SUMMARY

A large number of circulatory disorders such as thrombosis, myocardial infarction (heart attack), and pulmonary embolism have become some of the leading causes of mortality in modern day society, worldwide. In recent years, great emphasis has been laid on the discovery of various clot-dissolving agents, which can induce dissolution of fibrin clots *in vivo*. This has lead to the emergence of thrombolytic therapy, involving drugs like Urokinase (UK), Tissue plasminogen activator (tPA), Streptokinase (SK) and Staphylokinase (SAK). Among the various thrombolytic agents that are being used at present, streptokinase shows the best overall prospects as thrombolytic agents, especially for developing countries since other agents like UK, and tPA are known to have a lower half-life as compared to streptokinase and are much more expensive than SK.UK and tPA, which are physiological PG activators are 'direct' in action as they are enzymes in themselves and directly act on HPG, converting the zymogen into an active enzyme HPN, which is responsible for the dissolution of fibrin clots *in vivo*. In contrast to these SK, and SAK, obtained from the bacterial sources are 'indirect' PG activators, as they do not have any catalytic function of their own.

The indirect activators such as SK recruit circulating HPG in the blood stream to generate HPG activating potential. SK first combines with 'partner' HPG in an equimolar (1:1) manner to form a tight, enzymatically active SK.HPG 'virgin' activator complex, which rapidly converts into an SK.HPN activator complex. The mature SK.HPN activator complex then catalytically transforms 'substrate' HPG to HPN. HPN is a relatively non-specific serine protease with trypsin-like broad substrate specificity. However, upon complexation with SK, its specificity becomes restricted to the Arg 561-Val 562 scissile peptide bond in substrate HPG (Markus and Werkheiser, 1964). This 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). 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 undoubtedly vital to the successful design of improved SK-based thrombolytic drugs of the

future. In this context, different structural, modeling and solution based studies have suggested that SK along with partner HPG, seems to provide template (exosites) 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 complex (Wang *et al.*, 1998; Parry *et al.*, 2000; Boxrud *et al.*, 2000, 2001). However, although the solution based and structural studies extensively elucidate the various kinds of protein-protein interactions involved in SK.HPN activator complex formation or "zymogen activation", yet the identity and exact contributions made by the protein-protein interactions between the substrate HPG and the enzyme SK.HPN that are involved in substrate 'recognition' and 'turnover' still remains largely an ambiguity. Therefore, the major objective of the current study has been to identify the structure-function inter-relationships in SK and HPG in context with substrate HPG recognition and activation by understanding how different domains of SK interact with each other in modulating the substrate HPG turnover on one hand, and gain insight about the contributions made by different regions of substrate HPG in this activation phenomenon on the other hand. Such insights would help us in underlining the mechanistic differences among different HPG activators with respect to HPG activation phenomenon.

The recent structural and computer modeling studies have established a close structural homology between the single domain of SAK and isolated α-domain of SK in particular and all the three domains in general (Parry *et al.*, 1998, 2000; Esmon and Mather, 1998). Apart from this, mechanistically similar PG activator from *Streptococcus uberis* (SUPA) has also been isolated and found to possess a two-domain structural motif, which also shows significant structural homology with SK. Thus, a compelling need exists to glean insights regarding the similarities and dissimilarities in the 'design principles' between the three-domain SK, on the one hand, and the single- and two-domain structures of SAK and SUPA on the other, particularly in terms of the structure-function co-relations that underlie the inter-domain co-operativity between the individual SK domains.

In order to explore the inter-domain co-operativity in SK during human plasminogen (HPG) activation, isolated single domain constructs of SK *viz.* α, β and γ and bi-domain mutants of SK *viz.* αβ and βγ were constructed by rDNA approaches and

intracellularly expressed in *E.coli* BL21(DE3) cells. After two-step purification using HIC and DEAE-Ion exchange chromatography nearly 95 % pure protein was obtained. The far-UV CD spectra of these purified proteins showed that truncated derivatives of SK retained the native-like secondary structure. The truncated derivatives were then checked for their HPG activator activity using both HPN independent (Pathway 1) and HPN dependent pathway (Pathway 2). A very low HPG activator activity was observed in case of isolated α- and β-domains, which was in consonance with the recently reported low activator activity in isolated domains (Loy *et al.*, 2001). Though this activity was significantly raised in presence of HPN, suggesting that the activator activity observed in the isolated domains was HPN-dependent, yet it was 3-4 orders of magnitude lesser than that of native SK.PN. In comparison to the single-domains, the bi-domain constructs *viz.* αβ and βγ, showed higher HPN-dependent co-factor activity, 3.5 % and 0.7 % (SK.HPN *versus* HPG taken as 100 %) respectively. Interestingly, the low co-factor activity of the bi-domain as well as that of isolated single-domains could not be ascribed to the inability of these molecules to interact with HPG, since the physico-chemical studies using Resonant Mirror approach (carried out on real time interaction system; IAsys) showed that each of these constructs bind 'partner' HPG. The affinity of bi-domain constructs for partner HPG was found to be similar to that of native SK suggesting that the main reason for their low cofactor activity was not at the level of binary interaction, whereas the affinity of single-domains was observed to be considerably less, which might be the major contributory cause towards their very low cofactor activity. Subsequent analysis of the ternary interaction between the 'substrate' HPG with the immobilized SK/SK-domains.HPG binary complex revealed that substrate HPG docks onto the bi-domain constructs (αβ and βγ) with similar strength as it does onto the SK.HPN activator complex. However, ternary complexation with substrate HPG in case of isolated single domains could not be observed due to their weak binary complex formation with HPG. Overall analysis of steady state kinetics and physico-chemical studies thus, established that despite the attainment of near native docking potential, the co-factor activity in case of bi-domain constructs was not translated into the native SK.HPN like co-factor activity. This lack of correlation between the enzyme-substrate affinity and its catalytic turnover strongly suggest that these two attributes in case

of SK.HPN system are independent of each other indicating that there is probably an existence of some specific interactions between the macromolecular substrate (HPG) and SK.HPN activator complex at the post-docking stage that plays an important role in amplification of the low catalytic turnover of substrate HPG, associated with the bi-domain derivatives of SK.

Earlier study on SK (Dhar *et al.*, 2002) have pointed out that kringle domains of substrate HPG play an important role in HPG activation by SK.HPN activator complex as it was observed that catalytic domain of plasminogen (μPG), which is devoid of all its five kringle domains act as a poor substrate for the SK.HPN activator complex. Thus, when steady-state kinetics and physico-chemical studies were performed with the kringle-less derivative, μPG as substrate, it was observed that like SK, both the bi-domain constructs showed drastically compromised catalytic turnover (1 % of the native SK.HPN *versus* HPG) as well as 4-5 fold reduced affinity compared to that against full-length substrate HPG. This shows that when the kringle domains were removed from the macromolecular substrate, the catalytic advantage of even the three-domain native SK becomes compromised, suggesting that catalytic domain of macromolecular substrate acts as a poor substrate for the SK.HPN activator complex. Therefore, one of the main conclusions that can be drawn from the current study is that kringle domains of substrate HPG plays an important role in docking of the macromolecular substrate onto the activator complex but the docking alone is not the sole criterion for the full blown cofactor activity as it was observed that even the bi-domain constructs of SK can dock substrate HPG as efficiently as native activator complex, yet they fail to generate native-like co-factor activity. In other words, it is only when all the three domains of SK are collectively present, the ability to exploit the kringle domains of substrate HPG for maximum catalytic turnover is attained probably via the "long-range" protein-protein interactions, centered away from the scissile peptide bond (Arg 561-Val 562).

Though the present study in consonance with the earlier study (Dhar *et al.*, 2002) clearly demonstrates that interaction of the 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 *'exact'*

manner by which this happens and the exact contribution made by the different kringle domains in this catalytic process needs to be known. Thus, in order to gain further mechanistic insights into this kringle mediated HPG activation phenomenon; the steady-state kinetic and physico-chemical interaction studies with various truncated derivatives of HPG were explored. In this regard, different HPG derivatives having varied levels of kringle domains *viz.* K1-5, K1-3, K4, K5, miniPG were generated by limited proteolysis of HPG using enzymes such as plasmin and elastase. These proteins were then purified to the level of 90-95 % using the standardized chromatographic procedures.

The importance of the kringle domain(s) in SK.HPN mediated substrate HPG activation was further emphasized by carrying out steady-state kinetics and physico-chemical studies with miniPG (Kringle 5+catalytic domain) as substrate. It was observed that addition of only one kringle domain adjacent to the catalytic domain of substrate HPG enhances the catalytic efficiency of SK.PN activator enzyme by nearly 150-fold over that observed in case of kringle-less derivative, μPG, establishing firmly that the interaction between the kringle domain(s) of substrate HPG and the SK.HPN activator complex was crucial for substrate HPG activation. Interestingly, it was observed that the presence of K5 along with the catalytic domain of HPG generates the near native affinity in miniPG for SK.HPN activator complex, however, the catalytic turnover of SK.HPN against miniPG as substrate was only one-third of that observed against full-length HPG as substrate. This indicated that in case of SK.HPN system, merely a rise in substrate affinity does not automatically correlate with a proportionate increase in catalytic turnover. The apparent discrepancy in enzyme-substrate affinity and turnover, observed in case of substrate miniPG, strongly suggested that in case of SK.HPN system, substrate specificity and catalytic turnover might be mutually exclusive events. This discrepancy was further established by undertaking the detailed steady state kinetics and real-time physico-chemical studies, involving various site directed mutants of SK (altered in their kinetic attributes) and different derivatives of substrate HPG differing in terms of their kringle domains. The analysis of these results revealed that these kringle mediated long-range interactions are 'dual' in nature *i.e.* one that is involved in imparting 'substrate recognition' ability to the activator enzyme and the other in 'catalytic processing' of the substrate. It can

be reasoned that though the basal level of substrate HPG catalysis is imparted through the interaction of K5 of substrate with the SK.HPN activator complex, yet the amplification of this catalytic processing to the 'full-blown' level was achieved by involving the other four kringles (K1-4), probably in "post-docking" events that are involved in transformation of substrate HPG to product HPN followed by the release of product from the enzyme surface.

An attempt was further made in the current study to define this post-docking scenario by comparing the physico-chemical interactions in real-time between various substrate HPG derivatives and their corresponding product derivatives with different mutants of SK. These studies remarkably conclude that a distinct correlation between the catalytic rates (k_{cat}) of different enzyme-substrate combinations (as measured through steady-state kinetic studies) could be established with the ratios of the on-rates of substrate (k_{on} [substrate]) and its corresponding product binding (k_{on} [product]) to the enzyme surface. The inference that can be drawn from these studies is that though in the HPG activation phenomenon the rate at which the enzyme interacts with the macromolecular substrate ('residence time') was crucial but also important was the rate at which the enzyme releases the product from its surface after completion of catalysis. This is probably accomplished through the 'uncoupling' of product HPN from the SK.HPN enzyme surface. While the first rate is an outcome of initial enzyme-substrate interaction, the second likely is indicative of the product expulsion step that reflects the uncoupling of the long-range interactions through which the enzyme-substrate complex was stabilised, and substrate specificity was engineered into the proteolytic reaction in the first phase of the catalytic cycle.

Thus overall, it can be concluded that in case of SK.HPN system, although docking of the macromolecular substrate onto the SK.HPN activator complex is sufficient to explain the high *'substrate specificity'* (since even with the 1-2 % turnover of μPG, the reaction has become highly specific for the scissile peptide bond compared to that of free HPN, which is negligible), yet this docking is not sufficient to achieve a high catalytic turnover by itself. Note that this is in contrast to the case of the direct PG activators tPA and UK, where the presence or absence of kringles in the macromolecular substrate

docking act make only a marked difference in substrate turnover. The results of the current study reveal that apart from the long-range protein-protein interactions between the kringle domains of substrate HPG and the SK.HPN activator complex that impart the docking potential to the substrate HPG, the phenomenon that are clearly independent of this macromolecular substrate docking *per se* ("post-docking" events) are also crucial in amplifying the catalytic potentiation of substrate HPG to those associated with the native enzyme-substrate system. These post-docking events very likely involve the steps such as the catalytic processing of the substrate and release of the nascent product after 'decoupling' from the enzyme surface. These putative post-docking events in case of SK might involve some kind of a 'global' conformational change that might facilitate the release of the product from the enzyme surface and thus helps in attaining the catalytic rates of native co-factor SK.HPN activity against HPG. It is likely that such a conformational change in HPG is 'global' in character, since the rates with μPG alone or even with miniPG, are significantly lower than that achieved with full-length HPG, containing all the five kringle domains.

Such a mechanism of *substrate-assisted catalysis* that exploits long-range 'supra' catalytic center based interactions between substrate and enzyme encountered in SK, and to some extent in SAK (as became clear from the kinetic and physicochemical studies), is clearly distinct from the direct PG activators such as UK and tPA, which display catalysis with nearly the same rates irrespective of the presence or absence of kringle domains in their macromolecular substrate. Unlike UK and tPA where short-range interactions between the regions in and around the scissile peptide bond of substrate HPG and enzyme provides full substrate specificity and catalytic power, long-range protein-protein interactions involving the regions far away from the scissile peptide bond of the substrate HPG have been exploited by SK through evolutionary selection of a three-domain design to generate the highest degree of catalytic efficiency amongst all known HPG activators. The elucidation of the exact molecular events and epitopes involved in this process would greatly help in redesigning of existing proteases into efficient substrate specific HPG activator enzymes and also in the future de novo design of novel target specific proteolytic functionalities.