-state kinetic parameters have shown that kringles of substrate are important in modulating the catalytic turnover of the substrate where it has been observed that for kringle rich full length substrate plasminogen (natural HPG from residues 1-791) the catalytic turnover is approximately 100-fold higher than that of kringle less substrate plasminogen, namely μPG (from residues 543-791) in SK-HPN mediated catalysis (16, 17). Strikingly this ‘substrate-assisted’ proteolysis as observed for SK has not been observed for ‘direct’ plasminogen activators like tPA or Urokinase which do not discriminate between kringle rich and kringle less macromolecular substrates (18). Amongst all the HPG activators the catalytic power of the SK-HPN enzyme complex is the highest probably because of productive utilization of the long-range interactions between the activator and the substrate. Although several studies have pointed towards the role of substrate assisted catalysis by SK-HPN activator enzyme, the mechanistic insights into the exact catalytic event/s modulated by the substrate has not been elucidated so far. Comparison of the crystal structures of the zymogen/enzyme pairs of the serine proteases have shown that in most of these zymogens an activation loop is present in the catalytic domains. This activation loop has an activation “scissile” peptide bond (Arg<sup>561</sup>-Val<sup>562</sup>), which upon proteolytic cleavage results in conformational changes (19, 20). One working hypothesis is that conformational transitions which are originated in the activation loop and/or other adjoining regions such as the kringle domains following scissile bond cleavage in the nascent product plasmin may be ‘utilized’ in dis-engagement of the product from proteolytic enzyme (SK-HPN) and facilitating the decoupling of macromolecular complexation between the erstwhile substrate plasminogen and the activator enzyme at the end of each catalytic cycle.

In order to get an insight into the mechanism utilized by kringle rich and kringle less plasminogen derivatives as substrate, a FRET based spectroscopic approach has been utilized as an intermolecular proximity sensor on a stopped-flow apparatus to get real time visualization of the catalytic events happening on a millisecond time scale (21, 22). For its very high sensitivity and flexibility, FRET has been used extensively for studying protein-protein interactions (23-25). Experiments in this study have been designed in such a way that any change in the fluorescence signal in the catalytic chain of events starting from the initial enzyme-substrate docking, on the one extreme followed by catalytic transformation of the docked substrate into product plasmin through scissile peptide bond cleavage and finally to the ‘decoupling’ of the nascent product from the activator enzyme on the other can be confidently captured as distinct FRET signals. Using steady-state kinetics, active site titration studies and FRET based rapid mixing experiments; we have tried to selectively segregate different steps of the plasminogen to plasmin conversion and correlate the role of substrate kringles in its catalytic turnover by taking kringle rich and kringle less plasminogen derivatives as substrate.

Materials and methods

Materials
Full length human plasminogen was purchased from Roche Diagnostics GmBH, Germany. T7 RNA polymerase- promoter based expression vector, pET-23d and *Escherichia coli* strain BL21 (DE3) and Rosetta 2DE3 were procured from Novagen Inc. (Madison, WI). Custom-oligonucleotide primers used for sub-cloning and site directed mutagenesis were supplied by Integrated DNA Technologies. Phenyl Agarose 6XL, Lysine Sepharose 4B, SP-Sepharose™, DEAE-Sepharose™ and Superdex™ matrix used for different chromatographic processes were procured from Pharmacia Amersham-GE, Uppsala, Sweden. HIS-Select® Nickel Affinity gel from SIGMA. Chromozym®PL (tosyl-Gly-L-Pro-L-Lys-pNA) was procured from Roche Diagnostics, USA. L-lysine was purchased from Sigma Inc., St. Louis, USA. Fluorophores (5-[2-(2-iodoacetamido) ethylamino]-1-naphthalene sulphonylic acid (IAEDANS) and Floresceine-5-Maleimide (F5M)) were purchased from Molecular Probes, Portland, OR, USA. All other reagents used were of the highest analytical grade available.

**Expression and purification of recombinant SK and SK mutants**

SK cloned in pET-23d expression vector was expressed intracellularly as inclusion bodies under T7 phage RNA polymerase promoter. Following transformation in *E. coli* BL21 (DE3) cells, single colony was inoculated in 10 mL Luria-Bertani (LB) medium containing ampicillin 100 μg/ml for overnight at 37°C, 200 rpm. 5% v/v primary inoculum was added in 600 mL LB medium supplemented with 100 μg/ml ampicillin at 37°C, 200 rpm. 1mM isopropyl-1-thio-β-D-galacto-pyranoside (IPTG) was used to induce the culture when optical density at 600 nm (OD<sub>600</sub>) reaches 0.5-0.6 and incubated for 4 hours at 40°C, 200 rpm (26). Cells were then harvested and lysed by sonication (30 seconds on and off cycle at 4°C) using lysis buffer (100 mM NaCl, 10 mM Tris Cl pH 8.0 and 1 mM EDTA) and the pellet containing inclusion bodies was solubilized in 8 M urea. Denatured protein was refolded by 10-fold dilution with buffer-A (0.4 M NaCl, 20 mM phosphate buffer pH 7.5) before loading onto Phenyl Agarose 6XL beads and eluted in water. Protein thus eluted was further subjected to ion exchange chromatography on a DEAE-Sepharose column pre-equilibrated with 20 mM Tris-Cl, pH 7.5, and eluted under a linear gradient of 0.0-0.5 M NaCl. Protein purity was analyzed by 10 % SDS-PAGE, and the concentrations were determined by using UV absorbance at 280 nm.

Single site thiol mutant of SK (SK-L260C) was prepared by using QuickChange® mutagenesis kit from Stratagene Inc., USA using two complementary primers having desired mutation at the center, and the presence of mutation was confirmed by DNA sequencing after cloning in *E. coli*. Mutant was expressed and purified using same methodology as the wild type SK.

**Expression and purification of recombinant kringle rich LysPG and mutants**

Kringle rich plasminogen (LysPG) containing all the five kringle and the catalytic domain (residues 78-791) was cloned in pET-11a vector and expressed intracellularly as inclusion bodies under T7 phage RNA polymerase based promoter. Following transformation in Rosetta 2DE3 *E. coli* cells single colony was inoculated in 10 mL LB medium containing ampicillin 100 μg/ml and chloramphenicol 34 μg/ml for overnight at 30°C, 200 rpm. 5% v/v primary inoculum was added in 400 mL LB medium containing 100 μg/ml ampicillin and chloramphenicol 34 μg/ml at 30°C, 200 rpm. 1mM IPTG was used to induce the culture when OD<sub>600</sub> reaches 0.5-0.6 and incubated for 6 hours at 37°C, 220 rpm. Cells were then harvested and suspended in buffer-A containing 5 mM EDTA, 100 mM NaCl, 10 mM Tris-Cl, pH 8.0 and sonicated (30 seconds on and off cycle at 4°C). Following sonication the IB’s were harvested by centrifugation and the pellet thus obtained was washed with buffer-B supplemented with 2 M urea and 2% (v/v) Triton-X100. After washing, IB’s were again harvested by centrifugation and solubilized in 8 M urea and 20 mM BME. Denatured and reduced protein was subjected to *in vitro* refolding using refolding buffer composed of 50 mM Tris-Cl pH 9.0, 1 mM EDTA, 1 M urea, 20% glycerol, 2.5 mM BME, 50 mM NaCl, 1 mM GSH and 0.5 mM GSSG for 48 hours at 4°C. Refolded protein was then dialyzed against 100 mM NaCl, 20 mM Potassium Phosphate buffer pH 7.2, 50 M urea and 1 mM EDTA. Finally, protein was sequestered by affinity binding on Lysine-agarose and eluted under a linear gradient of 0-0.2 M EACA. Protein thus purified was further buffer exchanged using Sephadex G-25 beads against buffer-C containing...
Protein purity was checked on 10% SDS-PAGE.

Expression and purification of recombinant kringle less μPG and mutants

Kringless microplasminogen- μPG (residues 543-791) devoid of all the kringles and containing only catalytic domain was cloned in pET-11a vector and expressed intracellularly as inclusion bodies under T7 phage RNA polymerase based promoter. Following transformation in Rosetta 2DE3 E. coli cells single colony was inoculated in 10 mL LB medium containing ampicillin 100 μg/ml and chloramphenicol 34 μg/ml for overnight at 30 °C, 200 rpm. 5% v/v primary inoculum was added in 400 mL LB medium containing 100 μg/ml ampicillin and chloramphenicol 34 μg/ml at 30 °C, 200 rpm. 1 mM IPTG was used to induce the culture when OD600 nm reaches 0.5-0.6 and incubated for 6 hours at 37 °C, 220 rpm. Cells were then harvested and suspended in buffer-A containing 5 mM EDTA, 100 mM NaCl, 10 mM Tris-Cl, pH 8.0 and sonicated (30 seconds on and off cycle at 4 ºC). Following sonication IB’s were harvested by centrifugation and the pellet thus obtained was washed with buffer-B supplemented with 2 M urea and 2% (v/v) Triton-X100. After washing, IB’s were again harvested by centrifugation and solubilized in 8 M urea and 20 mM BME. Denatured and reduced protein was subjected to in vitro refolding using refolding buffer composed of 50 mM Tris-Cl pH 8.0, 1 mM EDTA, 1 M urea, 20% glycerol, 2.5 mM BME, 1 mM GSH and 0.5 mM GSSG for 48 hours at 4 ºC. Refolded protein was then dialyzed against 20 mM sodium acetate pH 5.0, 50 mM urea, 1 mM EDTA. Finally, protein was sequestered by cation-exchange chromatography on SP-Sepharose and eluted under linear gradient of 0-0.5 M NaCl in 20 mM sodium acetate pH 6.0 (16). Protein concentrations were determined by using UV absorbance at 280 nm and the protein purity was checked on 10% SDS-PAGE.

Preparation of HPN

HPN, is an active form of full length plasminogen- HPG. It was prepared by digesting HPG with urokinase covalently immobilized on agarose beads using a ratio of 300 Plough units/mg of HPG in reaction buffer comprising of 50 mM Tris-Cl, pH 8.0, 25% glycerol, and 25 mM L-lysine at 22 ºC for 10 hours under constant stirring (26).

Assays for studying the activation of plasminogen derivatives by SK

One stage colorimetric assay method was used to measure the kinetics of activation of plasminogen derivatives by SK-HPN activator complex in assay buffer (50 mM Tris-Cl pH 7.4, 100 mM NaCl) supplemented with 0.5 mM chromogenic substrate Chromozym®PL (38). Change in absorbance at 405 nm due to the hydrolysis of chromogenic substrate into p-nitroaniline was measured as a function of time (t) using Molecular Devices Versamax microplate reader at 22 ºC. The activator activities were obtained from the slopes of the activation progress curves as molar concentrations of p-nitroaniline generated following activation of plasminogen substrate into plasmin. Molar extinction coefficient of p-nitroaniline at 405 nm is 9.96 M⁻¹cm⁻¹.

Assays for determining the Steady-State Kinetic Constants for activation of plasminogen derivatives by SK-HPN activator complex
The kinetics of plasminogen activation by SK-HPN activator complex was determined by transferring preformed SK-HPN activator complex (1 nm final concentration) to the reaction wells containing varying concentrations of substrate plasminogens (HPG/ LysPG/ µPG) in assay buffer (50 mM Tris-Cl buffer, pH 7.5) supplemented with 0.5 mM chromogenic substrate Chromozym®-PL. The kinetic parameters of activation were then calculated from Michaelis–Menten (v versus S) plots using non-linear regression in sigma plot (version 10.0) (28).

Quantitative estimation of active site formation by SK-HPN activator complex in plasminogen derivatives

To monitor the active site formation in substrate plasminogen derivatives by preformed equimolar SK-HPN activator complex, a slight modification is made in the classical 4-Nitrophenyl-4-
guanidinobenzoate hydrochloride (NPGB) titration method (6, 29, 30). Here, preformed equimolar complex of SK-HPN (7.5 µM) was first made and then nearly equimolar substrate plasminogen (9.5 µM) was added just before the addition of 100 µM NPGB in 10 mM Na-phosphate buffer pH 7.2. The “burst” of p-
nitrophenol release due to acylation of an active center was monitored at 405 nm as a function of time using Molecular Devices Versamax microplate reader at 22 °C, molar extinction coefficient of p-nitrophenol at 405 nm is 18.5 mM⁻¹cm⁻¹.

SDS-PAGE based kinetics of peptide bond cleavage in substrate plasminogen by preformed equimolar SK-HPN activator complex

SDS-PAGE based kinetics of peptide bond cleavage in substrate plasminogen was performed to compare the rate of Arg⁵⁶¹-Val⁶⁶² scissile peptide bond cleavage in substrate plasminogen by preformed 1:1 equimolar SK-HPN activator complex both in the presence and absence of 100 µM NPGB inhibitor. Both full length- HPG and kringle less derivatives of substrate plasminogen (7.5 µM) were incubated with slight excess of preformed equimolar SK-HPN activator complex (9.5 µM) for different time periods of incubation at 25 °C in 50 mM Tris-Cl buffer, pH 7.5. Following incubation, the reaction was stopped by adding 1X reducing dye. Before analyzing the samples on 10% SDS-PAGE, samples were incubated in the boiling water for 5 minutes. For the reaction in the presence of inhibitor, NPGB was added to the reaction mix before adding the components of the binary and the ternary complex.

Design of fluorophore labeling sites and protein labeling

Single site, free cysteine mutants of SK, LysPG and µPG generated using standard site-directed mutagenesis methods (31) were derivatized with thiol coupling of exogenous fluorophores. Sites were chosen on the basis of their surface accessibility and their proximities during protein-protein interactions. Cysteine mutants generated for this study were derivatized with their respective fluorophores. (1) Labeling of single cysteine SK (SK L260C) with donor fluorophore IAEDANS. Cysteine mutant of SK (SK L260C) is derivatized with donor fluorophore IAEDANS. IAEDANS was dissolved in dimethyl formamide (DMF) just prior to use. SK L260 C was incubated with 10-fold molar excess of DTT for 30 minutes at room temperature under constant stirring prior to labeling, excess DTT was removed by Zeba spin desalting columns pre-equilibrated with labeling buffer (20 mM Tris pH 7.4, 200 mM NaCl and 2 mM EDTA). Concentration of the proteins thus obtained was determined spectrophotometrically at A₃₅₀ using its extinction coefficient. The protein sample to be labeled was then incubated with 10-fold molar excess of IAEDANS in labeling buffer with constant stirring at room temperature for 90 minutes in the dark. Unbound probe was separated from the labeled protein by passing reaction mix through Sephadex G – 25 gel filtration medium under dark conditions. Each fraction thus collected was analyzed for its labeling ratio using equation 1 and stored at -80 °C in dark tubes. (2) Labeling of free cysteine mutants of plasminogen derivatives (LysPg F583C and µPG F583C) with an acceptor fluorophore (F5M). Acceptor fluorophore F5M was dissolved in dimethyl sulfoxide (DMSO) just prior to use and the modification was carried out in labeling buffer (20 mM Tris pH 7.4, 200 mM NaCl and 2 mM EDTA). Samples to be labeled were quantified spectroscopically by A₃₅₀ and the two-fold molar excess of dye was added and the reaction was carried out for 90 minutes in the dark with constant stirring. Free/unbound dye was removed by passing
reaction mix through Sephadex G–25 gel filtration media under dark conditions and the fractions thus collected were analyzed for their labeling ratios (equation 1) and stored in dark conditions at -80 °C.

**Determination of dye to protein labeling ratio**

Dye to protein labeling ratio was calculated using following equation 1 (23):

\[
\frac{[\text{Dye}]}{[\text{Protein}]} = \left( \frac{A_{\text{max}}}{\varepsilon_{\text{dye}}} \right) / \left( \frac{A_{280} - A_{\text{max}} \times \text{CF}}{\varepsilon_{\text{protein}}} \right)
\]

Equation 1:

\[ [\text{Dye}] - \text{Molar concentration of the dye} \]
\[ [\text{Protein}] - \text{Molar concentration of the protein} \]
\[ A_{\text{max}} - \text{absorbance of protein: dye conjugate at } \lambda_{\text{max}} \text{ of the dye (for IAEDANS 336 nm, for F5M 494 nm)} \]
\[ \varepsilon_{\text{dye}} - \text{molar extinction coefficient of the dye, 1cm path length (IAEDANS 6100 cm}^{-1}\text{M}^{-1}, \text{F5M 83000 cm}^{-1}\text{M}^{-1}) \]
\[ A_{280} - \text{absorbance of the protein: dye conjugate at 280 nm} \]
\[ \text{CF} - \text{correction factor for the dye (IAEDANS 0.22, F5M 0.3)} \]
\[ \varepsilon_{\text{protein}} - \text{molar extinction coefficient of the protein, 1cm path length (SK 44762 cm}^{-1}\text{M}^{-1}, \text{LysPG cm}^{-1}\text{M}^{-1} 148970, \mu\text{PG 50500 cm}^{-1}\text{M}^{-1}) \]

The purity of each sample was assayed by comparing SDS-PGE gels of labeled and unlabeled protein samples.

**Determination of spectral properties of labeled proteins**

The spectral properties of the proteins were recorded from 400 nm to 650 nm on a Quattro fluorimeter with an excitation wavelength of 336 nm, slit width of 5 nm and a constant temperature of 25 °C. Samples were prepared by diluting stock solutions to the required concentration in the labeling buffer (20 mM Tris pH 7.4, 200 mM NaCl and 2 mM EDTA). Before the acquisition of emission spectrum, samples were allowed to equilibrate for 10 minutes. The efficiency of energy transfer (E) between the donor and the acceptor was determined from the emission spectra of the donor in the presence of acceptor at donor excitation wavelength from equation 2 (23):

\[
E = 1 - \frac{F(DA)}{F(D)}
\]

Equation 2:

Where F(DA) represents fluorescence of the donor in the presence of acceptor and F(D) is fluorescence of donor alone. Peak excitation/emission wavelength for donor is 336/490 nm and for acceptor is 494/518 nm respectively.

**Stopped-flow based mixing experiments**

Stopped-flow kinetic experiments were performed using Biologic rapid kinetics instrument equipped with four syringes (10 mL) set in a parallel fashion (SFM400). Fluorescence data were collected by a MOS-250 unit equipped with PMT450 (detector) fitted with a long pass filter (495 nm) (Semrock Inc.) and 15 nm fluorescence quartz cuvette, FC15. All the experiments were performed using labeling buffer (20 mM Tris pH 7.4, 200 mM NaCl and 2 mM EDTA) as running buffer in the flow lines and all protein dilutions were made in the same buffer. Four syringes (referred as S1, S2, S3 and S4) were filled with labeling buffer (S1 and S4), SK-HPN activator complex (S2), and substrate plasminogen (µPG or LysPG) (S3). 1:1 equimolar SK-HPN activator enzyme was made just before performing the experiment. The excitation wavelength was 336 nm, and the slit width of 4 nm was used. Fluorescence light emitted from the reaction mixture (520 nm) after transfer of energy from the donor to the acceptor was monitored after passing through an emission long pass filter (495 nm). Series of stopped-flow experiments were performed at 25 °C in the buffer. After rapid mixing, time dependent fluorescence intensity data were recorded from...
the PLP. For reactions in the presence of presence of NPGB inhibitor, SK-HPN activator complex was made in the presence of 10 molar excess of NPGB just before performing the experiment. Each averaged set of data collected was analyzed with the Biokine analysis programme using bi-exponential fit.

Results

Steady-state kinetics of activation of full length- kringle rich and kringle less substrate plasminogens

Steady-state kinetic analysis was performed to examine the role of substrate kringles in SK-HPN mediated catalysis (see materials and methods section). Native and recombinant Plasminogen derivatives with and without kringles attached to the catalytic domain (figure 1 for pictorial representation of substrate derivatives) were analyzed for their activation by preformed SK-HPN activator complex. Kinetic data thus obtained (Table 1) showed that full length plasminogen undergoes nearly two orders of magnitude higher catalytic turnover as compared to that of µPG as substrate under substrate saturation conditions, whereas the apparent affinity (Kₘ) of the two plasminogen derivatives as substrate were approximately five-fold higher for µPG in comparison to full- length plasminogen as substrate, which is in consonance with our earlier observations (13, 16, 37). Previously reported studies using surface plasmon resonance-based biosensor have also shown differential binding constants for the activator and kringle rich/ kringle less substrate derivatives (13, 37). These results clearly show that there are two independent phenomenon which are modulated by kringles of the substrate, one being its enzyme-substrate docking ability (14-16) and the second being its subsequent catalysis. Importantly, this mode of enzyme-substrate interaction as seen for SK, has been observed to be different from physiological activators (uPA) which activates µPG and HPG with almost same catalytic rates (16). With an inquisitiveness to better understand this physiologically important molecular machinery, in the present study, we have specifically focused on unraveling the key mechanistic steps utilized by the enzyme centered on the substrate kringles so as to potentiate catalysis by therapeutically significant SK-HPN activator enzyme complex.

Analysis of the pre-steady-state through NPGB “bursts” during a single-turnover cycle monitored by active site titration

Active site titrant NPGB gives a near-quantitative estimation for the absolute active site concentrations in given SK-HPN enzyme complex solutions (29). To investigate whether the generation of active center in the substrate plasminogen by SK-HPN activator complex is substrate kringles dependent or not, NPGB based active site titration was performed on full length HPG and kringle less µPG as substrates (see materials and methods). The amplitude of a substrate activation burst as observed for SK-HPN activator complex and HPG as substrate was found to be similar to that of SK-HPN activator complex and µPG as substrate (figure 2). This clearly indicates that under pre-steady-state conditions where a slight excess of an enzyme was taken over the substrate, the rate of active site generation in the latter was independent of the presence or absence of kringles around the catalytic triad of the substrate plasminogen.

SDS-PAGE based kinetics of peptide bond cleavage in substrate plasminogens by the preformed equimolar SK-HPN activator complex

SK-HPN mediated plasminogen activation cleaves single chain substrate plasminogen at Arg⁵⁶¹-Val⁵⁶² scissile peptide bond giving bi-chain product plasmin, comprising of a light and a heavy chain. This scissile bond is present in the activation loop (residues 558-566) which is common for both kringle rich and kringle less plasminogen. Cleavage of 92 kDa kringle rich HPG gives ~83 kDa HPN with characteristic two bands corresponding to 60 kDa heavy chain and 23 kDa light chain on 10% SDS-PAGE (32), (figure 3B). Similarly, cleavage of 28 kDa kringle less µPG as substrate gives µPN of molecular weight ~23 kDa corresponding to light chain, (see figure 3D). The SDS-PAGE based analysis of the samples obtained after incubation of substrate plasminogen (7.5 µM) with a slight excess of activator enzyme (9 µM) for different time periods ranging from 10 seconds to 1 hour has shown that for both kringle rich and kringle less substrate derivatives the rate of peptide bond cleavage or the appearance of cleaved product on 10% SDS-PAGE is similar. For both the substrates, cleavage of the scissile peptide bond happens within first 60
seconds of incubation, indicating that the presence or absence of kringles adjacent to scissile peptide bond
328 does not affect its cleavage by the preformed SK-HPN activator enzyme.

329 Further, to check the capability of an active site titrant NPGB that forms a rapid and irreversible
330 stable acyl-enzyme intermediate with proteolytically active enzyme (6, 30), to block the scissile bond
331 cleavage in substrate plasminogen, incubations of activator enzyme with substrate plasminogen derivatives
332 were performed in the presence of 10-fold molar excess of NPGB (see materials and methods; also, figures
333 3A and 3B). SDS-PAGE profile of the samples thus obtained after different time periods of incubation has
334 shown that the inhibitor blocks the peptide bond cleavage of substrate plasminogens completely for the
335 given time periods of incubation.

336 Thus, the results validated the use of NPGB effectively to freeze the reaction at ternary complex
337 stage under experimental conditions where slightly excess of enzyme / activator complex was taken over
338 the substrate concentration, and confirmed the use of NPGB to specifically differentiate the signals obtained
339 before substrate docking, and steps consequent to that i.e. peptide bond cleavage in the substrate, followed
340 by de-coupling (dissociation) of the nascent product from the activator complex.

341 Design of labeling sites, protein labeling and functional characterization of constructs
342 As per available structural information of SK and plasminogen proteins (1, 5, 33, 34), the sites of
343 fluorophore labeling were chosen in such a way that these were surface accessible and the donor and
344 acceptor labeled FRET pairs are proximal enough in the ternary complex (activator enzyme and substrate),
345 so that these can be used as a spectral ruler to monitor the proximity of the complex (21). Accordingly, a
346 single cysteine mutant of SK (L260C; where leucine at 260 position was substituted with cysteine) was
347 constructed for thiol specific IAEDANS as a donor dye having peak excitation wavelength of 336 nm and
348 peak emission wavelength of 490 nm. This site for substitution in SK was present on the tip of a fully
349 exposed hairpin loop – 250 loop in the β domain. Prior studies have shown the role of this loop as a putative
350 binding site for substrate plasminogen through substrate kringles mediated interactions (14, 15) (figure 4A).
351 Similarly, a single and free cysteine containing mutants (F583C; where phenylalanine at 583 position in the
352 catalytic domain was substituted for cysteine) of the two substrate plasminogens were constructed for thiol
353 specific F5M as an acceptor dye having peak excitation wavelength of 494 nm and peak emission
354 wavelength of 518 nm (figure 4B and 4C). This site of substitution in plasminogens is present on the 37-
355 loop of catalytic domain, comparison of crystal structures of zymogen (μPG) and active (μPN) proteins
356 have shown that the 37 loop of the catalytic domain remains unchanged during zymogen to active protein
357 conversion (19). Single site thiol mutants thus designed were generated by site directed mutagenesis and
358 the constructs were expressed recombinantly in E. coli expression system (see materials and methods).
359 Following expression, proteins were labeled with their respective fluorophores (see materials and methods)
360 (figure 4D and 4E) and dye to protein molar labeling ratios for each protein – fluorophore pairs were
361 determined using equation 1. The labeling ratio for SK-IAEDANS pair has been observed to be 0.9 -1.2
362 whereas for plasminogen- F5M pair it has been observed to be in the range of 0.75 -0.85.

363 Fluorophore labeled thiol derivatives, thus obtained, were compared to their wild type constructs
364 for functional properties by steady state kinetics as described under materials and methods section.
365 Comparison of the steady-state kinetic parameters has shown that both thiol substitution and fluorophore
366 based covalent modification of the proteins do not have any significant effect on their kinetic behavior
367 (Table 2), indicating that thiol mutants thus generated and their covalent modifications through fluorophore
368 labelling does not alter the inherent catalytic behavior of the proteins and the modified proteins thus
369 generated can be treated similar to that of wild type proteins in terms of their catalytic properties.

370 Observation of fluorescence behavior of the labeled proteins
371 The labeled proteins thus obtained were further studied for their spectral behavior (see materials
372 and methods). Comparison of emission spectra of donor labeled SK alone (SK-D*) and its 1:1 equimolar
373 binary complex with partner HPN (SK-D*-HPN) at excitation wavelength of 336 nm showed that there
374 was no significant quenching of the donor fluorescence by the partner molecule (HPN) at binary complex
375 formation stage (figure 5A). In the absence of any crystallographic insights on the binary complex of SK

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and full length plasmin as a partner, the results confirm the non-interference of partner (HPN) kringles on the spectral properties of the donor labeled cofactor SK.

From figure 5B, excitation and emission profiles of acceptor (494 nm) and donor (490 nm) labeled proteins have shown a considerable spectral overlap, which validates the use of given donor and acceptor fluorophores as a FRET pair under given experimental conditions. Further to validate the use of donor labeled cofactor SK (SK-D*) and acceptor labeled substrate plasminogen derivatives (LysPg-A* or μPG-A*) as FRET pair to be used as a spectral ruler to monitor different steps of catalysis, fluorescence spectra of the complexes were collected in the presence of an irreversible active site inhibitor – NPGB (figure 5C). From the fluorescence spectra of donor alone (SK-D*) and the donor in the presence of acceptor (either SK-D*-LysPg-A* or SK-D*-μPG-A*), the efficiency of energy transfer (E) is determined from the decrease or the quenching of the donor fluorescence in the presence of acceptor (see materials and methods) using equation 2, and was observed to be approximately 0.30 and 0.35 for kringle less and kringle rich substrate derivatives respectively (figure 5D).

As FRET efficiency is a function of distance and falls off with sixth power of distance between the donor and the acceptor molecules (23), any small change in the distance between the FRET pairs following catalytic events can be sensed by a significant change in the FRET efficiency. The observation of significant FRET efficiency between SK-D*-HPN as an activator enzyme and its equimolar complex with plasminogen derivatives as substrate (either LysPg-A* and μPG-A*) suggests that the FRET model thus developed for the ternary complex between the activator and the substrate can be safely used to follow the reaction dynamics during kinetics.

Pre steady-state Stopped-flow studies

After the proteins were labeled with their respective fluorophores and the FRET model was established between the activator enzyme and the substrate plasminogens, rapid mixing experiments were performed using stopped-flow apparatus (see materials and methods). This experiment was designed to get real-time visualization of the events happening during SK-HPN mediated catalysis of substrate plasminogen(s) on a millisecond time scale and to selectively identify the role of substrate kringles in its catalysis. Concentrations of the activator complex and the substrate were used such that the catalytic events from the single cycle turnover can be observed, hence during the formation of an active ternary complex between slightly higher concentrations of activator (3.5 μM) were mixed with substrate plasminogen 3 μM (either LysPG or μPG). It should be noted that the concentrations of both the activator and the substrate are taken higher than their apparent Kₘ, such that it favors ternary complex formation. Figure 6 shows the pictorial representation of events following the sequential addition of the components of the ternary complex (SK-HPN and LysPG). Docking of a substrate with the activator enzyme brings the FRET pairs proximal enough for the transfer of energy from the donor to the acceptor and a consequent increase in acceptor fluorescence. Post-docking there is scissile peptide bond cleavage in the docked substrate leading to its catalytic transformation into product plasmin and finally the release of the nascent product from the activator complex leading to dissociation of FRET pairs and the concomitant decrease in acceptor fluorescence. By monitoring the change in acceptor fluorescence, association and dissociation of the ternary complex can be readily followed. The components of the active ternary complex were mixed together with instrument dead time of 2.6 milliseconds and the fluorescence traces corresponding to emission from the acceptor fluorophore at donor excitation wavelength of 336 nm were recorded. Following rapid mixing, it was observed that acceptor fluorescence signal started decaying immediately for kringle rich LysPG as substrate and in around 300 seconds for kringle less μPG as substrate. Interestingly, with both the substrate derivatives initial phase of fluorescence gain by the acceptor, corresponding to transfer of energy from the donor to the acceptor during substrate docking was not observed. Presumably, docking of the substrate to the activator enzyme is a very fast step happening within the instrumentation dead time of 2.6 milliseconds, henceforth could not be captured under given experimental conditions where substrate is saturated with activator enzyme ([E] > [S]).

Following enzyme and substrate docking, there is cleavage of scissile peptide bond in the substrate derivatives. SDS-PAGE based kinetics of scissile bond cleavage has already shown that for both kringle
rich and kringle less plasminogen derivatives the rate of bond cleavage is similar. So the observable
differential decay in the acceptor fluorescence signal can be attributed to the final or the product release
step of the catalysis leading to dissociation of the FRET pairs and the concomitant decrease in the acceptor
fluorescence.

Rate constants determined from bi-exponential fitting of the Stop flow fluorescence traces give slow and fast kinetic constants corresponding to \(k_{\text{fast}}\) and \(k_{\text{slow}}\) (Table 3). The kinetic constants for the slower
or the rate determining step has been observed to be 100-fold higher for kringle rich (0.50 s\(^{-1}\)) derivative as
compared to that of kringle less (0.0056 s\(^{-1}\)) substrate plasminogen (figure 7B and 7D). Comparison of
kinetic constants obtained from stop flow traces with that of steady-state kinetic parameters have shown
that the kinetic constants of the slower step are corresponding well with that of turnover rate constants from
the steady-state studies. Results thus obtained conclude that the slower or the rate determining step of
plasminogen catalysis corresponds to dis-engagement of the ternary complex or the dissociation of the
nascent product from the activator enzyme.

Determination of stop flow traces in the presence of NPGB inhibitor (figure 7C and 7E) further
confirms the formation of a stable ternary complex for the given time scale of data collection. It also
confirms that any significant change in the fluorescence signal during the scan can be attributed to the “post
docking” and “post scissile bond cleavage” step of catalysis. From SDS-PAGE analysis and activity assays
in the presence of NPGB it has been shown that NPGB blocks the peptide bond cleavage in the substrate
and the consequent conversion of zymogen into active protein plasmin, further absence of any significant
fluorescence change in acceptor in the presence of NPGB indicates that the trigger required for product
decoupling is provided by the scissile bond cleavage in the substrate by the activator enzyme and is relayed
through the kringles of the substrate because for kringle less substrate delayed decay in acceptor
fluorescence has been observed as compared to that of kringle rich plasminogen derivative. Figure 7A
represents the kinetics of activation of substrate plasminogen derivatives (LysPG and \(\mu\)PG) by preformed
activator complex SK-HPN under substrate saturating conditions both in the presence and absence of NPGB
inhibitor. Observations thus obtained further validates the absence of any catalytic activity in the presence
of inhibitor and shows differential processing of both the substrate derivatives by the activator complex.

Discussion

Amongst all plasminogen activators (as in SAK, tPA, uPA), it has been observed that SK-HPN
activator complex has highest catalytic power likely due to productive utilization of long-
range interactions
between the activator and the enzyme (16, 18). Studies have shown that as compared to uPA, SK undergoes
substrate assisted catalysis where it has been demonstrated through steady state kinetics that presence of
kringles adjacent to the catalytic domain in substrate plasminogen play a significant role in potentiating its
catalysis, whereas for uPA both kringle rich and kringle less substrate derivatives are processed with almost
same rates (11-17). Further analyses of kinetics data obtained from the SK-HPN mediated activation of
kringle rich and kringle less plasminogen derivatives have shown that there is only a 5-fold difference in
Michalis constant \(K_m\) whereas \(k_{\text{cat}}\) shows two orders of magnitude difference amongst two substrate
derivatives, with catalytic turnover of \(\mu\)PG approximately 2\% as compared to that of full length HPG. These
observations from steady state kinetics studies reveal that following enzyme-substrate docking, kringles
adjacent to catalytic domain in substrate plasminogen, even though they are distinctly apart from the
targeted scissile peptide bond cleavage site, likely play a very significant role in potentiating its catalysis
in SK-HPN mediated activation. Thus, a compelling need exists to explore the mechanistic steps utilized
by this machinery in potentiating catalysis to about 100-fold by utilizing these long-range interactions.

In the present study, therefore, we have segregated this system into different steps of catalysis.
Following enzyme-substrate complex formation, or, at the “post docking” step there is chemical
transformation of substrate into product which in this case is the generation of an active center in the
substrate plasminogen after scissile peptide bond cleavage. Following this chemical transformation step
there is a product release step in the catalysis cycle. We developed event specific methods to pointedly
determine the role of substrate kringles during these different steps of catalysis. In order to understand the
possible role of substrate kringles in the first, namely the chemical transformation step (1) NPGB based
active site titrations were performed to quantitate proteolytic active centers generated in substrate plasminogen derivatives by SK-HPN, and (2) SDS-PAGE based time-order studies were performed to compare the rate of peptide bond cleavage in both the substrates (with and without kringles) by the SK-HPN. Finally, to obtain a time dependent and spatial visualization of the events following enzyme substrate complex formation leading up to product dissociation a (3) FRET based rapid mixing experimental approach was utilized.

In the studies reported herein, quantitative estimation of active centers generated following enzyme-substrate complex formation have shown that for both kringle rich and kringle less substrate derivatives the generation of SK-HPN mediated active center is similar, indicating that kringles adjacent to catalytic domain have little or no influence on post-docking active center generation in the catalytic domain of the substrate. Further, SDS-PAGE based studies have revealed that the rate of Arg561-Val562 scissile peptide bond cleavage is similar for both the substrate derivatives, further confirming that kringles do not have any significant role in the chemical transformation of substrate plasminogen into the product (plasmin) following enzyme substrate complex formation. Further, we obtained time dependent and event specific information on catalytic events following enzyme- substrate complex formation up to enzyme-product dissociation, by first developing an intermolecular FRET model between the activator enzyme and the substrate plasminogen using donor and acceptor fluorophore pairs, respectively. This is for the first time that insights into the macromolecular associations and dissociations were captured for this catalytic machinery and the kinetic information so obtained derived to specifically determine the rate limiting steps of catalysis. The FRET model, that we established successfully, was used to determine rapid reaction kinetics of SK-HPN mediated activation of kringle rich and kringle less substrate plasminogens using stopped-flow based rapid mixing experiments under pseudo- first order conditions where an excess of enzyme (i.e. the preformed equimolar SK-HPN) was taken to carry out a single turnover of substrate to its product within millisecond time resolution. Fluorescence traces obtained from rapid mixing of donor labeled activator and acceptor labeled substrate derivatives have shown that kringles contribute significantly in decoupling the nascent product from the activator complex following substrate to product conversion. Rate constants determined from stopped-flow traces show that kinetic constant for the slowest (rate determining) step is approximately 100-fold lower for kringle less substrate than that of kringle rich substrate plasminogen, and thus elegantly correlates with the respective steady state parameters for catalytic turnover. Interestingly, from the stopped-flow data it is observed that it is the complex dissociation or product decoupling step which is the rate determining step in the catalytic scheme. This data has clearly shown that substrate kringles provide the critical “catalytic switch” by enhancing the rate of product release by nearly two orders of magnitude compared to kringle less substrate.

One plausible explanation for this mechanism can be that following scissile bond cleavage, strain in the activation loop is released which induces conformational changes in the catalytic domain which are further transduced through the kringles present adjacent to the catalytic domain. These relatively large conformational changes in the nascent product smoothly facilitate the dissociation of the latter from the enzyme- product complex (35). By contrast, in case of kringle less substrate, it seems logical that no such relay of signals is possible, due to the absence of kringles adjacent to catalytic domain, resulting in observable delayed release of nascent product from the activator complex. To further validate that this trigger to initiate complex dissociation is initiated by scissile bond cleavage, experiments were performed in the presence of NPGB inhibitor which blocks the SK-HPN active site. In the presence of NPGB, the substrate docking step is not affected as evidenced from the FRET signal, but it completely blocks the peptide bond cleavage in substrate plasminogen and fixes the reaction at the ternary complex stage (SK-HPN-substrate plasminogen). Indeed, this is validated from the activation assays, fluorescence signal and SDS-PAGE profile for ternary complexes in the presence of NPGB inhibitor. The results thus obtained strongly establish that for the release of substrate from the activator complex, a trigger is provided by the scissile bond cleavage and the signal thus originated in the catalytic domain is further relayed through the kringles of the substrate (19, 35) to facilitate the release of product from the activator complex, thereby uncoupling the initial enzyme-macromolecular substrate complexation across relatively large surface areas in both entities.
Domain truncation and mutagenesis studies have already established the role of long-range protein-protein interactions between the domains of SK and the kringles of plasminogen in accelerating plasminogen activation (36, 37). The role of various surface exposed exosites in SK cofactor in modulating the catalytic turnover of the substrate plasminogen has been well explored (11,13,15,17,38). The SK mediated activation of substrate plasminogen probably involves various conformational organizations and re-organizations at the cofactor, partner and substrate levels. Apart from mutagenesis studies cited above, we have obtained direct evidence of distinct conformational alterations in each catalytic entity from Small-angle X-ray scattering (SAXS) based studies as well (Ashish, Sharma, Sahni et al., Unpublished). That these conformational transitions, are the sole driving force for the catalytic turnover of the macromolecular substrate by the SK-HPN activator enzyme is strongly suggested by the present results (which are in sharp contrast to, say, the ATP driven conformational-catalytic enzyme systems in the genetic machinery of eukaryotic cells) and provide a tantalizing peep into the catalytic mechanism whereby the SK attaches itself to human plasmin (a non-specific protease) and converts it into an extremely specific enzyme (39, 40).

In the present study, thus, for the first time, we have reported the role of substrate plasminogen kringle domains in the ‘decoupling’ of the product from the activator complex and thereby controlling the rate limiting or the product-release step of catalysis. Unpublished data from the lab have shown that there are also certain ‘hot-spots’ in the cofactor-SK, which assist the substrate kringles in the release of the product from the activator complex, thus providing evidence that this ‘machinery’ is very tightly regulated both at the activator and the substrate level. With a better understanding of mechanistic approaches utilized in this system, and further identification of the exact interacting sites in SK as well as the kringles of plasminogen substrate, one can potentially re-engineer the system to accelerate the activation reaction, leading to the successful designing of improved SK based bio-therapeutics of the future.

Abbreviations

HPG, Human Plasminogen; PG, Plasminogen; µPG, microplasminogen; SK, Streptokinase; HPN, Human Plasmin; µPN, microplasmin; CD, Catalytic Domain; FRET, Fluorescence Resonance Energy Transfer; NPG, 4-Nitrophenylguanidinobenzoate hydrochloride; K_m, the Michaelis-Menten constant; k_cat, rate of catalysis at substrate saturation; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IAEDANS, 5-[2-(Iodoacetamido) ethylamino]-1- naphthalene sulphonic acid; F5M, Floresceine-5-Maleimide; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis, BME- β-mercaptoethanol, EDTA-Ethylenediaminetetraacetic acid, EACA- Epsilon-aminocaproic acid.

Author Contributions

GS conceived and supervised the project. VS designed and executed all the experiments. Both GS and VS analyzed the corresponding results and prepared the manuscript. SK contributed in initial set up of the experiments.

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Competing Interests

The authors have declared that they have no conflicts of interest with the contents of the article.
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Table 1. Steady-state kinetic parameters for the activation of plasminogen derivatives by equimolar SK-HPN activator complex. The kinetic parameters for activation of full length human plasminogen - HPG, recombinant native like kringle rich LysPG and recombinant kringle less µPG were determined at 22 °C in 50 mM Tris-Cl, pH 7.4, 100 mM NaCl and 0.5 mM chromogenic substrate as described under materials and methods. Data represents the mean of three independent determinations.

| Activator protein | Substrate | K_m  (µM) | k_cat (min⁻¹) | k_cat/K_m (min⁻¹ µM⁻¹) |
|-------------------|-----------|-----------|---------------|------------------------|
| SK-HPN            | HPG       | 0.40 ± 0.05 | 11.01 ± 0.50  | 27.50 ± 2.00           |
| SK-HPN            | LysPG     | 0.40 ± 0.08 | 8.50 ± 0.60   | 19.76 ± 0.02           |
| SK-HPN            | µPG       | 2.00 ± 0.10 | 0.18 ± 0.03   | 0.13 ± 0.03            |

Table 2. Comparison of steady-state kinetic parameters for activation of plasminogen derivatives (labeled/unlabeled) by equimolar SK-HPN activator enzyme (labeled/unlabeled). The kinetic parameters for activation of recombinant kringle rich LysPG and recombinant kringle less µPG by SK-HPN activator enzyme were compared with their fluorophore labeled derivatives LysPG-A*, µPG-A* and SK-D*-HPN at 22 °C in 50 mM Tris-Cl, pH 7.4, 100 mM NaCl and 0.5 mM chromogenic substrate as described under materials and methods. Data represents the mean of three independent determinations.

| Activator protein | Substrate | K_m  (µM) | k_cat (min⁻¹) | k_cat/K_m (min⁻¹ µM⁻¹) |
|-------------------|-----------|-----------|---------------|------------------------|
| SK-HPN            | µPG       | 2.00 ± 0.10 | 0.18 ± 0.03   | 0.13 ± 0.03            |
| SK-HPN            | LysPG     | 0.43 ± 0.08 | 8.50 ± 0.60   | 19.76 ± 0.02           |
| SK-D*-HPN         | µPG-A*    | 1.80 ± 0.30 | 0.15 ± 0.02   | 0.08 ± 0.00            |
| SK-D*-HPN         | LysPG-A*  | 0.40 ± 0.05 | 8.20 ± 0.50   | 20.50 ± 0.80           |
Table 3. Rate constants of different conformational transitions during donor labeled SK-D*-HPN mediated activation of acceptor labeled substrate plasminogen derivatives (µPG-A*/LysPG-A*). Fluorescent traces corresponding to rapid mixing reactions were fit by a biexponential model and rate constants corresponding to a fast step (k_\text{fast}) and the rate limiting slower step (k_\text{slow}) were determined. The table also compares the stopped-flow parameters thus obtained with that of steady-state parameters.

| Substrate          | k_\text{fast} (-1 s\(^{-1}\)) | k_\text{slow} (-1 s\(^{-1}\)) | k_\text{cat} (s\(^{-1}\)) |
|--------------------|-------------------------------|-------------------------------|--------------------------|
| Kringle rich       | 127.0 ± 0.8                   | 0.5                           | 0.14 ± 0.01              |
| LysPG-A*           |                               |                               |                          |
| Kringle less       | 8.0 ± 1.0                     | 0.0056 ± 0.0009               | 0.0025 ± 0.0005          |
| µPG-A*             |                               |                               |                          |

Figure Legends

Figure 1. Pictorial representation of different plasminogen derivatives used in this study. (A) Full length Human plasminogen – HPG (residues 1-791) showing five kringles (K1, K2, K3, K4 and K5) attached sequentially to the catalytic domain (CD). (B) Kringle rich LysPG (residues 78-791) containing five kringles attached sequentially to the CD. (C) Kringle less µPG (residues 543-791) having CD alone.

Figure 2. NPGB based active site titration of plasminogen derivatives by SK-HPN activator complex. Preformed equimolar SK-HPN (7.5 µM) activator enzyme was added to assay buffer containing 10 mM Na-phosphate buffer, pH 7.2. Slightly more than equimolar substrate plasminogen derivative/s (kringle rich (HPG) or kringle less (µPG)) at 9.5 µM final conc. was added to reaction buffer followed by immediate addition of 100 µM NPGB. The “burst” of p-nitrophenol release was monitored spectrophotometrically at 405 nm (see materials and methods section for details). The curves shown represent molar concentration of p-nitrophenol generated following NPGB hydrolysis by SK-HPN alone (solid triangles), SK-HPN with HPG as substrate (solid squares) and SK-HPN with µPG as substrate (open spheres).

Figure 3. SDS-PAGE based kinetics of SK-HPN mediated peptide bond cleavage in kringle rich and kringle less plasminogen substrate. For all the profiles M represents prestained marker (kD) from puregene, lane 1: 9.5 µM SK (48 kDa) alone, lane 2: 9.5 µM HPG (60 and 23 kDa) alone, lane 3: 9.5 µM SK-HPN binary complex, lane 4: 7.5 µM substrate plasminogen alone (92 kDa for kringle rich PG and 28 kDa for kringle less µPG), lane 5: ternary complex (7.5 µM substrate plasminogen with 9 µM SK-HPN) after 10 seconds of incubation, lane 6: ternary complex after 1 minute of incubation, lane 7: ternary complex after 2 minutes of incubation, lane 8: ternary complex after 5 minutes of incubation, lane 9: ternary complex after 10 minutes of incubation, lane 10: ternary complex after 30 minutes of incubation, lane 11: ternary complex after 60 minutes of incubation. (A) kinetics of peptide bond cleavage in kringle rich substrate in the presence of 100 µM NPGB inhibitor. (B) kinetics of peptide bond cleavage in kringle rich substrate without NPGB inhibitor. (C) kinetics of peptide bond cleavage in kringle less substrate in the presence of 100 µM NPGB inhibitor. (D) kinetics of peptide bond cleavage in kringle less substrate without NPGB inhibitor. Incubations were performed at 25 °C and the reaction was stopped by adding 1X reducing dye.

Figure 4. Cartoon representation of fluorophore labeling sites on protein and the reaction scheme for fluorophore labeling at free thiol group of cysteine. (A) Full length streptokinase showing all three
domains (α, β and γ) reconstructed using Modeller software and 1BML pdb file as a template. Residue in yellow sphere, L260C (where Leucine 260 is mutated to cysteine) represents labeling site. (B) Catalytic domain of µPG, PDB ID 1Qrz, showing fluorophore labeling site F583C (where Phenylalanine 583 is mutated to cysteine) as a yellow sphere, residues in red (R561, V562) shows sites for scissile peptide bond cleavage. (C) Full length LysPG (showing all the kringles and CD) reconstructed using Modeller software and 1DUU pdb file as template. Residue in yellow sphere, F583C (where Phenylalanine 583 is mutated to cysteine) represents labeling site, residues in red (R561, V562) shows sites for scissile peptide bond cleavage. (D) Reaction scheme for the labeling of acceptor fluorophore F5M at free thiol group of cysteine-583 position of plasminogen derivatives (E) Reaction scheme for the labeling of donor fluorophore IAEDANS at free thiol group of cysteine-260 position of streptokinase.

Figure 5. Fluorescence spectra of the fluorophore labeled proteins. (A) Fluorescence emission spectra of donor (IAEDANS) labeled SK alone (3.5 µM) (solid squares) and its 1:1 equimolar complex with HPN (open squares) at an excitation wavelength of 336 nm. (B) Curves represent excitation profile of donor labeled SK (black solid lines), acceptor labeled µPG (red solid lines), acceptor labeled LysPG (green solid lines) and emission profile for donor labeled SK (black segmented lines), acceptor labeled µPG (red segmented lines) and acceptor labeled LysPG (green segmented lines). (C) Spectral profile showing quenching of donor fluorescence (at 3.5 µM) in the presence of acceptor labeled substrate plasminogen derivatives (3 µM) and 10 molar excess of NPGB inhibitor (30 µM) at donor excitation wavelength of 336 nm. Curve 1- represents donor labeled SK alone, curve 2- donor labeled SK in the presence of acceptor labeled µPG and curve 3- donor labeled SK in the presence of acceptor labeled LysPG. (D) Determination of FRET efficiency as calculated from the quenching of the donor fluorescence in the presence of acceptor. For all the spectra, temperature was maintained at 25 °C and the buffer used was 20 mM Tris-Cl pH 7.4, 200 mM NaCl and 2 mM EDTA.

Figure 6. Schematic model interpreting rapid mixing experiments. (A) Preformed equimolar donor labeled SK-D*-HPN activator enzyme was mixed with acceptor labeled kringle rich LysPG-A* (or kringle less µPG-A*) as a substrate, forming a ternary complex. This ternary complex formation brings the two fluorophores (represented as red and blue flags) in close proximity resulting in energy transfer from the donor to the acceptor fluorophore (shown as yellow color in the figure). Following substrate docking, there is a catalytic transformation of substrate to product plasmin through scissile peptide bond cleavage and finally product release, resulting in the decoupling of newly formed product HPN (or µPN with µPG as substrate) from the complex and consequent dissociation of FRET pairs. (B) In the presence of active site inhibitor NPGB, there is a formation of ternary complex but the catalytic steps following complex formation (i.e. peptide bond cleavage and the product release) are blocked. Color code: green -SK; orange- HPN as a binary partner; purple- kringle rich Plasminogen as a substrate molecule; black- NPGB; red flag- donor fluorophore IAEDANS, blue flag- acceptor fluorophore F5M.

Figure 7. Stopped-flow traces for activation of substrate plasminogen derivatives by preformed activator enzyme. (A) Assay curves showing activation of kringle rich and kringle less substrate plasminogen by 1 nm preformed equimolar SK-HPN activator complex in the presence and absence of NPGB inhibitor, 2 µM LysPG (solid squares) and 2 µM µPG (solid spheres) without NPGB and 2 µM LysPG (open squares) and 2 µM µPG (open spheres) in the presence of 10 molar excess of NPGB inhibitor. Assay curves represents molar concentration of p-nitroaniline generated following hydrolysis of Chromozym®PL. Reaction was carried out in assay buffer (50 mM Tris-Cl pH 7.5, 100 mM NaCl) supplemented with 0.5 mM Chromozym®PL, (see materials and methods). Data represent the mean of three independent determinations (B) Fluorescence traces for the rapid mixing of donor labeled activator enzyme with acceptor labeled kringle rich substrate plasminogen under pseudo first order conditions where a slight excess of enzyme (3.5 µM) over substrate (3 µM) is taken. The mixture was excited at 336 nm and the emission from the acceptor (520 nm) following energy transfer from the donor was monitored after passing.
through an emission long pass filter 495 nm (C) Fluorescence traces for the rapid mixing of donor labeled activator enzyme in the presence of 10 molar excess of NPGB with acceptor labeled kringle rich substrate plasminogen under similar experimental conditions. (D) and (E) represents fluorescence traces for the rapid mixing of donor labeled activator enzyme with acceptor labeled kringle less substrate plasminogen under similar reaction conditions in the absence and presence of NPGB, respectively. Fluorescence traces thus obtained were then fitted to a bi-exponential model using Bio-kine32 V4.66 software (shown as a thin red line superimposed on each relaxation curve) and the kinetic constants were determined.
(A) Activator Enzyme (SK-D⁸-HPN) + Substrate (LysPG-A⁸) → Ternary complex (Activator Enzyme-Substrate) → Activator Enzyme + Product (Plasmin)

(B) Activator Enzyme (SK-D⁸-HPN) + NPGB inhibitor + Substrate (LysPG-A⁸) → Ternary complex (Activator Enzyme-Substrate) → No Reaction
