Thrombolytic therapy with SK has been well established, and has proved to be of immense advantage in the clinical management of diverse complications of the circulatory system, particularly in myocardial infarction. Although much is known about the basic biochemistry of SK action, very little information is available on the structural basis of PG activation by this protein. This information is vitally needed to design improved versions of this drug. Hence, the present study was aimed at the identification and characterization of the structurally and functionally important areas/regions in SK involved in the interaction of SK with PG, so that an insight could be obtained of the mechanism of SK action.

To meet the requirements of relatively large quantities of highly purified SK for this study, two different purification procedures were developed. One, involving a combination of conventional chromatographic methods viz., HIC (hydrophobic interaction chromatography) and ion-exchange was found to be quite amenable to scale-up to 10 liter levels fermentation. The other, based on a novel affinity-based procedure using, for the first time, animal plasmin(ogen)s as affinity ligands, was also standardized. The ability of SK to form a strong 1:1 stoichiometric complex with either PG or PN was exploited to design affinity matrices comprising of plasmin(ogen)s from various animals (e.g., rabbit and porcine species) as affinity ligands. Both these methods (conventional as well as affinity based) could purify extracellular SK from *S. equisimilis* H46 A culture broth to apparent homogeneity with very high specific activities (around 200,000 IU/mg), with overall yields ranging from 65-70 percent.

As a first step towards the understanding of the molecular mechanism of SK action, two principal contact sites in SK involved in interaction with HPG were localized using a limited proteolysis approach. Employing Western blotting with radiolabeled PG after SDS-PAGE of limited tryptic digest of SK, three fragments of MW 7 kD, 19 kD and 31 kD were found to possess PG-binding ability. Each of these fragments was then isolated by reverse phase HPLC and characterised with respect to its sequence, as well as its PG-binding properties by ELISA. These analyses revealed that there exist two distinct, high-affinity, independent HPG binding sites in SK. One of the sites was located in the core region of SK i.e. SK143-293, and the other one was shown to be present in the N-terminal region (residues 1-59) of SK. With the help of a synthetic peptide, the N-terminally located PG-binding-site was further localised to the region 37-51 of SK. This stretch contains a short sequence (LTSRPA) that is also present in the PG-binding domain of human fibronectin.

In order to locate the "core", HPG binding site in SK, delineation of the PG binding region in a tryptic fragment of SK derived from the central region (SK143-293) which could bind as well as activate HPG (albeit at reduced levels in comparison to the activity of the native, full-length protein), was carried out. Since this fragment was refractory to further controlled proteolysis, recourse to a synthetic peptide approach (peptide walking) was undertaken wherein the HPG interacting properties of sixteen overlapping 20-mer peptides derived from this region of SK were examined systematically. Only four peptides from this set viz., SK234-253, SK254-273, SK274-293 and SK263-282, together representing the contiguous sequence SK234-293, displayed HPG binding ability. This was established by a specific HPG binding ELISA as well as by dot blot assay using ^{125}I-labelled HPG. These results showed that the minimal sequence with HPG binding function resided between residues 234-293. None of the synthetic SK-peptides was found to activate HPG either individually or in combination, but in competition experiments where each of the peptides was added prior to complex formation between SK and HPG, three of the HPG binding peptides (SK234-253, SK254-273 and SK274-293) strongly inhibited the generation of a functional activator complex by SK and HPG. This indicated that residues 234-293 in SK participate directly in intermolecular contact formation with HPG during the formation of the 1:1 SK-HPG complex. Two of the three peptides (SK234-253 and SK274-293), apart from interfering in SK-HPG complex formation, also showed inhibition of the amidolytic activity of free HPN by increasing the K_m by approximately 5-fold. A similar increase in K_m for amidolysis by HPN as a result of complexation with SK has been interpreted to arise from the steric hinderance at or near the active site due to the binding of SK in this region. The results suggested that SK234-253 and SK274-293 also, like SK, bound close to the active site of HPN, an event that was reflected in the observed alteration in its substrate accessibility. By contrast, while the intervening peptide (SK254-273) could not inhibit amidolysis by free HPN, it showed a marked inhibition of the activation of 'substrate' PG (human or bovine plasminogen) by activator complex, indicating that this particular region is intimately involved in interaction of the SK-HPG activator complex with substrate plasminogen during the catalytic cycle. This finding provides a rational explanation for one of the most intriguing aspects of SK action i.e. the ability of the SK-HPG complex to selectively catalyze the activation of substrate molecules of PG to PN, while free HPN alone cannot do so. Taken together, the results strongly support a model of SK action in which the segment 234-293 of SK, by virtue of the epitopes present in residues 234-253 and 274-293, binds close to the active center of HPN (or, a cryptic active site, in the case of HPG) during the intermolecular association of the two proteins to form the equimolar activator complex; the segment SK254-273 present in the center of the core

region then imparts an ability to the activator complex to selectively interact with substrate PG molecules during each PG activation cycle.

It is known that the low plasminogen (PG) activating ability of several truncated fragments of the multi-domain protein, streptokinase (SK), namely SK64-386, SK60-414, SK60-387 and SK60-333, all of which encompass the centrally located high-affinity PG binding domain, is enhanced upto several hundred-fold by N-terminal SK-fragments (e.g., SK1-59 or SK1-63) which, by themselves, are completely inactive. The mechanism of this complementation by the N-terminal region of SK and its role in SK-PG interactions was investigated using a synthetic peptide approach. The addition of either natural SK1-59 or chemically synthesized SK16-59 at saturation (400-500 fold molar excess) generated amidolytic and PG activation capabilities in equimolar mixtures of HPG (human plasminogen) and a derivative of SK devoid of the N-terminal region (either SK60-414 or SK56-414, prepared by expression of truncated SK gene fragments in *E. coli*) that were approximately 1.2 and 2.5 fold, respectively, of that generated by equimolar mixtures of native SK and HPG. Although in the absence of SK1-59, equimolar mixtures of SK56-414 and HPG could generate almost 80 percent of amidolytic activity, albeit slowly, less than 2 percent level of PG activation could be observed under the same conditions. These results indicated that the contribution of the N-terminal region lay mainly in imparting in SK56-414 an enhanced ability for PG activation, since SK56-414 could not generate this property in either the zymogen (HPG) or its amidolytically activated form (HPN) in the absence of the N-terminal fragment. A series of truncated peptides derived from the amino-terminal region (SK16-51, SK16-45, SK37-59, SK1-36, SK16-36 and SK37-51) were synthesized and then tested for their ability to (i) complement equimolar mixtures of SK56-414 and HPG for the generation of amidolytic and PG activation functions, (ii) inhibit the potentiation of SK56-414 and HPG by SK16-59, and (iii) directly inhibit PG activation by the 1:1 SK-HPG activator complex. Although SK16-59, SK16-51 and 16-45 displayed ability to generate amidolytic potential in HPG in the presence of SK56-414, this potential was found to be preserved even in the smaller SK-peptides viz. SK37-59 and SK37-51. This capability in SK37-59, however, could be abolished by specifically mutating the sequence -LTSRP- present at position 42-46 in native SK, suggesting the involvement of this locus in the generation of amidolytic activity in HPG. While SK16-51 retained virtually complete ability for potentiation of PG activation in comparison to SK16-59 or SK1-59, this ability was reduced by approximately 4-fold in the case of SK16-45, and completely abolished upon further truncation of the C-terminal residues to SK16-36 or SK1-36. Remarkably, however, these peptides displayed strong inhibition of PG activation by the

native activator complex in the micromolar range of concentration. On the other hand, fragments SK37-51 and SK37-59, which also could not directly potentiate PG activation by SK56-414, did not show any inhibition of the PG activation by native activator complex. The inhibition of PG activation by fragments SK16-36 or SK1-36 could be competitively relieved by increasing the concentration of substrate PG in the reaction, suggesting that this region in SK contains a site specifically directed towards interaction with substrate PG. This conclusion was substantiated when potentiation of PG activating ability was found to be considerably reduced in a peptide (SK25-59) in which the sequence corresponding to this putative locus (residues16-36) was truncated at the middle. A sensitive dot-blot assay using radioactive HPG, as well as a specific ELISA, showed that SK1-36/SK16-36 could indeed bind HPG, and that this binding, although relatively weaker, was independent of that shown by fragments SK37-59 or SK37-51. Taken together, these findings strongly support a model of SK action wherein the HPG binding site resident in the region 37-51 helps in anchoring the N-terminal domain to the strong intermolecular complex formed between HPG and the region 60-414, whereas that located in and around residues 16-36 is involved in the relatively low-affinity interaction of the activator complex with substrate PG. Thus, the region 16-36 of SK helps in conferring the characteristic substrate specificity to the activator complex by imparting an enhanced ability to selectively interact with substrate PG molecules during the catalytic cycle.

In conclusion, this study has yielded fundamental insight into the mechanism of action of SK. This is expected to help in the future design and construction of therapeutically improved, second-generation derivatives of this important clot-dissolving drug.