SUMMARY

According to world health statistics report, in comparison to other NCD, Non Communicable Diseases (e.g. diabetes, cancer, and stroke) the proportion of deaths from Cardiovascular Diseases (CVD) is greater. As per study, in India CVD became the leading cause of mortality. According to an estimate from The Global Burden of Disease study, in India age-standardized CVD death rate is 272 per 100000 population which is higher than the global average of 235 per 100 000 population. As per an estimate by 2030 three out of every five cardiac patients in the world will be from India (Gupta et al., 2016; Prabhakaran et al., 2016).

CVD is a class of diseases that involves heart or blood vessels and includes myocardial infarction, pulmonary embolism, deep venous thrombosis and stroke. Commonly used medication for these clinical conditions are thrombolytics or clot dissolving drugs such as tissue Plasminogen Activator (tPA), Urokinase (UK) or Streptokinase (SK). Amongst these, SK is a commonly used thrombolytic in developing economies for its low cost and potent thrombolytic properties. SK is a bacterial protein that converts HPG to HPN which cleaves fibrin as a part of clot lyses process in vivo (Bajaj and Castellino, 1977; Banerjee et al., 2004).

The Sahni lab at CSIR- IMTECH, Chandigarh has contributed significantly in the area of structure-function analysis and engineering of the protein cardiovascular drug – Streptokinase in the last 20 years or so. Apart from the generation of cost-effective process technology for first generic indigenous clot buster (Natural SK, brand named 'STPase'), manufactured and marketed by Cadila Pharamaceuticals Ltd., to recombinant Streptokinase, produced by Shasun Drugs, Chennai under several brands, the group patented and licensed India's first Bio-pharmaceutical protein product (Clot Specific Streptokinase)- now in Human Phase II Clinical trials. Recently, the fourth generation 'Anti-thrombotic' Clot buster has also been patented and licensed out and is now in

pre-clinical phase of development. The group has contributed significantly by producing technology for India's first indigenous clot bluster drug, natural streptokinase (Patent Application No.: IN 3448DEL2015; IN 06563DELNP2011; IN 0837DEL2008; IN 1727DEL1994; IN 0159DEL2003; IN 0160DEL2003; IN 3825DEL1998).

In order to enhance the therapeutic properties of SK, one needs to have an in-depth understanding of the molecular events controlling SK mediated activation of Plasminogen. Dr Sahni's group have also made a very significant contribution in understanding structure-function relationship of SK.HPN mediated Plasminogen activation (Aneja et al., 2009; Aneja et al., 2013; Chaudhary et al., 1999; Dhar et al., 2002; Jayaraman, 2012; Joshi et al., 2012; Maheshwari et al., 2016; Nihalani et al., 1998; Nihalani et al., 1997; Sawhney et al., 2016a; Sawhney et al., 2016b; Sundram et al., 2003; Yadav et al., 2011; Yadav et al., 2008; Yadav and Sahni, 2010).

SK acts as a protein cofactor since it does not have an enzymatic activity by itself, and binds to HPG to form a high-affinity stoichiometric complex, which undergoes conformational transition/s that are not fully identified, to form SK.HPG* (a pre-activated complex) that selectively cleaves Arg^{561}-Val^{562} scissile peptide bond in substrate Plasminogen, converting it into its active form HPN, Plasmin (Bode and Huber, 1976; Boxrud et al., 2000; Jackson and Tang, 1978; McCance and Castellino, 1995; McClintock and Bell, 1971; Renatus et al., 1997; Summaria et al., 1982; Wang et al., 2000; Wang et al., 1998).

With the advancement in the field of protein engineering, rational designing of enzymes with new and improved functions have gained a lot of interest amongst researchers. The present work is devoted to get further insights and understanding of catalytic steps in SK-mediated activation of substrate Plasminogen, which can be potentially exploited for designing bio-therapeutics with improved properties.

Studies have shown various structural elements in SK referred to as "exosite" which are located relatively away from the active site in SK.HPN activator

complex and play an important role in substrate recognition, docking and probably in the processing of substrate to product as well (Aneja et al., 2009; Aneja et al., 2013; Chaudhary et al., 1999; Dhar et al., 2002; Tharp et al., 2009; Yadav et al., 2008). Since, SK dependent activation of Plasminogen is mediated through conformational organizations and reorganizations taking place at both substrate and the activator level we cannot rule out the significance of conformational dynamicity in SK mediated catalysis as this catalysis is solely dependent on these motions. Also, the idea that specific and directed protein motions mediate catalytic turnover in the pathways of enzyme catalyzed reactions has gained considerable support in the recent years (Kempner, 1993; Kohen, 2014; Wolf-Watz et al., 2004).

In the present work, we attempted to explore through simple biochemical experiments, the possibility of coupling the intrinsic motions of the protein (streptokinase) with its catalytic efficiency as a partner with plasmin(ogen) using site directed mutagenesis and MD simulation approach.

As discussed already, SK binds to HPG to form a high-affinity stoichiometric complex, SK.HPG* (a pre-activated complex) (McClintock and Bell, 1971; Summaria et al., 1982). Once this stable binary complex is formed, docking of substrate Plasminogen is mediated through interactions between kringle K5 of incoming substrate Plasminogen and 250 loop of SK in SK.HPG* binary complex, forming HPG.SK.HPG* ternary complex (Dhar et al., 2002; Tharp et al., 2009). Following ternary complex formation there is cleavage at R_{561}-V_{562} scissile peptide bond (Robbins et al., 1967) converting single chain Plasminogen substrate into bi chain Plasmin product. On conversion into product, substrate HPG likely undergoes conformational transitions which facilitate the release of product (Linde et al., 1998; Parry et al., 1998; Peisach et al., 1999; Wang et al., 2000; Wang et al., 1998) from the activator complex.

At one level, we made an attempt to correlate the flexibility of the 250 loop of SK with the catalytic efficiency of SK. Accordingly the amino acid residues

along the hinges, or flanks, of the loop have been substituted with Glycine or Proline residues. We then screened *in silico* followed by *in vitro* a set of mutants thus generated, for their catalytic efficiency and conformational flexibility using steady state kinetics and MD simulations, respectively. Amongst the various loop mutants of SK thus generated, SK.G1 mutant where residues along the hinges (at 250 and 260th position) were substituted to Glycine showed measurably higher flexibility *and catalytic efficiency* as compared to that of wild type SK. SK.P3 mutant of SK, where three residues along the hinges (at 249,250,251 and 259,260,261 position) were substituted to Proline, not only reduced the flexibility factor of the loop, but also resulted in a concomitant lower catalytic efficiency (50% lower catalytic efficiency) compared to the wild type SK. Steady state analyses have shown that the mutants retained native-like affinity for the substrate Plasminogen, indicating that the mutation does not significantly affect the 'docking' of the substrate into the activator complex. Further, the mutants were screened for their capability to generate an active center in the partner (HPG) molecule, and it has been observed that all the mutants showed similarity in their capability to generate active center in the partner molecule in a way similar to native SK. Results so far obtained have shown that SK loop mutants are similar to wtSK in their capability to generate an active center in the partner molecule as shown by NPGB based titration and docking of the substrate Plasminogen to the wtSK/ loop mutant SK activator complex as shown by steady state kinetics studies. mutants were then compared to wtSK for the next step of catalysis i.e. peptide bond cleavage in the substrate HPG molecule to generate a bi-chain product i.e. HPN using SDS PAGE based analysis, results have shown that for all the mutants rate of scissile peptide bond cleavage is similar to that of wtSK.HPN activator complex.

Now, since the mutants have shown similarity to wild type in their capability to generate an active center in the partner molecule, docking of substrate to the activator complex and processing of scissile peptide bond in the substrate molecule, we hypothesized that this difference in the catalytic efficiency of the

mutants towards the processing of substrate Plasminogen can be attributed to differential post-cleavage step i.e. the release of product from the activator complex. In order to get an insight on the post–cleavage events of catalysis, we have developed a FRET based model system to get an in-depth understanding of events happening post cleavage. SK was labeled with donor fluorophore and PG was labeled with the acceptor fluorophore, the positions of the fluorophores were selected in a way that the change in FRET efficiency following complexation as in case of ternary complex formation and dissociation of complex upon release of product from the activator complex can be attributed to different events of catalysis under pre-steady state conditions. Since the process occurs over a faster time scale than that accessible by manual mixing in regular cuvettes, and we can miss the information using steady state fluorescence measurements, the experiments were optimized on a rapid mixing set up. We believe that this approach will further strengthen our conclusions that the flexibility of the critical 250 loop of SK as altered through site directed mutagenesis along the hinges of the loop caused increase or decrease in substrate turn-over by the streptokinase-Plasmin complex.

Although, we have tried to explore inter-relationship of the motions in the co-factor with that of catalysis, we cannot rule out the (additional) possibility of the contribution/s of substrate level dynamicity in SK mediated processing of the substrate Plasminogen. Indeed, there are reports (Joshi et al., 2012) which suggest the role of substrate kringles in differential processing of the substrate by the SK.HPN activator complex. Studies have shown that sequential addition of kringle 5 and kringle 4 to the isolated catalytic domain (CD) of Plasminogen resulted in an increase in the catalytic efficiency from 25% (in case of CD alone) to nearly 80% (in case of midiPG which contains two of the five kringle domains of native himan plasminogen); also, the reports have shown that kringle 5 of Plasminogen is very important for affinity between substrate and enzyme (Tharp et al., 2009), and also it has been observed that addition of kringles beyond kringle 5 does not result in any further changes in the affinity of the

substrate towards the activator complex. MD simulations of the midi-PG was chosen to study substrate level conformational dynamicity, and these showed that the linker region connecting two kringles together (k4-k5 linker) is a highly flexible structure. Amino acid composition analysis showed that the linker constitutes a prominent stretch of Proline residues. We have sequentially substituted these Prolines to Alanines through site directed mutagenesis and analyzed the mutants thus generated for their catalytic properties. It has been observed that this substitution has a synergistically decreasing effect on catalysis. With the p4a4 mutant, where all Prolines were substituted to alanine, only 30% of native activity was retained as compared to that of wild type midi PG. The PG mutant was further checked for its activation by using physiological activator tPA as a Control, to rule out the possibility of any perturbation in the active center milieu of the Plasminogen, due to the mutation/s incorporated. Comparisons in the activation of midi PG wild type/mutants by SK and tPA have shown that the linker mutant exhibited SK-dependent behavior different from its nearly full, native like activation with tPA. To rule out the possibility that the mutation resulted in any proteolytic susceptibility in the mutant as compared to that of wild type substrate midi PG, and which may be responsible for its apparent lower catalytic efficiency as substrate, we performed SDS-PAGE gel based proteolytic susceptibility test of the mutant and compared that with wild type. It has been observed that both the mutant and the wild type showed closely similar behavior. Further MD simulations of the mutant and its comparison with wild type showed that Proline to Alanine mutations in p4a4 mutant of midi Plasminogen introduced flexibility factor to the k4-k5 linker, connecting two kringles together.

Although not conclusive at this stage, we can summarize from the present work that the k4-k5 inter-kringle linker provides the two kringle domains optimum conformational flexibility needed for SK.HPN mediated activation of Plasminogen. Disrupting the inter kringle conformational freedom, as in case of linker mutants, can significantly alter the catalytic processing of the substrate

by the activator complex. We hypothesize that the kringle-specific conformational changes induced in Plasminogen substrate are somehow 'sensed' by the SK.HPN activator complex. Thus the flexibility plays a significant role in the catalytic processing of the substrate either by altering the rate of peptide bond cleavage or by modulating the product release step of catalysis. Which one of these is critical we cannot confirm at this point of time, and needs to be explored further.

With the growing requirement of having better thrombolytic drugs with enhanced catalytic potential it is important to have an in-depth understanding of the molecular events controlling SK.HPN mediated catalysis of Plasminogen substrate. The present study will provide evidence to couple the conformational dynamicity of the protein with its catalytic efficiency and the development of FRET based model will help in identifying those hot spots in the protein which plays an important role in regulating the post cleavage step/s of catalysis e.g. product release. Identification of those hot spots and their exploitation for rational designing of enzymes with desired properties using protein engineering based approaches will be the main aim in future for the lab. The present study is a step in that direction.