Shekhar Kumar (2009). ***Studies on concerted structural transitions and protein-protein interactions in plasminogen activation by bacterial plasminogen activator.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Thrombotic occlusive diseases are manifested in several disorders that have significant morbidity and mortality, including acute myocardial infarction, pulmonary embolism, deep venous thrombosis, and stroke. The most common interventions for these clinical conditions are thrombolytics or clot-dissolving drugs, such as streptokinase (SK), tissue plasminogen activator (tPA) or urokinase (UK). Among these, SK remains the drug of choice particularly in the poorer economies because of its low relative cost and potent thrombolytic properties.

Although, the world has benefitted hugely from conventional thrombolytics but due to their limited efficacy and potentially life-threatening side effects these drugs require attention for development of a new generation of improved clot dissolvers. For example, recent advances in the field of thrombolytic therapy propose the use of human plasmin (HPN) and its truncated derivatives for the treatment of ischemic strokes and arterial thrombosis (Zivin *et al.,* 1999, Lapchak *et al.,* 2002; and Lewandowski *et al.,* 2001, Marder and Stewart, 2002; Novokhatny *et al.,* 2004). The site-specific administration and or localized action of these potential drugs can potentially avoid the risk of bleeding that is often associated with other systemic human plasminogen (HPG) activators, used currently to dissolve clots. However, the development of human plasmin (HPN) based therapies is hampered by limited availability of HPG/HPN since commercially viable production of plasmin(ogen) is often beset by limitations posed by heterologous expression systems. In particular, the complex, multidomain structure of HPG that is stabilized by several intramolecular disulfide linkages, presents an additional challenge to develop efficient *in vitro* refolding methods for their high-level recombinant protein production from bacterial sources. Thus, any future development of HPN-based direct thrombolytic therapy requires commercially viable methods that yield HPN and its functional fragments from suitable recombinant sources. In this work, we present an easy and efficient method to produce three different derivatives of HPG viz. *microplasminogen* (abbreviated as microPG; catalytic domain devoid of all kringles),

miniplasminogen (abbreviated as miniPG; catalytic domain alongwith kringle 5) and *midiplasminogen* (abbreviated as midiPG, catalytic domain linked to Kringle 4 & 5) from *E. coli* inclusion bodies (IBs). Highly optimized conditions for refolding of these proteins were deduced from a systematic approach that utilized a matrix-based screen for different refolding buffers made from a combination of reagents. This optimized method also proved useful in refolding of variants of microPG and miniPG which were subsequently modified with sulfhydryl reactive polyethylene glycol (PEG) reagents which imparts beneficial properties such as increased *in vivo* half life and reduced α_2-antiplasmin inhibition. High yield of refolded population and its easy purification assures an unabated supply of recombinantly expressed HPG forms that would facilitate their utilization for testing in *in vitro* and *in vivo* studies before they qualify for clinical use.

In addition to development of direct thrombolytic therapies, parallel efforts are also needed to improve therapeutic properties of SK; however, this would require a firmer understanding of the molecular action of SK. Similar to all other plasminogen activators SK also converts HPG to HPN, which in turn, cleaves fibrin as part of the clot lysis process. SK, originally a bacterial protein, acts as a "molecular parasite" for HPN, and restricts the latter's trypsin-like broad substrate specificity to a highly specific one for the Arg 561-Val 562 scissile peptide bond in substrate HPG. Therefore, SK not only activates HPG but also governs the functionality of the bound HPN to recruit more HPG. The remarkable alteration of the macromolecular substrate specificity of HPN by SK as a result of the latter's 'protein co-factor' property, has been a subject of intense investigations in recent time (Nihalani *et al.*, 1998; Boxrud *et al.*, 2000; Dhar et al. 2002; Sundram et al., 2003; Boxrud *et al.*, 2004; Boxrud and Bock, 2004). Thus, deciphering the molecular details of this mechanism and associated structure-function co-relations, whereby SK modulates the substrate preference of the active site of plasmin(ogen) after complexation with the latter, is not only vital to the successful design of improved SK-based thrombolytic drugs of the future but would also add to our understanding the role

enzyme systems. In this context, a number of structure-function studies have suggested that SK, along with partner HPG, seems to provide a template on which the substrate molecule can optimally dock through protein-protein interactions, resulting in the optimized presentation of the HPG activation loop at the active center of the enzymatic on SK-mediated HPG activation which indicated that interactions with extended macromolecular recognition sites (exosites) rather than the active site of HPN are the principal determinants of binding affinity and covalent specificity for the macromolecular substrate (Chaudhary *et al.,* 1999; Boxrud *et al.,* 2000; Dhar *et al.,* 2002). Earlier solution studies indicated that the 250-loop of the β-domain of SK constitutes such an "exosite" with which substrate kringle(s) can potentially interact and thus promote the initial low-affinity binding of SK and catalytic domain to a more avid binding (Dhar *et al.,* 2002). However, the nature of this interaction and the exact role and identity of participating kringle(s) remained unelucidated. In the present study, this issue has been addressed through a multi-site fluorescence resonance energy transfer (FRET) based approach for measuring various prospective enzyme-substrate interaction sites in a stabilized ternary complex. The FRET studies enabled us to measure the intermolecular distances between one fixed location in the SK 250-loop and five different epitopes/sites spread over the miniaturized substrate derivative, miniplasminogen (miniPG, catalytic domain along with kringle 5). The distance geometry obtained through FRET measurements were utilized to select and validate most suitable computer modeled structure wherein substrate miniPG was docked onto the known X-ray diffraction structure of the SK.μPN enzyme complex. The comparison of the selected ternary interaction model on the basis of experimentally determined distances from FRET experiments allowed the most proximal placing of kringle 5 onto the 250-loop of the β-domain of SK, thus providing the first evidence that

it is indeed Kringle 5 that specifically interacts with the 250-loop of the SK β-domain. These observations prove convincingly that interactions involving the 5th kringle domain of substrate are intimately involved in the mechanism of operation of at least one of the macromolecular substrate-specific exosites in the SK-plasmin(ogen) activator complex.

The transient conformational changes associated with SK based HPG activation have remained elusive for several years until the recent studies of Boxrud and Bock, 2004 that elegantly utilized active site fluorescently labeled HPG to gain insights about the kinetic mechanism of HPG activation, especially the early phases of 1:1 complexation of SK with HPG. However, these studies relied on a fluorophore that was conjugated via a tripeptide bound to the active site of HPG, which raises a possibility that active site labeling could itself affect the natural conformational changes on the activation pathway during the maturation of the SK.HPN activator complex, thus artifactually compromising those present in an 'uninhibited' system. Accordingly, to avoid any interference in SK binding to the HPG active site, we developed a different approach where the IAEDANS, a conformation-sensitive fluorophore (Lackowicz, 1999) was attached to one of the surface exposed loops (the 88-97 loop of the SK α-domain) of SK to monitor structural transitions during the conformational activation of HPG as well as in the transformation of the initial SK.HPG complex to mature SK.HPN activator complex. The rationale to place the fluorophore in the α-domain was its suggested involvement in the conformational activation pathway of HPG under physiological conditions (Sazonova et al., 2004). In addition to this, recent solution kinetics studies in our lab have also confirmed the role of 88-97 loop of the α-domain in HPG activation (Yadav *et al.,* 2008). These observations suggested that α-domain likely harbors a capability to interact and utilize both kringles and serine protease domains for activation of HPG. However, any direct evidence of such a process and its kinetic relevance to the conformational activation of HPG remain unelucidated. In the present study, we examined the 1:1 complexation of fluorescently labeled SK with HPG/HPN or their kringle-less derivatives. Binary complexation studies of

once SK combines with HPN. An increase in fluorescence intensity was observed, albeit of lower magnitude even in case where only serine protease domain (μPN) of HPN was used to make an enzyme-cofactor complex, suggesting that the catalytic domain constitutes at least one of the regions in plasmin(ogen) that interacts with the 88-97 loop of SK α-domain. Stopped-flow transient kinetic studies of equimolar complexation of labeled SK with HPG or HPN revealed a two-phase kinetics composed of an early, fast phase of fluorescence enhancement followed by a slow phase. Rate constants characteristic of early fast phases of SK complexation with HPG or HPN were insensitive to inhibition by lysine analogue (εACA), thereby suggesting that the very initial steps of binary complexation are kringle-independent. The slow phase of fluorescence enhancement was found to be originating from kringle rearrangements since this phase selectively exhibited a strong susceptibility to impedance by εACA. Moreover, this slow rearrangement step was not observed when the kringle-less derivatives, such as μPN (catalytic domain of HPN) and μPG (catalytic domain of HPG) were employed to make the binary complex with SK. Rate constants obtained from rapid-kinetics studies were utilized to propose reaction schemes for conformational activation of HPG and formation of the mature SK.HPN activator complex.

Though the present study in consonance with the earlier study (Dhar *et al.*, 2002) clearly demonstrates that the interaction of kringle domains of the macromolecular substrate (HPG) with the SK-plasmin(ogen) activator complex plays a very important role in activation of substrate HPG by improving the enzyme-substrate reactions, yet, the mechanistic insights regarding the overall catalytic phenomenon i.e. dynamic events such mystery so far. Thus, in order to gain further insight into kringle-mediated enzyme-

substrate interaction, we used steady-state and rapid kinetics methods employing truncated substrate HPG derivatives with varying kringle numbers. Steady-state kinetic parameters for the activation of different substrate HPG derivatives viz. microPG, miniPG midiPG when compared with full-length HPG revealed the significant contribution made by kringle(s) in catalytic turnover (k_{cat}). It was observed that catalytic turnover by the SK.HPN activator for the kringle-less derivative, microPG, was very less (only 1-2 %) as compared to that against native full-length substrate HPG, however, upon addition of a single kringle, as in case of miniPG, the activity shoots up by nearly 30-fold. Addition of one more kringle, as in case of midiPG, further lifted the activity to levels equivalent to nearly $3/4^{th}$ of the full length HPG. However, affinity parameter (K_m) for both miniPG and midiPG was undistinguishable from the full length HPG although the K_{cat} for both substrates remained significantly less than the full length HPG. This apparent non-correlation between the catalytic efficiency on one hand and enzyme substrate affinity, on the other, is seemingly in contrast with the established model of HPG activation by SK and other bacterial HPG activators whereby the docking of the catalytic domain of substrate onto the HPG activator complex is supposed to impart both specificity and catalytic drive to the HPN active center. In order to further dissect the individual steps that control the catalytic efficiency, we developed an intermolecular FRET system specifically to probe the pre-steady state kinetics of catalytic conversion of HPG by SK.HPN activator complex. For this, a donor fluorophore (IAEDANS) labeled SK was complexed with HPN to be used as an enzyme complex for acceptor labeled (QSY® 35 iodoacetamide) derivatives of microPG, miniPG and midiPG. Stopped-flow FRET studies

cycle. Kinetic fitting of the fluorescence traces provided characteristic rate constants that were correlated with the two crucial post-docking events *viz.* scissile-peptide bond cleavage and the product release. The observed rate constants associated with the catalytic events further suggest that the product release step is a major contributor towards the high catalytic turnover rates of the SK.HPN enzymatic system as opposed to substrate-binding associated with the docking step.

The results of the present study reveal that the multi-point protein-protein interactions operating between the substrate HPG and the cofactor SK are crucial not only for conformational activation of HPG but also to make SK.HPN a highly efficient enzyme. It was also evident that long-range protein-protein interactions between the kringle domains of substrate HPG and the SK.HPN activator complex was actually operating through a mechanism that assists in rapid 'decoupling' of nascent product from the enzyme's surface. It appeared that this might involve a 'global' conformational change, a process that may be imminent upon sensing a signal from 'kringle transduced' structural transition(s) originating from scissile peptide bond's cleavage thus, mechanically aiding in product release.

Overall, it can be concluded that the concerted structural transitions and protein-protein interactions are being utilized in the SK mediated plasminogen activation to achieve high order catalysis. The information obtained from these studies could dissect the components of such a wonderful albeit complicated molecular machine and may help in modular design of much efficient future molecules with tailor-made specificities.