SUMMARY

The present treatise is devoted to the exploration of the mechanism of catalysis of human plasminogen activation by the streptokinase-plasmin(ogen) complex. The complex, also referred to as an "activator complex", is a highly specific protease with attractive properties, both from an enzyme mechanism point of view, as well as its medical importance as a widely used "clot buster", or thrombolytic drug.

Thrombolytic therapy is used for patients with circulatory diseases such as acute myocardial infarction, ischaemic stroke, deep venous thrombosis etc. (Abdelouahed et al., 1995) to dissolve pathological blood clots. This phenomenon is known as fibrinolysis, which is mediated by thrombolytic agents that convert the inactive zymogen, plasminogen (PG), or profibrinolysin, in blood circulation, by specifically cleaving the "target" scissile peptide bond between residues Arg561 and Val562 in PG, to its active serine protease enzyme form, plasmin (PN) or fibrinolysin, which dissolves the blood clot into soluble degradation products. Plasminogen activation by the bacterial activators like Staphylokinase (SAK) and Streptokinase (SK) is mechanistically distinct from that by the physiological activators urokinase (UK) and tissue plasminogen activator (tPA). While UK and tPA are enzymes, and directly act on the scissile peptide bond of HPG, thus mediating the specific cleavage of HPG to convert the substrate into product-plasmin, the bacterial activators like SAK and SK are not enzymes and the term "kinase" is a misnomer for the bacterial activators. Rather, these bacterial activators are 'cofactor' proteins which bind equimolarly to HPG or HPN with high affinity, to confer substrate specificity to the bound HPN in the complex, such that the otherwise proteolytically indiscriminate active centre of plasmin converts to a specific one, that specifically cleaves the substrate's single-chain to form the bi-chain product, HPN.

Although there has been considerable exploration towards an understanding of the biology of thrombus formation, one cannot ignore the necessity to more critically explore insights whereby one can design an improved drug for the treatment of thrombotic disease which will specifically target the thrombosis without affecting the hemostasis of the body system. To address these issues, one needs to obtain an in-depth understanding of the structure-function correlations associated with a given thrombolytic agent. Among all the bacterial and physiological PG activators, SK is

the drug of choice especially in the developing countries because of its relatively lower cost and higher *in vivo* half life compared to the expensive UK and tPA. We can thereby extend this knowledge on SK and develop clinically approved SK-based drugs especially for the economically poor nations by bringing to them more affordable thrombolytics. In the past, WHO reports have repeatedly highlighted the need to address the severity of this serious problem in both affluent and developing societies.

Considerable efforts have already been made in the last few decades to acquire deeper mechanistic insights into the underlying mechanism of protein 'cofactor' role of SK in catalysis (Markus and Werkheiser, 1964; Reed et al., 1995; Lin et al., 1996; Nihalani et al., 1997; Nihalani et al., 1998; Esmon and Mather, 1998; Boxrud et al., 2000; Dhar et al., 2002; Sundram et al., 2003; Yadav et al., 2008; Aneja et al., 2009; Yadav et al., 2011; Aneja et al., 2013; Gladysheva et al., 2007). Taken together, these studies have shown the role of SK in non-proteolytic activation of zymogen plasminogen (Wang et al., 2000b; Gladysheva et al., 2007), in high affinity binding to partner PG (Nihalani and Sahni, 1995; Reed et al., 1995; Nihalani et al., 1997; Nihalani et al., 1998) and in efficient processing of substrate PG (Lin et al., 1996; Yadav et al., 2008; Aneja et al., 2009; Yadav et al., 2011). However deciphering the molecular details of the mechanism and associated structure-function interrelationships whereby SK modulates the substrate preference of the active site of plasmin(ogen) after it forms an equimolar complex with plasmin(ogen) is complicated due to the large size of the multi-domain proteins involved in the activation process and also the flexible regions in these proteins which makes crystallization of the ternary HPG.SK.HPG complex difficult.

Studies in our laboratory (Dhar et al., 2002; Yadav et al., 2008; Aneja et al., 2009; Yadav et al., 2011) have revealed the structural elements in SK that contribute towards substrate specificity and processivity to the SK.HPN complex. SK expresses "new" extended sites away from the active site of the SK.HPN complex termed as "exosites" which have critical roles in substrate recognition and docking, substrate specificity and also probably in processing of the substrate to product. Elegant demonstration of extended interactions via "exosites" between the SK.HPN activator enzyme complex and the macromolecular substrate HPG has been made using steady–state kinetic methods (Dhar et al., 2002; Yadav et al., 2008; Tharp et al., 2009;

Aneja et al., 2009; Yadav et al., 2011). These studies have deciphered surface-exposed loops in SK which although are located far away from the enzyme active site, these extended sites or exosites have important role in catalysis. Though the 250-loop has a role in substrate-enzyme affinity and imparts specificity to the SK.HPN activator complex towards substrate (Dhar et al., 2002), the other surface-exposed loops in SK like 88-97 loop, 170 loop, coiled-coil region (Yadav et al., 2008; Aneja et al., 2009; Yadav et al., 2011) probably also play major roles in recognition and uncoupling of the substrate after scissile peptide bond cleavage even though they have minimal role in substrate-enzyme affinity per se.

Inspite of the fact that the catalytic domains of the serine proteases of coagulation cascade show high homology among themselves and with the proto-typical digestive protease trypsin (Rose and Di Cera, 2002) still they show a very narrow and distinctive specificity towards the protein substrate (Bode et al., 1997) unlike trypsin which can cleave indiscriminately after virtually every exposed lysine and arginine in any protein (Roach et al., 1997). The concept of "exosites" in blood coagulation enzymes was introduced in 1977 by John Fenton (Bing et al., 1977). Blood coagulation proteases (enzymes) exhibit high specificity for recognition of substrate, inhibitor or effector molecule via exosites on surface regions of the catalytic domains, located away from the active site (Fenton et al., 1988; Krishnaswamy, 2005). For example, thrombin, a key enzyme of the blood clotting cascade, has a trypsin-like specificity and cleaves at the Arg residues of substrate but differs from trypsin action by selective cleavage at specific Arg residues through interactions, via exosite(s) (I or II or both) which act as recognition site(s), positioned away from the active site (see Figure 2.6, and 2.7) and regulates a network of activities. Factor D is a complement serine protease, responsible for initiating protease action in the alternative pathway of complement activation, which exhibits induced fit mechanism of specificity (Narayana et al., 1994). Though it exhibits similar structural fold as serine protease, there are critical loops that play role in catalysis and substrate specificity. Factor D has a distorted catalytic triad and a somewhat deformed S1 pocket; it circulates in this self-inhibited inactive form and gets activated only in the presence of its natural substrate C3b-complexed factor B (Page et al., 2005). On the contrary, in the coagulation cascade there are additional cofactor-mediated protein-protein interactions (as explained in Figure 2.8) to improve the specificity towards

substrate. Blood coagulation probably arose during the process of evolution from the complement system and immune response/host defense mechanism (Krem and Di Cera, 2001). In the complement cascade, MASP-2 CCP exosite interaction is proposed to have role in correctly orienting the protease towards C4 substrate and with this interaction, there is a chain of domain movements and conformational adjustments (in both the enzyme and the substrate) which stabilise the R-loop (containing the scissile bond) conformation for efficient cleavage at the scissile bond (Kidmose et al., 2012). Thus, in principle, exosites function to confer specificity in the protease for the substrate via enzyme-substrate affinity and aid in the optimal stereochemical docking of the substrate towards the enzyme's active site cleft for efficient cleavage at the scissile peptide bond.

The solved crystal structures of the SK.μPN complex and the isolated β-domain of SK (Wang et al., 1998; Wang et al., 1999b) indicate that there are multi-site protein-protein interactions between all the three domains of SK and the partner microPN, but still large surfaces are available to interact with the oncoming macromolecular substrate with definite specificity. This high resolution structure, however, gives no definitive clue as to the molecular details of the contributions that the SK.HPN complex makes towards substrate recognition and processivity via multi-site protein-protein interactions.

It has been shown by several groups (Summaria and Robbins, 1976; Shi and Wu, 1988; Wang et al., 1999a) that the isolated light chain of PG i.e. μPG is capable of forming 1:1 equimolar complex with SK that shows nearly the same extent of HPG activator activity as the complex between full length HPG and SK, giving the clear indications that kringles are not important for the formation and functioning of 1:1 equimolar complex between SK and HPG. Instead, the kringles are extremely important in SK-mediated catalysis (Joshi et al., 2012). It has been shown long back (Shi et al., 1990; Sundram et al., 2003) that the extent of activation of μPG as a substrate by activator complex was much less as compared to activation of full length HPG by the SK.HPN activator complex. Recent mechanistic studies (Tharp et al., 2009; Joshi et al., 2012) have strongly pointed towards the important role of the kringle domains of substrate HPG in catalysis as well. The kringle domain, although apparently some distance away from the scissile peptide bond in the catalytic domain of the substrate, are required for efficient turnover by the SK.HPN activator complex.

However, it is not very clear how exactly they contribute towards catalysis. Studies by Dhar et al., 2002 do clearly establish the interaction of the substrate HPG kringles with the 250-loop of SK in the activator complex, even though which kringle/s precisely is involved was not established. Later, Joshi et al. (2012) identified the important role of kringles 4 and 5 in potentiating the catalysis. The exact conformational juxtaposition/involvement however is not known because the 'actors' involved, namely SK.HPN and substrate HPG, are large multidomain proteins not yet amenable to direct structural analysis by NMR or crystallography. Even the X-ray crystal structure of the full length HPG has only recently been solved (Law et al., 2012) (see Figure 2.15) even though the structures of a number of individual domains of HPG were established earlier, for example, the structure of kringle 1 (Rejante and Llinás, 1994a; Rejante and Llinás, 1994b; Mathews et al., 1996), kringle 4 (Atkinson and Williams, 1990; Mulichak et al., 1991) and kringle 5 (Chang et al., 1998) with various small molecular weight bound ligands were determined by NMR and X-ray crystallography. But a lack of structural detail to explain the mechanism of interaction of SK.HPN with substrate kringles currently exists. While the three-dimensional crystal structures of isolated kringle 4 (Mulichak et al., 1991), kringle 1 (Wu et al., 1994a), kringle 5 (Chang et al., 1998) and catalytic domain (Wang et al., 2000a) and also recently full length HPG (Law et al., 2012) have been determined, one still lacks the information on the exact interactions of the substrate kringles in catalysis by the activator complex.

In the absence of the HPG.SK.HPG full-length substrate-enzyme ternary complex, the observed results from various laboratories, including our's, can be combined to envision a scenario of a SK protein-cofactor mediated catalysis wherein both substrate docking and turnover are mediated by the selective interactions between the SK of the activator complex and the kringles in the substrate PG. The involvement of the kringle domains of PG in substrate recognition by the activator complex can be judged from the long-observed decrease in microplasminogen activation, which is devoid of all the kringles, by SK and also from the significant fall in the ternary complex (HPG.SK.HPG) formation in the presence of ε-amino-n-caproic acid (Young et al., 1998), an agent that binds to kringles and alters their conformational states. Hence, the recognition of the role of kringles of the substrate by the SK in the SK.HPN activator complex to enhance enzyme-substrate collision

and docking, is logical; however, whether these kringles are involved in subsequent catalytic steps such as stereochemical positioning, active centre alterations, product release etc, are intriguing possibilities, that need to be pointedly explored.

Though X-ray crystallography is the most direct approach to structure solving at atomic-level detail, it is sometimes difficult to obtain crystals of large protein complexes. An alternative powerful (even though of less resolution) technique is that of Förster Resonance Energy Transfer (FRET). FRET, as a spectroscopic ruler in the distance range 10-80 Å, has been effectively used to study biological macromolecules and assemblies, and finds applications in various complex biological systems. The classical, and also the most prevalent, use of FRET is to measure distances between specific and known sites in protein structures and protein assemblies in solution, and to determine the geometrical disposition of proteins (Clegg, 1995). FRET is a long-studied physical phenomenon involving a non-radiative energy transfer from an excited donor chromophore to the acceptor chromophore by intermolecular long-range dipole-dipole coupling (Förster, 1946; Förster, 1951; Clegg, 1995). Stryer and his co-workers had found that the energy transfer rate is dependent on the sixth power of distance and on the overlap integral (Stryer and Haugland, 1967; Haugland et al., 1969). Hence, as a spectroscopic ruler, resonance energy transfer can be used for making long-range distance measurements in biology (Stryer, 1978). If the probe linker arm is flexible, there is enough dynamic averaging and in such a situation there is no significant effect of the orientation factor arising from the probe per se, on the accuracy of average distance measurements. Hence, one can reliably obtain average distances from resonance energy transfer measurements. Förster Resonance Energy Transfer, though a relatively lower resolution method compared to X-ray crystallography (XRC) or nuclear magnetic resonance (NMR) spectroscopy to obtain structural information, is a highly sensitive detection technique to obtain crucial insights into specific molecular interactions.

In the present study, we have used a catalytically inactivated, non-turnover "stable", SK.HPN (macromolecular) complex to map its interaction with substrate, by steady-state FRET, employing the SK β-domain exosite ("250-loop") as one "flag" and various substrate plasminogen domains in the ternary complex as the other "flags". We first formed an equimolar complex of SK with active-site inhibited HPN followed by addition of another potent inhibitor NPGB (p-nitrophenyl p'-

guanidinobenzoate) before adding the macromolecular substrate. In this situation, the SK.HPN complex would dock the substrate HPG and form a stable ternary complex that would not catalyze any product (HPN) formation. The focus of this study was to map substrate PG-activator enzyme interactions, with particular emphasis on the possible interactions between the SK 250-loop situated in the β-domain, and the functional domains of full-length substrate (LysPG i.e. HPG derivative with N-terminal peptide removed but having all the five kringles and the catalytic domain intact).

Both SK and PG were expressed recombinantly in *E.coli* to introduce unique cysteine residues for the site-specific incorporation of thiol-specific fluorescent probes to facilitate the spectroscopic study of the relative positioning of the two proteins in the complex. We have mapped the position of Leucine-260 in the 250-loop of SK β-domain in relation to the different sites on substrate miniPG (catalytic domain+kringle5)/LysPG (HPG with N-terminal peptide removed) and of these, the site on the kringle-5 on one of its face (carrying the lysine binding sites) was found to be the closest. The 37 loop in the catalytic domain has a direct role in activation process (Wang et al., 2000a). The active-site is located in a cleft which intersects perpendicularly with the junction of the two subdomains (N-domain and C-domain) (Wang et al., 2000a). The surface loops, like the 37 loop (residues 581-585) and the calcium binding loop (residues 620-630) occupy the N-subdomain, while the autolysis loop (residues 686-695) occupies the C-subdomain. These are the main loops, that surround the active site, which we chose for the study. The autolysis loop contributes to the structure of the activation pocket. From our FRET distance results, we saw that all the chosen acceptor sites residing in the serine protease domain resulted in comparatively low energy transfer, which is indicative of their relatively distal positioning from SK 250-loop of β-domain compared to LBS face of the fifth kringle in the substrate. Further, the experimental steady-state FRET measurements with isolated catalytic domain as substrate showed close agreement with the SK.μPN.μPN ternary model (see Figure 4.16 and Table 4.3-A for details). FRET between points on SK (position 260) and the catalytic domain of PG (in microPG i.e. PG containing catalytic domain alone or in miniPG or LysPG having mutation in the above mentioned catalytic domain loops) showed no significant positional change of PG relative to SK. Since the transfer efficiency did not change appreciably, it could be

surmised that the region did not shift significantly relative to SK subsequent to substrate docking.

Typically the exogenous fluorescent labels in biological systems have some degree of rapid motional averaging which reduces the fluorescence anisotropy. Fluorescence polarization measurements tell us about the angular relationship between the donor and the acceptor transition dipoles during the excited state lifetime of the donor (Stryer and Haugland, 1967). The term, κ^2 is the relative orientation of the donor's emission transition dipole and the acceptor's absorption transition dipole. κ^2 may vary between 0 and 4 and cannot be easily determined experimentally. The uncertainty in κ^2 affects the precise distance determination. To cope with this, emission anisotropy is used to determine the rotational freedom of the donor and the acceptor. The steady-state emission anisotropy calculated for the labeled SK and the labeled LysPG (see Table 4.1 A, and B) were found to be well below fundamental anisotropy or the value for the totally immobilized fluorophore reported in the literature. Since the donor probe and the acceptor probe attached to the above mentioned proteins exhibited considerable rotational mobility, it also justified the use of $\kappa^2 = 2/3$ in the calculation of the critical transfer distance, R_0. Table 4.2 shows the fluorescence anisotropy of fluorophore IAEDANS attached to the 250-loop in stable equimolar binary and ternary complexes. It can be seen that there is a slight increase in the anisotropy of the probe attached to the 250-loop of SK in the "stable" binary complex (without substrate) and in the ternary complex with miniPG or LysPG or HPG as substrate and thus the 250-loop retains considerable flexibility even when SK is bound to the partner and the substrate. This suggests that the interaction of the 250-loop of SK with the substrate might be transient in nature.

From the efficiencies of energy transfer (E) and $R_0(2/3)$ values (i.e. Förster critical distance value when κ^2 has been assumed to be 2/3 for the given dye pair), the relative distance between the donor and the acceptor was calculated to determine the distance estimates (see Table 4.3 A, B, and C), and a model was constructed (as shown in Figure 4.17) depicting the relative spatial proximity relations between the probes attached to the thiol groups and the geometrical disposition of the substrate's fourth and fifth kringle domains in the complex. The present work clearly demonstrated that the distances determined by FRET with microPG as substrate were in good agreement with the distances derived from the ternary SK.μPN.μPN model

(modeled based on SAK ternary X-ray crystal structure) in terms of the relative positioning of the residues.

Quantitative determination of distances through steady-state FRET allowed us to build a ternary interaction model based on the obtained FRET distances. In this model, the sites of interaction between cofactor SK and the docked substrate can be envisioned based on actual solution-phase FRET. The obtained results showed a near-proximal placing of the 250-loop of the β-domain of SK and the kringle-5 and kringle-4 domains of substrate LysPG. Interestingly, we also observed that the kringle surface interacting with the SK 250-loop in the ternary complex is different in the case of the two structurally homologous kringles - kringle 4 and kringle 5. This study has thus provided us the first glimpse of the kringle structure-function correlations in the SK.PN.PG ternary complex.

Aneja et al., (2013) have shown that there is a "synergism" among the various surface-exposed exosites of streptokinase (see Figure 5.8) during catalysis. These exosites occupy spatially discrete locations in SK, namely 88-97 loop in the alpha domain, the 170 loop in the beta domain, and a discrete coiled coil region in the gamma domain that cooperate to generate the high rates of PG activation by SK, known to be the highest among all other bacterial and physiological PG activators. When the catalytic activities of single, double and triple exosite mutants altered in the 88-97 and 170 loops, and the coiled coil region of SK, respectively, were undertaken, a synergistic decline in k_{cat} was observed without any significant effect in enzyme-substrate affinity (K_m), thus strongly indicating that these exosites are the substrate processing sites and contribute specifically towards substrate turnover rather than just the binding of the substrate at the active centre of plasmin (see Table 5.2, and 5.3). It may be realized that although these exosites have little contribution towards enzyme-substrate affinity, even the relatively weak/soft contacts that they make with the substrate may aid in the optimal presentation of the scissile peptide bond in substrate HPG for cleavage. In addition, these may also help in the uncoupling of the substrate from the SK.HPN complex after the scissile peptide bond gets cleaved. Thus, it is important to resolve how these SK exosites are playing their role in actually enhancing the rate in HPG activation by the SK.HPN complex.

In general, steady-state kinetics constitute a relatively indirect approach to gauge an overall picture of enzymatic action and makes certain simplifying

assumptions, whereas single cycle reactions (transient state kinetics or the pre-steady state kinetics) are more direct methods to determine the events occurring at the active site of the enzyme during the different steps of catalysis. These methods allow one to study an enzymatic reaction on the time scale of a single enzyme turnover after the addition of substrate. This involves taking enzyme in excess or nearly stoichiometric with substrate to avoid multiple cycles of catalysis. Since our enzyme system has K_m (a steady–state kinetic parameter) in the micromolar range, we met an essential criterion in performing single turnover kinetics by taking the enzyme concentration in excess of the K_m of the substrate for the catalytic reaction of SK.PN with HPG. Thus single turnover kinetic studies provide valuable and additional information beyond that of steady-state kinetics since in these experiments all of the substrate binds to the enzyme's active site and undergoes reaction to form transient intermediates and finally, product (plasmin, in our case) after a single turnover catalytic cycle (Johnson, 2013).

The reagent, NPGB (p-nitrophenyl p'-guanidinobenzoate) has been described in literature for active site acylation in several proteases, and hence used to titrate the active site formation in the SK.HPG complex (Chase and Shaw, 1969; McClintock and Bell, 1971; Wohl, 1984). Neither SK alone nor HPG alone can give the NPGB "burst" observed at 410 nm, but if SK and HPG are premixed in 1:1 stoichiometry, the characteristic burst due to p-nitrophenol release gives yellow colour and a rapid increase in absorption (Wohl, 1984). As seen in Figure 5.10, which shows native-like zymogen activation for SK loop mutants, indicating that they have pathway I capability to expose the active site in the partner zymogen.

In our lab, studies of SK/SK loop mutants (Aneja et al., 2013), and their steady-state kinetics have shown that with increasing the mutational load (single exosite, double exosite etc), the rate of appearance of product gets adversely affected. With the attempt to explore the exact role of SK exosites in the totality of catalysis, single cycle reaction conditions were set up for the single and double exosite mutants. By steady- state kinetics, these mutants were upto two orders of magnitude slower in catalytic rates as compared to native SK. Since NPGB can acylate the plasmin active site to give NPGB "burst" in the 0.5 minute preincubated equimolar SK.HPG complex, NPGB was added to each of the ternary complex formed between SK/SK exosite mutants.HPN complex and HPG to look specifically at the early phase of

catalysis at the surface of the active site before substrate gets converted into product plasmin. The pre-steady state NPGB burst along with colour formation due to product plasmin reaction with NPGB and consequent color generation was observed for the single and double exosite mutants of SK as in case of wild SK (see Figure 5.11 A, B, C, D, and E). The observation of a pre-steady state burst of product formation irrespective of exosite mutations signifies that the product formation at the active site of the various activator enzymes were the same. This is sharply in contrast with the results of multiple-turnover, steady-state kinetics, where the exosite mutations resulted in sharp decline in k_{cat} values. The corollary of this observation is that product release step must be rate limiting. If the burst would have been absent, it would have suggested that the rate of scissile peptide cleavage per se were adversely affected in the exosite mutants. Further 10% SDS-PAGE was run to examine the rate of scissile peptide bond cleavage of substrate HPG by SK/SK loop mutant.HPN complex. Densitometric analysis of SDS-PAGE (see Figures 5.12, and 5.13) showed that the half life ($t_{1/2}$) for the substrate disappearance into product was rapid i.e. the first 50% substrate depletion occurred fast in a single turnover catalytic cycle, within 10-15 seconds, for wild SK and single exosite mutant(s) and took ~30 seconds for double exosite mutant(s). Our experimental observation using wild SK and synergy k_{cat} mutants (single and double exosite mutants) suggests that scission rate is not the rate determining step under single cycle condition. This is starkly different from the results of steady-state kinetics which show that single exosite mutant has a nearly 10-fold drop in activity as against wild type SK, and the double exosite mutant shows 75-80 fold drop in activity. The exosite mutants which have 75-80 fold decline in steady-state kinetics, however, do not solely affect the k_{cat} through slower rates of scission of the substrate as revealed in the single cycle turnover experiment, suggesting that their altered rates of HPG activation arise from a greater time scale at steps subsequent to cleavage, very likely product expulsion, which would involve uncoupling from the large macromolecular surfaces.

Since the rates of cleavage under single turnover conditions between native SK and exosite altered mutants does not show much major change it clearly suggests that substrate recognition or positioning of the substrate for cleavage is not the rate determining step as the rate of cleavage is only minimally affected at these steps. This points towards the need to explore step(s) subsequent to cleavage as these SK exosites

might be contributing towards the catalytic efficiency of the machinery via the product release step of catalysis.

The present study provides a strong basis for further detailed investigations into the selected SK exosite mutants which have k_{cat} effects for finding out their role in post-cleavage event/s such as product expulsion during catalysis, which is not understood at present. This can be persued by combining rapid kinetic studies with sensitive fluorescence signals in a Stopped Flow machine. The understanding of how the switch in substrate preference of HPN is brought in the molecule as the SK binds to it has both fundamental and applied importance and is likely to open future vision to understand the working of similar 'protein-cofactor' systems in fibrinolysis and other biological phenomena.