SUMMARY

Protein Engineering Studies of Streptokinase

Acute coronary ischemic syndromes and strokes are caused by thrombosis in arteries where obstruction leads to ischemia of the heart or brain, respectively. Thrombolytic therapy has become a cornerstone of treatment for acute myocardial infarction and the development of SK as a thrombolytic agent in the developed world earlier, and now in India, has given a fresh lease to life, especially because of its low cost and high potency as a HPG activator. However, it is imperative to have a detailed insight into the structure-function aspects of SK and its mechanism of action in order to design and develop more efficacious molecules or molecules with novel properties.

Although named as a 'kinase', SK is an inert molecule by itself, but becomes active once it binds to HPG in a 1:1 stoichiometric complex, thereby forming the 'activator complex'. This activator complex then further acts on other 'substrate' HPG molecules to convert them into HPN. Unlike free HPN, however, which is essentially a trypsin-like protease with broad substrate-preference, the SK.HPN complex displays an extremely narrow substrate specificity. This remarkable modification of the macromolecular substrate specificity of HPN by SK is currently believed to be due to 'exosites' generated on the SK.HPN complex (Nihalani *et al.*, 1998; Boxrud *et al.*, 2000). However, although solution and structural studies have suggested that SK along with partner HPG, seems to provide a template on which the substrate molecule can dock through protein-protein interactions, resulting in the optimized presentation of the HPG activation loop at the active center of the complex, the identity of these interactions, and their exact contributions to the formation of the enzyme-substrate intermediate/s still remain largely an ambiguity.

In order to clearly identify a structural epitope/element that has a role in conferring substrate HPG affinity onto the SK.HPG activator complex, the three-dimensional structures of the isolated three domains of SK were superimposed through computer modeling studies that revealed the presence of a distinct, solvent-exposed flexible loop in the β domain (residues 254-262; 250-loop) of SK that is absent in the other two otherwise structurally homologous domains. A deletion mutant, $SK_{del254-262}$ was constructed, using overlap extension-PCR reaction that was cloned in the pET-23d expression vector. Both $SK_{del254-262}$ as well as native SK proteins were expressed intracellularly in *Escherichia coli* BL21 (DE3) cells after induction with IPTG and purified using a rapid purification method. The eluted proteins were more than 95 %

pure, as analyzed by SDS-PAGE. To check whether the deletion of the 250-loop resulted in changes in secondary structure, the far-UV CD spectra of SK and the mutant ($SK_{del254-262}$) were studied. No significant differences were observed. In addition, the far-UV CD spectra of the β domain alone and β domain containing the deletion ($\beta_{del254-262}$) were also checked to obviate any 'averaging' effect of local changes due to the CD contributions from rest of the two domains of SK in the full-length molecule. For this, the isolated β domain with the deletion at the same site was constructed. The cDNAs encoding for the β domain alone and $\beta_{del254-262}$ were then expressed in *Escherichia coli* and purified from IBs to more than 95 % homogeneity by ion-exchange chromatography. Interestingly, no discernable changes were observed in the secondary structure of $\beta_{del254-262}$ with respect to $\beta_{wild-type}$, indicating that neither the overall secondary structure of SK nor that of the β domain alone had been perturbed by the deletion of the nine-residue loop from the protein.

When the mutant was checked for HPG activator activity, significant decrease in the rates of substrate HPG activation by the mutant ($SK_{del254-262}$) were observed. In order to explore whether the deletion of the 250-loop resulted in any significant alteration in the affinity of the mutant with partner HPG, the kinetics of interaction between immobilized HPG and SK were compared with that of $SK_{del254-262}$ by the Resonant Mirror approach using a semi-automated instrument for measuring protein-protein interactions in real time. To examine the effect of immobilization chemistry on the values of kinetic constants, preliminary experiments were performed by immobilizing SK/$SK_{del254-262}$ onto both carboxy-methyl dextran cuvettes (using a amino-coupling protocol recommended by the manufacturers) and onto biotin cuvettes in which biotinylated HPG was immobilized on streptavidin captured on this cuvette. The kinetics of association and dissociation of HPG with immobilized SK/$SK_{del254-262}$ indicated that the mutant exhibited an affinity that was not significantly different from that of SK. Remarkably, both immobilization techniques yielded similar binding constants for SK and $SK_{del254-262}$, indicating that the coupling procedure *per se* does not significantly interfere in the binding interaction between SK and HPG.

In order to find out the principal cause of fractional loss in activity, the mutant was checked whether it could open the active site in partner HPG. It was found that $SK_{del254-262}$ could indeed open the active site and generate amidolytic activity, [illegible] other. In order to find out

suggesting that the lower activity was not due to any significant effect on the ability to activate partner HPG at the binary complexation stage.

A steady-state kinetic analysis of $SK_{del254-262}$ revealed that its low HPG activator activity arose primarily from a 5-6 fold increase in K_M for HPG as substrate, with little alteration in k_{cat} rates. Under these conditions, the k_{cat} for the small peptide substrate tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate was essentially unchanged indicating that the primary characteristics of the active site remained unaltered.

A new Resonant Mirror based assay was devised for the real-time kinetic analysis of the docking of substrate HPG onto pre-formed binary complex of biotinylated HPG and SK/$SK_{del254-262}$. Remarkably, the observed increase in the K_M for the macromolecular substrate was proportional to a similar decrease in the binding affinity for substrate HPG, as obtained in case of $SK_{del254-262}$, although under those conditions the 1:1 affinity of $SK_{del254-262}$ for partner HPG was minimally changed.

In contrast, kinetic studies on the interaction of the two proteins with μPG (having just the catalytic domain of HPG without any of the five kringles) showed no difference between formation of ternary complex with μPG by preformed equimolar complexes of HPG with either SK or $SK_{del254-262}$, nor between the rates of activation of μPG into μPN under conditions where native HPG was activated differentially by SK and $SK_{del254-262}$, clearly indicating a kringle-mediated mechanism for the role of the 250-loop of SK in substrate docking/recognition.

The involvement of kringles was further indicated by a hyper-susceptibility of the $SK_{del254-262}$.HPN activator complex to EACA inhibition of substrate HPG activation in comparison to that of the native SK.HPN activator complex. Further, 'ternary' binding experiments on the Resonant Mirror showed that the binding affinity of K1-5 of HPG to the preformed binary complex of the 250-loop deleted mutant and HPG was reduced by about 3-fold in comparison to that of SK.HPG. Overall, these observations identify the 250-loop of SK as an important structural determinant of the inordinately stringent substrate specificity of the SK.plasmin(ogen) activator complex and demonstrate that this structural epitope in the β domain promotes the binding of substrate HPG to the activator via the kringle/s during the HPG activation process.

The elucidation of the crystal structures of SAK and SK has highlighted the remarkable structural homology between SAK on one hand, and each of the three domains of SK, especially the α domain of SK, on the other. In order to find out

whether the α domain of SK displays cofactor activity similar to that of SAK, $\alpha_{wild\text{-}type}$ was purified to near-homogeneity using two chromatographic steps, *viz.* Ni^{+2}-IMAC followed by ion-exchange chromatography and then checked for its ability to activate substrate HPG. The wild-type α domain was found to exhibit only 0.002 % activity when compared to that of SK. To explore whether this meager activity was genuine activity or was due to the known proteins in *Escherichia coli* that have been shown to possess HPG activation ability, lysates of *Escherichia coli* cultures transformed with the pET-23d vector alone, without SK α gene, were subjected to exactly the same purification procedure employed for $\alpha_{wild\text{-}type}$. The purified $\alpha_{wild\text{-}type}$ protein showed higher cofactor activity than the 'mock' purified proteins.

When the cofactor activity of $\alpha_{wild\text{-}type}$ generated after mixing with equimolar amounts of HPN was examined, a 25-fold increase in activity was observed compared to the activity measured with HPG alone, indicating that the activity was HPN-dependent. In order to investigate whether the cofactor activity of $\alpha_{wild\text{-}type}$ was an under-estimation because of HPN-mediated proteolysis, the susceptibility of $\alpha_{wild\text{-}type}$ to HPN-mediated proteolysis, if any, was checked; this showed that $\alpha_{wild\text{-}type}$ was highly prone to proteolysis. The principal target of scission was found to be Lys59-Ser60 peptide bond in SK α, by N-terminal amino acid sequencing. With a view to minimize the proteolysis, Lys59 was mutated to an Ala residue. This construct (α_{K59E}) was found to be highly stabilized against HPN-mediated proteolysis and interestingly, showed a 8-fold increase in cofactor activity, compared to that of $\alpha_{wild\text{-}type}$. However, the cofactor activity measured was still much lower than that of SAK.

With a view to further enhance the cofactor activity of α_{K59E}, the structures of the α domain of SK and SAK were compared; this revealed the presence of an eight-residue loop in SAK, which was absent in SK α. The loop was then grafted into SK α (α_{loop}) by the overlap extension PCR-based method. The resultant construct, termed α_{loop}, showed a 2-fold higher cofactor activity than α_{K59E}. A detailed kinetic analysis showed a 5-fold decrease in K_M over that of α_{K59E}. In order to optimize the stereo-chemical contacts of the SAK loop with the rest of the α domain, random PCR-mutagenesis in the flanks encompassing four amino acid residues on either side of the loop in α, was carried out. The library of these mutants was expressed in a secretory vector, and nearly ten thousand clones were screened, using the *in situ* radial

caseinolysis plate assay (Malke and Ferretti, 1984). Five hundred 'hyperactive' clones were selected on the basis of larger zones of clearance on the agarose plates layered with casein and HPG. The best of these 'hyperactive' mutants were then transferred from the secretory vector to pET-23d, a T7 RNA polymerase promoter-based expression vector, and the proteins were purified by Ni^{+2}-IMAC followed by ion-exchange chromatography. When these combinatorial mutants were assayed for their cofactor activities, they were found to exhibit ~ 3-6 fold higher activity than α_{K59E}. One of the combinatorial mutants (termed α_{CM}), which showed ~ 6-fold higher activity than that of α_{K59E} was then analyzed further. The steady-state kinetics of α_{CM} indicated that α_{CM} displayed overall catalytic efficiencies nearly that of SAK.

The HPG-binding interactions of $\alpha_{wild\text{-}type}/\alpha_{K59E}$ and α derivatives were then checked using the Resonant Mirror based approach, in which each of these proteins was allowed to interact with biotinylated HPG. It was found that the interactions at the binary level were not greatly altered for these constructs. However, when attempts were made to study ternary interactions between substrate HPG and $\alpha_{wild\text{-}type}/\alpha_{K59E}/\alpha$ derivatives/SAK pre-complexed with immobilized HPG, reliable binding constants could not be determined in case of α_{K59E} because of the weak nature of the binary complex formed between α_{K59E} and HPG. On the other hand, the binary complex of α_{CM} and HPG was stable even after washing with buffer, and therefore, binding constants for its ternary interactions with substrate HPG could be determined. The equilibrium dissociation constant of the ternary interaction in case of SAK was found to be 4-fold higher than that of α_{CM}, an effect that was also observed in a corresponding increase in affinity (K_M) for substrate HPG in case of the combinatorial mutant.

When cofactor assays and Resonant Mirror-based ternary interaction studies were carried out with μPG as substrate, no discernable differences were detected in case of $\alpha_{wild\text{-}type}/\alpha_{K59E}$ and α_{CM}, under conditions where substantial differences were observed, when HPG was used as the substrate. These observations strongly point out the role of kringles in substrate recognition and overall catalytic enhancement.

To explore whether the higher levels of cofactor activity observed in the α derivatives over that of $\alpha_{wild\text{-}type}$ were translated into an increased ability to dissolve fibrin clots, fibrin-clot lysis assays were carried out, using these proteins.

Remarkably, this miniaturized SK α-based HPG activator was capable of *in vitro* fibrin clot lysis, thereby making it a possible candidate for *in vivo* therapeutic use.

These results clearly point out the critical role played by the eight-residue loop in SAK, which, upon transposition into the α domain of SK, and, further optimization by localized random mutagenesis, promotes substrate recognition and turn-over via the kringle domains of HPG. The fact that protein-grafting followed by random mutagenesis successfully transformed an *intrinsically inactive α domain* into a module that exhibited catalytic efficiency nearing that of SAK, and also carried out clot-dissolution *in vitro* provides new insight into the evolutionary process associated with the bacterial PG activators, and paves the way for the design and development of miniaturized clot-buster proteins with greater therapeutic value.