

The development of economically viable thrombolytic therapy has been possible with the extensive and successful use of streptokinase (SK) as the drug of choice, especially in the third world countries, for the treatment of various clot-related diseases. However, the need exists for the creation of better and more effective streptokinase, possessing longer plasma half-life, more efficient reperfusion capacity, and lesser side effects due to greater clot-specificity. A crucial and obligatory step in the development of such improved variety of molecules lies in the thorough understanding of the fundamental structure-function correlation underlying the functioning of SK. SK first binds to plasmin (PN), a serine protease, in equimolar ratio to form a tight SK-PN complex. It functions by modulating and restricting the substrate specificity of this 'partner' PN from a broad spectrum serine protease to a molecule, devoted to the activation of plasminogen (PG) to plasmin. Despite intense research for the past three decades to decipher the molecular basis behind functioning of the molecule, the mechanism adopted by SK, especially with respect to 'substrate' PG activation by the SK-PN complex, still remains unclear. This treatise attempts to unravel some of the functional aspects of this enigmatic protein and identifies some of the critical residues that play cardinal role in functioning of SK.

As a prelude to the in-depth study of the structure-function inter-relationships in SK, especially with respect to (a) the identification of critical residues in the central β domain of SK that are involved in its interaction with plasminogen, and (b) the co-operation amongst the three structurally homologous domains of SK in the development of PG activating capability, we cloned, over-expressed, and purified SK and its various mutant derivatives. These derivatives of SK included the individual domains (α , β and γ), their native-like two-domain combinations ($\alpha\beta$ and $\beta\gamma$), as well full-length SK with several point mutations in the 'core' region of the β -domain (residues 230-290) [previously identified in our laboratory by Peptide Walking studies to play a key role in plasminogen activation], by site-specifically altering two charged residues at a time to alanines. The development of a rapid, efficient and generalized methodology for the purification of SK and its derivatives utilizing a 2-step procedure viz., purification on HIC (Phenyl-agarose), followed by a polishing step on ion-exchange chromatography, greatly facilitated the structure-functional studies. Hydrophobic interaction chromatography on Phenyl-agarose column turned out to be a particularly useful and powerful technique, since the desired protein purified to over 80 % levels in one rapid

ACC. No. 17H-130

step owing to the removal of most contaminating, unwanted, intrinsic *E. coli* proteins at this stage. The purification procedure also yielded structurally intact proteins as assessed by CD and various functional studies. The detection of the expected secondary structure/s in SK and its various derivatives, (calculated from the crystal structure) corroborated the utility and effectiveness of this purification strategy for isolation of structurally intact molecules to be used in future functional studies.

The possible role of the central β domain of SK (residues 151-287) in PG activation was probed using the purified site-directed mutants in the 'core' region of SK. The thirteen different mutants were screened for altered ability to activate equimolar 'partner' human PG, or altered interaction with substrate PG resulting in an overall compromised capability for substrate PG processing. Of the eight initial alanine-linker mutants of SK, one mutant viz. SK_{KK256.257AA} (abbreviated SK-D1) showed a roughly twenty-fold reduction in PG activator activity in comparison to wild-type SK expressed in *E. coli* (rSK). Five other mutants were as active as rSK, with two [SK_{RE248.249AA} and SK_{EK281.282AA}, referred to as SK(C) and SK(H), respectively] showing specific activities approximately one-half and two-third, respectively, that of rSK. Unlike SK(C) and SK(H), however, SK(D1) showed an extended initial delay in the kinetics of PG activation. These features were drastically accentuated when the charges on the two Lys residues at positions 256 and 257 of rSK were reversed, to obtain SK_{KK256.257EE} [abbreviated as SK(D2)]. This mutant showed a PG activator activity approximately ten-fold less than that of SK(D1). Remarkably, inclusion of small amounts of human plasmin (PN) in the PG activation reactions of SK(D2) resulted in a dramatic, PN dose-dependent rejuvenation of its PG activation capability, indicating that it required pre-existing PN to form a functional activator. The steady-state kinetic parameters for HPG activation of its 1:1 complex with human PN revealed that although it could form a highly functional activator once 'supplied' with a mature active site, the K_m for PG was increased nearly 8-fold in comparison to that of rSK-PN. This observation, juxtaposed with the observed stereo-chemical arrangement of Lys256.267 at the tip of a flexible loop in the β -domain strongly points to an important role of this loop in general and these residues in specific, for the 'capturing' substrate PG by the activator complex. SK mutants carrying simultaneous two- and three-site charge-cluster alterations viz., SK_{RE248.249AA;EK281.282AA} [abbreviated SK(CH)], SK_{EK272.273AA;EK281.282AA} [SK(FH)], and

SK_{RE248.249AA;EK272.273AA;EK281.282AA} [SK(CFH)] showed additive/synergistic influence of multiple charge-cluster mutations on HPG activation when compared to the respective 'single-site' mutants, with the 'triple-site' mutant [SK(CFH)] showing absolutely no detectable HPG activation ability. Nevertheless, like the other constructs, the double- and triple-charge cluster mutants retained a native-like structure and affinity for complexation with partner PG. These results provide the first experimental evidence for a direct assistance by the SK β -domain in the docking and processing of substrate PG by the activator complex, a facet not readily evident probably because of the flexibility of this domain in the X-ray crystal structure of the SK- μ PN complex. These studies also establish the unique mechanism of SK action, namely the modulation of the substrate specificity of PN (a broad-spectrum serine protease) through cofactor assisted binding of substrate PG at the active site of the equimolar complex.

SK functions as a 'protein-cofactor' to the enzyme by binding near the active site of PG/PN and exhibiting exosites for docking of 'substrate' PG in a stereochemically productive orientation. In order to test whether structural homology of the three domains of SK with the unidomain activator SAK bestows upon the three domains of SK, PG activating function, each of the SK domains was expressed in *E. coli*, purified and analyzed for activity. But none of the individual domains or their two-domain combinations showed any substantial activity, over the intrinsic PG activator capability existent in the background *E. coli* membrane protein that has been previously reported (Lundrigan and Webb). Surprisingly however, the absence of activity in these domains could not be ascribed to the inability in these molecules to interact with PG, since not only did each of these bind PG (with affinities in the low μ M range), but the pre-formed domain-PG binary complex could also bind another PG molecule ('substrate' PG) to form a ternary complex. When the covalently attached two-domain complex were examined for various PG interacting and activating functions, the presence of ternary complex, as well as kringle-mediated, substantial, and easily measurable activity in the two-domain covalent combination, $\alpha\beta$ (3.5 %) and $\beta\gamma$ (0.5 %) was detected. These observations revealed the absence of any direct correlation between docking of substrate and generation of activity. However, a correlation was evident between the generation of activity in the truncated molecules and ability to competitively inhibit PG activation by urokinase (UK), a facet corroborated by the absence of activity, and ability to inhibit UK

in the independent domain moieties, and presence of activity and ability to inhibit UK mediated PG activation by the bi-domain construct. Since PG activation by UK is known to occur by virtue of binding near the active site and acting on the catalytic domain of PG, we infer that PG activation arises only after acquiring capability to recognize the catalytic domain of 'substrate' PG, which the unidomain molecule are largely incapable of. Thus our investigations reveal that 'functionally relevant docking' of substrate in SK requires not only the direct binding of activator molecule to the catalytic domain of 'substrate' PG, but also the existence of kringle dependent interactions, a property minimally requiring two-domain in tandem for catalytic functioning.

In conclusion, this study unequivocally demonstrates the critical role played by the β -domain in capturing substrate PG molecules and thus provide additional information that the X-ray studies could not reveal. It also identifies the most critical feature required for the generation of PG activating function/s in an SK-like molecule is the ability to seek the catalytic domain and the kringle domain/s simultaneously, which only two-domain combinations are capable of performing. These results thus pave the way for further work directed at understanding structural interactions of the various SK domains in the process of recognizing the kringle and catalytic domain during substrate docking on the one hand, and its concerted release upon conversion to product, on the other. A molecular understanding of this process would clearly aid in designing more effective PG activator derivatives of this life-saver protein.