SUMMARY

The physiological plasminogen activators (pPAs), such as urokinase (UK) and tissue plasminogen activator (tPA), can activate any mammalian PG molecule irrespective of its source organism, indicating that these protein-protein interactions are conserved due to the mutual co-evolution of interaction partners (Goh, Bogan et al. 2000; Gladysheva, Turner et al. 2003). In contrast, members of the pathogenic bacterial genera *Streptococcus* and *Staphylococcus* secrete co-factor proteins, such as streptokinase (SK) and staphylokinase (SAK), respectively, also known as non-physiological plasminogen activators (nPAs), which activate PG in a species-specific manner. In case of SK, which is the main subject of the present study, initial SK.HPG complex matures to a SK.HPN activator complex which then catalytically transforms 'substrate' HPG to HPN. The mechanism of action of SK activation of the partner molecule HPG/HPN has been an investigative issue since last few decades and extensive biophysical and biochemical studies have been done. The main focus of the studies has been mainly to decipher the mode of interaction of the domains of SK with the partner molecule. The mutagenesis and deletion studies with various truncated versions of SK defined few regions in the three domains of SK that are crucial for binding of partner HPG (Young, Shi et al. 1995; Parrado, Conejero-Lara et al. 1996; Fay and Bokka 1998; Young, Shi et al. 1998).

Site-specific mutagenesis is a useful method for validating functional residues of protein and thus provides new insights into the structure-function mechanism of proteins, especially when the crystal structure data on a protein is already available. Through site directed mutagenesis, few distinct 'exosites' which are important in SK-mediated HPG activation and interaction of substrate HPG have been identified (Chaudhary, Vasudha et al. 1999; Dhar, Pande et al. 2002; Yadav, Datt et al. 2008; Aneja, Datt et al. 2009). Random mutagenesis has earlier been used to define very critical residues in proteins with known structural information and also the ones with no solved structure (Hermes, Blacklow et al. 1990; Holm, Koivula et al. 1990; Shibata, Kato et al. 1998; Ward, Field et al. 2004). In this study we have isolated and examined a series of random-mutant clones of the COOH-terminal half (spanning some part of β-domain and complete γ-domain) of SK (residues 210-414) to find out new exosite, as there is a little information regarding the specific role of this region of SK (Young, Shi et al. 1995; Parrado, Conejero-Lara et al. 1996; Wu, Shi et al. 2001). The C-terminal half of SK (residues

210-414) was selected and an error prone PCR was done to deliberately introduce mutations in this segment. The results described above strongly indicate the presence of a substrate interacting region in the γ-domain of SK. The region spanning residues 314-347 in SK is a catalytically important locus that primarily contributes toward processing of substrate HPG by the SK.HPN complex. This has been elucidated by results of kinetic studies. As a result, in the present study the significance of the "upstream" section of the coiled-coil region of SK in substrate HPG activation has been unambiguously demonstrated. Taking into account the present results of our steady-state kinetic studies, there clearly seem to be two functionally distinct loci, one encompassing residues roughly 345-380 of the SK γ-domain, which faces towards the partner PN and might be involved in formation and/or activation of the 1:1 activator complex, and another encompassing the region from residues roughly 320-340, which seems to be interacting with substrate PG in which, through mutagenesis, critical residues have been shown to be functionally important in enzyme-substrate interactions. From the thermal factors of the SK.PN structure (Wang, Lin et al. 1998), it also appears that the peptide backbone bearing the constellation of charged residues in the coiled coil region of the domain of SK, especially Asp322, Arg324, Asp328, Asp325, Arg330, Asp331, Lys332, and Lys334 is highly flexible and can potentially participate in an extended network of salt bridges and hydrogen bonds within the coiled-coil region or with partner and/or substrate HPG. A priori, a salt bridge between Arg330 of SK and Glu714 of the methionine loop of substrate μPG seems to provide a rigidifying anchor to facilitate optimal interaction with substrate. The present study, as also recent studies in the case of other exosites in SK (Wang, Lin et al. 1998; Wang, Tang et al. 1999; Boxrud, Fay et al. 2000; Yadav, Datt et al. 2008), has convincingly shown that this region is indeed catalytically important through its interaction with the substrate catalytic domain and is not important in zymogen activation per se. Based upon the observations reported in this study and earlier findings (Dhar, Pande et al. 2002; Yadav, Datt et al. 2008; Aneja, Datt et al. 2009; Tharp, Laha et al. 2009), it can be envisioned that the substrate HPG interaction sites are not centered at one or two epitopes in SK, but rather, these seem to be strategically "scattered" over all the three domains of SK. Thus, the present work along with previously gleaned understanding on SK exosites opens a new and interesting paradigm for the catalytic mechanism of action of this medically important co-factor protein (Dhar, Pande et al. 2002; Yadav, Datt et al. 2008; Aneja, Datt et al. 2009; Tharp, Laha et al.

2009). Earlier, the importance of exosites, especially in providing substrate specificity via facilitated docking of the latter, is well demonstrated in blood coagulation proteinases, especially prothrombinases (Boskovic and Krishnaswamy 2000; Huntington 2005; Bock, Panizzi et al. 2007). In the case of SK too, it seems reasonable to assume that the different exosites in SK probably modify the substrate specificity by providing additional docking sites for an enhanced presentation of the scissile peptide bond in substrate HPG onto the enzyme active site through their precise interactions onto cognate "receptor" sites in the catalytic domain of substrate HPG.

SK is an important thrombolytic agent that is still being used world-wide to treat acute myocardial infarction and ischemic stroke especially in cost-conscious societies (Marder and Stewart 2002). It is an indirect fibrinolytic agents; it requires HPG as a substrate for thrombolysis. Presence of thrombus-associated HPG is in limited supply (Henkin, Marcotte et al. 1991) and this results in suboptimal lysis of retracted thrombi and incomplete reperfusion of occluded vessels. Thus, one of the major limitations in successful development of the therapeutic approach is a lack of easily available supply of HPG/HPN and its truncated derivatives. Moreover, various truncated and/or mutated derivatives of HPG are required for structural studies especially in order to define inter-domain interactions in the SK-HPG 'system'. Expression of MicroPG and MiniPG has been demonstrated from various sources such as *Pichia pastoris* (Nagai, Demarsin et al. 2003), baculovirus (Wang, Lottenberg et al. 1995) and *E. coli* (Medynski, Tuan et al. 2007). Different truncated forms of HPG in *Pichia pastoris* are also reported (Joshi, Nanda et al. 2011) but to make mutations and expression is cumbersome in *Pichia pastoris*, also non-mammalian glycosylation and heterogeneity in glycosylation imparts polydispersity in the protein, which limits its usability in defining precise structural information. Hence, to devise a platform wherein almost all the forms of HPG (truncated or mutated) can be expressed and isolated, and which retain the activity and conformations as of the native HPG, was extremely important. This can then be used to explore the SK-PG system and to sequentially decipher the role of individual domains of PG and the sequential conformational changes in structure of SK and PG by solution-based biophysical methods, such as SAXS. This information can lay the basics for the design of new thrombolytics.

The present study demonstrates the successful expression of recombinant HPG derivatives as IBs in *E. coli*, and their successful refolding with native-like intact functional properties. Importantly, the refolding protocols have been simplified to obtain an unified protocol for the refolding of truncated as well as full length recombinant HPGs. Circular dichroism data of HPG and rGluPG show similarity in secondary structural contents in these proteins, which further confirms that the protocols evolved for recombinant HPG expression and *in vitro* refolding are indeed optimal. Kinetic parameters for the activation of rMicroPG, rMiniPG, rMidiPG rK3PG, rLysPG and rGluPG were compared to HPG (blood plasma-derived HPG). These activity analyses established the functional authenticity and parity of the refolded proteins with native HPG. The kinetic parameters suggested that efficient utilization of the different HPG derivatives as substrates for SK.HPN enzyme complex is dependent on presence of kringles. To the best of our knowledge, this is the first study in literature on successful, high-yield refolding of *E. coli* IB derived rK3PG, rLysPG and rGluPG.

After the establishment of the kinetic parameters, the conformational studies of rPG were carried out. The presence of millimolar amounts of EACA changes the conformation of PG from close to open, which cause an increase in the volume of molecule. The results of our DLS experiments clearly suggest that there indeed occurs a change in the size of rGluPG and HPG when EACA is added. This result is in accordance with the previous report based on SAXS experiments of HPG (Mangel, Lin et al. 1990). The secondary structure of PG does not change during the conversion of closed to open form (Mangel, Lin et al. 1990). This indicates that shape of PG is formed by the domain interaction and this interaction gets abolished upon conversion to the open form. The effect of substrate conformation can also be studied with the steady state kinetics using SK.HPN as an activator for the PG. It was observed that the rate of catalysis increases in the absence of NaCl since the substrate (truncated derivatives of rPG) is in open conformation. Furthermore, previous studies have also shown that the rate of catalysis increases when full length HPG is in open conformation (Collen 1980; Chibber and Castellino 1986; Gaffney, Urano et al. 1988).

The present study has also explored the events that take place during SK-mediated PG activation. SAXS-based approach was taken to gain information about the macromolecular conformations, flexibility, shape and assembly state of SK and PG

either individually or in complex, in solution. Inactive mutants of PG i.e. rGluPG (S741A), rMidiPG (S741A) and rMicroPG (S741A) were made to make stable gesbinary/ternary complexes with SK. The measured SAXS data of SK, HPG, HPN, rPG their binary and ternary complexes confirmed the globular scattering nature and absence of any observable aggregation in the protein samples. This is the first study which describes structure of full length SK, HPN and rPGs (rGluPG and rMidiPG). Furthermore, the current study also establishes the structure of the binary and ternary complexes of SK with PG which is vital for mechanistic insights into SK-mediated PG activation.

In the present study, we have been able to obtain the modeled structure of SK which shows that SK in solution adapts a structure resembling an extended string of beads with D_{max} and R_G 130Å and 41Å, respectively (Fig. 5.8, Table 5.1). The SK and MicroPG binary complex suggests a conformational shape change in SK from elongated flexible string of beads molecule to 'open C' shape (Fig. 5.8 and Fig. 5.9), where the three domains of SK form a crator into which the catalytic domain of substrate HPG gets docked. In the ternary complex of SK.MicroPG.MicroPG, SK further gets collapsed to a 'closed C' shape (Fig. 5.10). Due to its flexible nature, SK undergoes sequential and temporal conformational changes from extended form when alone, to an 'open C' shape in binary complex, and further, to a 'closed C' shape in the ternary complex (Fig. 5.12).

Our SAXS experiments also show that HPG, HPN and rGluPG are all right-handed spiral molecules (Fig. 5.11). Furthermore, it has been determined from the present study that HPG is spiral in shape with D_{max} and R_G of 130Å and 38Å, respectively. Our observations are in agreement with the results reported by other groups (Mangel, Lin et al. 1990; Ponting, Holland et al. 1992). But these earlier studies lacked the solution-based structure of HPG which has been established recently (Law et al. 2012). However, some penalities appear as some parts of crysral lie outside the SAXS cage when the crystal structure of blood-derived HPG is placed inside the SAXS cage (Law et al., 2012). The occurance of penalties clearly suggests that crystal parameters and SAXS data provided by Law et al. have some discrepancies. However, in the present study, the blood-derived HPG modeled structure and SAXS cage are superimposable. Additionally, we have also established the SAXS-based structure of *E. coli*-derived rGluPG. rGluPG (non-glycosylated) is also a right-handed spiral structure but is more compact than HPG.

It undergoes sequential conformational changes from a spiral and compact structure when alone, to elongated K5 and catalytic domain in binary and ternary complexes of SK and PG. Moreover, kringle 4 and kringle 5 of substrate PG come closer to the β-domain of SK when in the SK.HPN.rGluPG ternary complex (Fig. 5.10).

The results of our SAXS experiments show that HPN undergoes conformational changes when it forms a binary complex with SK. The valley inside HPN gets broaden up to accommodate SK inside its valley (Fig. 5.9). Furthermore, upon interaction of this SK.HPN binary complex with substrate PG, HPN further undergoes conformational change. For instance, it attains inverted question mark "¿" shape when MicroPG was used as a substrate and it becomes "L" shaped when GluPG was used as a substrate (Fig. 5.9, Fig. 5.10 and Fig. 5.12).

Collectively, from the SAXS based structural analysis of individual proteins, their binary and ternary complexes it can be concluded that SK along with partner HPN and substrate GluPG all together undergoes sequential conformational changes during the process of catalysis (Fig. 5.12).