Introduction

'Cardiovascular disease' is a generalised term for a number of specific heart diseases affecting the heart and/or blood system especially veins and arteries that channelize the blood flow to and from the heart. These heart diseases are world's leading killers, claiming millions of lives every year including all age groups. The occlusion of a coronary or cerebral artery by a blood clot results in the final interruption of the blood flow. Perhaps prevention of atherosclerosis will have the most important effect on this process; yet for many, in whom prevention is too late, dissolution of the thrombus with plasminogen activator therapy is an attractive method of restoring blood flow. Plasminogen activators activate plasminogen to plasmin \( i.e. \) the main enzyme of the fibrinolytic system of the blood that finally leads to the dissolution of the blood clot to maintain coronary artery recanalization.

1.1 Haemostasis

Humans have evolved an intricate network of tightly regulated pathways to maintain a continuous blood flow throughout the body under physiological conditions for its proper functioning, a condition known as haemostasis (Colman et al., 2006). This haemostatic system is well equipped to handle a vascular injury by plugging it with a blood clot to prevent the uncontrolled blood flow. A blood clot is a mass of platelets entangled in a network of fibrin threads. The clots are formed inside the blood vessels when required and subsequently degraded in a highly controlled way. So the formation of the blood clots \( i.e. \) thrombosis and the dissolution of the blood clots \( i.e. \) thrombolysis, inside the body go in a balanced way by the interplay of a number of molecules participating in the coagulation cascade and the anticoagulant pathways. However, the formation of these clots, if unregulated, can pose a real threat to the body by interrupting the blood supply to various organs.

It has been found that accumulation of white blood cells during inflammation, in the walls of the arteries leads to the development of soft vulnerable plaques which if released can promote blood clotting. This is the main triggering event of the common cardiovascular diseases such as coronary artery disease leading to myocardial infarction, cerebrovascular disease causing stroke which have been a major cause of morbidity, mortality and disability in the industrialised countries. The use of the plasminogen activators for dissolution of the blood clots has now become an attractive therapy for
restoring the blood flow in the blood vessels (Collen and Lijnen, 1991). These therapeutics activate the main enzyme of the fibrinolytic system \textit{i.e.} plasminogen to plasmin that in turn digests the fibrin threads of the clot to soluble degradation products.

\textbf{1.2 Coagulation cascade}

Blood coagulation system basically operates as a ‘molecular machine’ where the cellular elements like blood platelets and white blood cells etc. interact with proteins, enzymes, cofactors and substrates for assembling effective macromolecular complexes thereby generating fibrin molecules (Spronk \textit{et al.}, 2003, Davie, 1995, Davie \textit{et al.}, 1991). Traditionally the coagulation cascade has been divided into intrinsic (contact activation) and extrinsic pathways (tissue factor stimulation) however, the boundary line between the two pathways is quiet blurred \textit{in vivo} (Colman \textit{et al.}, 2006, Davie, 1995, Schenone \textit{et al.}, 2004, Hoffman and Monroe, 2005). In the extrinsic pathway, tissue factor (TF), a membrane bound glycoprotein is the key player as a trigger of the coagulation system. On a vascular injury the molecule is expressed on the vasculature cells like monocytes and forms a complex with factor VIIa on a phospholipid surface to activate both factor X and IX which ultimately leads to the generation of thrombin from prothrombin (Versteeg HH, 2001). Initially a very small amount of thrombin is generated because of the inhibitory action of TFPI (tissue factor pathway inhibitor) on factor Xa. However, during propagation phase thrombin activates the other factors such as factor V, VII and XI (Osterud and Rapaport, 1977) to increase its own synthesis. In the intrinsic pathway, factor XI activates factor IX which alongwith factor VIII and Ca$^{2+}$ ions makes the ‘tenase complex’ to finally activate factor X following the common pathway thereafter for thrombin generation. Thrombin catalyses the conversion of fibrinogen to fibrin and leads to the clot formation (Fig. 1.1).

\textbf{1.3 Fibrinogen and Fibrinolysis}

Fibrinolytic system is the counterpart of the coagulation system and thus plays a regulatory role in haemostasis. Thrombin plays a central role in haemostasis by regulating its own synthesis and acting on a number of substrates participating in the coagulation including fibrinogen, factors XIII, V, VIII, XI, platelet membrane thrombin receptor (Coughlin \textit{et al.}, 1992a, Coughlin \textit{et al.}, 1992b, Colman \textit{et al.}, 2006). Its positive feedback effect leads to an increase in the tenase and prothrombinase complexes
Intrinsic System (PTT) | Extrinsic System (PT)
---|---
Kallikrein | Prekallikrein
Factor XII | Kallikrein
Factor XI | Factor X
Factor IX | Factor VIII
Factor X | Factor VII
Factor XIII | Factor XIII
Prothrombin | Thrombin
Fibrinogen | Fibrin

Common Path

Figure 1.1: Coagulation cascade
which trigger the thrombin burst and this burst finally increases the rate of fibrinogen conversion to fibrin for clot formation. Fibrinogenolysis is the conversion of fibrinogen to fibrin by the action of thrombin enzyme. The key molecule of the fibrinolytic system is plasminogen (Pg) which is converted to its active form plasmin (Pm) and then degrades the fibrin threads of the blood clots into soluble fragments. Partial degradation of fibrinogen exposes a number of lysine residues which further enhances its interaction with Pg through lysine binding sites (Suenson et al., 1984). The activation of Pg to Pm is catalysed through limited proteolysis by Pg activators such as urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), present physiologically. Thus, the fibrinolysis constitutes a repair mechanism for limiting clot formation (Fig. 1.2).

Under normal physiological conditions, the fibrinolytic system remains in a dynamic equilibrium by the controlled interactions of the activators and inhibitors of fibrinolysis and any imbalance of this equilibrium can lead to bleeding or thrombotic disorders.

1.3.1 Plasminogen-Plasmin (Pg-Pm) system

Pg-Pm system is the central apparatus for the dissolution of the blood clots and thus maintaining the regular blood flow in the blood vessels. Human Pg is synthesised in liver (Raum et al., 1980, Saito et al., 1980) as an 810 amino acid protein and during its secretion, 19 amino acids are cleaved for releasing 92 kDa (791 amio acids) mature protein (Castellino and Plopis, 2005). The primary structure of Pg was first reported in 1978 through elastase mediated limited proteolysis (Sotrup-Jensen, 1978). The schematic representation of the structure of Pg is shown in Fig. 1.3. The plasminogen molecule consists of an N-terminal pre-activation peptide, five kringle domains (named after a Danish breakfast role), namely K1-K5 and a C-terminal catalytic serine protease domain. The native human Pg is known as Glu-Pg because of the presence of a glutamate residue at the N-terminus and is converted to Lys-Pg through the cleavage of Lys77-Lys78 bond. Pg circulates in blood plasma at a high concentration (100-150 mg/litre) with a half life of 2.8 days (Collen D., 1972) and is converted to its active form Pm when required. The conversion of Pg to Pm is catalysed by physiologically present Pg activators (Violand and Castellino, 1976) (uPA and tPA) that specifically cleave Arg561-Val562 bond (Robbins et al., 1967, Summaria et al., 1967) to yield catalytically active serine protease.
Figure 1.2: Fibrinolytic system
Figure 1.3: Schematic representation of primary structure of human Pg. The kringle domains containing lysine-binding sites are marked as K1-K5. The amino acids forming the active site are indicated, (Ser740, Asp645, His602). Disulfide bonds are represented by dashed lines (Adapted from Lahteenmaki K. et al 2001).
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The molecule of Pm consists of a heavy chain of 65 kDa from Glu1-Arg561, containing five disulfide-bonded triple-loop kringle structures of approximately 80 amino acids each and a light chain consisting of a catalytic domain of 230 amino acids. The two chains are linked to each other through two disulphide bonds. His602, Asp645 and Ser740 form the catalytic triad of the catalytic domain which shows a considerable structural and functional homology to trypsin and other serine proteases. All the kringle domains exist as tripled looped independent modules with similar type of structures but are different in their lysine binding properties where the K1 and K4 domains show the strongest and K3, the least or no binding affinity for lysine or lysine analogues (Castellino and Ploplis, 2005). These lysine binding domains are not unique to Pg but are also present in other proteins involved in coagulation and fibrinolysis such as tPA, urokinase-plasminogen activator (uPA), prothrombin, apolipoprotein etc. The kringle domains facilitate the binding of Pg to various molecules such as fibrinogen (Suenson and Thorsen, 1981), bacterial and mammalian cell surfaces (Berge and Sjobring, 1993, D'Costa and Boyle, 1998, Wistedt et al., 1998). These kringles, thus, increase the rate of conversion of Pg to Pm by interacting with more and more lysine residues of fibrin exposed during its partial degradation at the time of initial phase of clot lysis. The binding of lysine residues to kringles induces major structural changes from a closed form to an extended flexible conformation which is more susceptible for activation by Pg activators (Mangel et al., 1990). In addition to these anchoring sites, serine protease domain of the molecule i.e microplasmin (μPm) is the main contributor in the function of Pm in various physiological and pathological processes such as tissue remodelling in cell migration, macrophage invasion in inflammation (Ploplis et al., 1998), embryogenesis, angiogenesis, keratinocytes accumulation, tumour cell growth and invasion (Ranson et al., 1998).

The complete three dimensional structure of Pg/Pm is not available yet although the structural data for all the kringles and catalytic domain is available (Atkinson and Williams, 1990, Mulichak and Tulinsky, 1990, Mulichak et al., 1991, de Vos et al., 1992, Wu et al., 1994, Battistel et al., 2009, Chang et al., 1998). The three dimensional structure for angiotatin (i.e. Kringle 1+2+3) has been solved by X-ray crystallography (Abad et al., 2002) (Fig.1.4). A structural model of human Pg with known and overlapping structures is presented in Fig. 1.5. The catalytic domain of human Pg (Fig. 1.6) can recruit various cofactors or adapters such as SAK and SK, which change the
Figure 1.4: Ribbon diagram representation of human angiostatin consisting of Pg kringles (K1, K2, K3). Each kringle contains three disulfide bridges (yellow).
Figure 1.5: Top and bottom 3-D structural model of human Pg based on overlapping 3-D structures of Pg fragments. Each kringle is shown in grey, the central Trp residue in each LBS is shown in magenta, the protease domain is shown in green, and the active site residues are shown in red (Adapted from Scheller J. et al. 2010).
Figure 1.6: Ribbon diagram representation of the catalytic domain of human Pg determined by X-ray diffraction (1DDJ). The active site residues are highlighted in green sticks.
substrate specificity of Pm. The unusual conformational changes induced on activation have been recently described through X-ray crystallography (Wang et al., 2000b).

1.3.2 Inhibitors of the fibrinolytic system

1.3.2.1 Plasminogen Activator inhibitor-1 (PAI-1)

PAI is a 52 kDa glycoprotein produced by endothelial cells but also secreted to some extent by other tissue types such as adipose tissue. PAI-1 is the member of SERPIN (serine protease inhibitor) family (Xue et al., 1998) and inhibits the physiologically present Pg activators i.e. uPA (Lin et al., 2011) and tPA as a principal inhibitor of fibrinolytic system. In addition to this, PAI-1 has also been shown in various cell types such as hepatocytes, platelets megakaryocytes and smooth muscle cells (Simpson et al., 1991). PAI-1 is present in low concentration in plasma (Urden et al., 1987) and under normal physiological conditions, remains in latent form after being secreted initially as an active molecule.

1.3.2.2 Plasminogen Activator inhibitor-II (PAI-2)

PAI-2 is another fibrinolytic inhibitor present mainly in monocytes/macrophages which is a potent inhibitor of uPA and to a lesser extent of tPA. It was first demonstrated in human placenta (Kawano et al., 1968, Kawano et al., 1970) and its concentration increases during the pregnancy period.

As a part of their mechanism of action these serpins undergo an unusual conformational change from a metastable 'relaxed state' to a more stable 'cleaved state' on interaction with the protease (Harrop et al., 1999, Jankova et al., 2001, Xue et al., 1998).

1.3.2.3 α2-Antiplasmin

α2-Antiplasmin is the principle inhibitor of plasmin enzyme in plasma. It is a single chain glycoprotein with a molecular weight of 70 kDa and contains about 13% carbohydrates (Collen, 1976, Moroi and Aoki, 1976, Mullertz and Clemmensen, 1976, Lijnen and Collen, 1985). During circulation, plasmin and antiplasmin react rapidly with each other at a rate constant above 10^-7 M^-1 s^-1 (Wiman and Collen, 1978). It also
interferes with the adsorption of Pg to fibrin and undergoes crosslinking with α-chains of fibrin during clotting. The carboxy terminus of α2-antiplasmin binds reversibly to lysine binding site of Pm.

1.3.2.4 α2-Microglobulin

α2-microglobulin is a large glycoprotein in blood plasma with a molecular weight of 725 kDa. It consists of four identical chains of 160 kDa and contains about 8% carbohydrate (Christensen and Sottrup-Jensen, 1984, Sottrup-Jensen et al., 1984). α2-microglobulin is a general protease inhibitor which acts on different types of proteases including serine-, cysteine-, aspartic- and metalloproteinases. α2-microglobulin is a “second line” inhibitor of many components of the fibrinolytic system. α2-microglobulin acts as a scavenger protease inhibitor (Cummings and Castellino, 1984) when extensive activation of the fibrinolytic system has occurred, leading to the generation of large amount of Pm since α2-antiplasmin cannot neutralize all the Pm.

1.3.3 Plasminogen activators

Pm is a broad spectrum protease that preferably cleaves peptide bonds next to lysine or arginine residues (Castellino, 1981, Parry et al., 2000). The conversion of Pg to Pm is a highly specific cleavage reaction at Arg561-Val562 position catalyzed by various Pg activators. On the basis of their mechanism of action, Pg activators can be classified as direct or indirect Pg activators. Direct Pg activators are the serine proteases which directly cleave Arg561-Val562 bond of Pg to convert it into Pm. The typical examples include uPA and tPA. Indirect Pg activators are not capable of activating Pg directly, but they serve as cofactors by forming a complex with Pg/Pm which finally cleaves Pg in a highly specific manner e.g. SAK and SK. On the basis of their source of origin, the Pg activators may be prokaryotic (bacterial) and eukaryotic.

1.3.3.1 Direct Pg activators

1.3.3.1.1 Tissue type plasminogen activator (tPA)

Human tPA is a single chain multidomain serine protease synthesized by various cell types such as endothelial cells and keratinocytes and is present in a concentration 5-10 μg/l in plasma. tPA is a large glycoprotein of 527 amino acids (68-70
kDa) having 7% carbohydrates. Half life of tPA in plasma is 5 minutes only and is present mainly as its complex with PAI-1. Limited plasmin hydrolysis of Arg275-Ile276 peptide bond converts the molecule to a two chain activator in which heavy (35 kDa) and the light chains (25 kDa) are held together by one inter-chain disulphide bond (Cys264-Cys395) (Jornvall et al., 1983). tPA molecule contains four domains: (1) a 47-residue-long amino terminal region (F-domain) homologous to the finger domains mediating the fibrin affinity of fibrinonectin; (2) residues 50 to 87 (E-domain) homologous to human epidermal growth factor; (3) two regions comprising residues 87 to 176 and 176 to 267 (K1 and K2 domains) that show a high degree of homology with the five kringles of Pg; and (4) a serine proteinase domain (residues 276 to 527) with active-site residues His322 (57), Asp371(102) and Ser478(195) (Ranby et al., 1982, Collen and Lijnen, 1991). tPA is a poor enzyme in absence of fibrin, but fibrin strikingly enhances the activation rate of Pg (Hoylaerts et al., 1982, Rijken et al., 1982, Samama et al., 1982) whether it is a single chain or double chain tPA. Pg activation by tPA involves the formation of weak ternary complex i.e. tPA-fibrin-Pg, with minimum catalytic activity. The resultant Pm generated at the fibrin surface then cleaves fibrin, exposing new carboxy terminal lysine residues that enable more tPA and Pg to bind via lysine binding sites present on specific kringles on the activator and its substrate. This positive feedback mechanism can regulate fibrinolysis, ensuring that Pm activity is targeted to fully assembled fibrin clot (van Zonneveld et al., 1986). The single chain proenzyme form of tPA is the only known serine protease to have full functional activity without proteolytic activation (Wallen et al., 1982). Crystal structure studies have shown that Lys156 of single chain tPA forms a salt bridge with Asp194 which promotes an active site conformation without cleavage (Tachias and Madison, 1996, Tachias and Madison, 1997, Renatus et al., 1997a). Both single and double chain tPA are nearly equal in their activity.

The three dimensional structure of the double module of tPA i.e. ‘FN1+EGF-like’ was determined by NMR spectroscopy (1TPG) (Smith et al., 1995) (Fig. 1.7a). The crystal structure of catalytic domain of human tPA in complex with low molecular weight inhibitor (dansyl-EGR-chloromethyl ketone) (Lamba et al., 1996) shows a typical trypsin-like fold (Fig. 1.7b).
Figure 1.7: A) Ribbon diagram representation of double module of human tPA comprising the fibronectin type I (FN1) domain and the epidermal growth factor-like (EGF-like) domain (ITPG). The disulfide bridges are shown in red sticks. B) 3D structure of the catalytic domain of human tPA in complex with the LMW inhibitor dansyl-EGR-chloromethyl ketone (IBDA). LMW inhibitor shown in green dots is covalently bound to His57 and Ser195 in the active site cleft (green sticks).
1.3.3.1.2 Urokinase-type plasminogen activator (uPA)

Although tPA and uPA have the same biological function i.e. activation of Pg to Pm, but both of these activators play different roles. tPA is mainly involved in haemostasis by generating Pm for fibrin clot degradation while uPA is involved in Pm generation for pericellular proteolysis, such as tissue remodelling and cell migration. uPA is a single chain multidomain glycoprotein of 55 kDa with 2% carbohydrates. uPA was initially described in urine at a concentration of 200-300 µg/l. In addition to pericellular activity, the probable function of uPA is to keep urinary tract free of fibrin deposits. This activator is synthesised in lungs, kidney, keratinocytes and endothelial cells. Single chain human uPA is converted to its two chain active form by the cleavage of Lys158-Ile159 bond held together by a disulphide bridge (Cys148-Cys279), catalysed by Pm, kallikrein or cathepsin (Goretzki et al., 1992, Ichinose et al., 1986, Cubellis et al., 1986, Estreicher et al., 1989) etc. The A-chain of 157 amino acids, has one EGF-like and one kringle domain while the B-chain of 253 amino acids comprises the serine protease part. As for its mechanism of action, like tPA, two chain uPA activates Pg following Michaelis-Menten kinetics (Violand and Castellino, 1976). The initial step in activation is the cleavage of Arg561-Val562 peptide bond in Glu1-Pg, forming Glu1-Pm. This form of Pm autocatalytically cleaves the Lys76-Lys77 peptide bond in the Glu1-Pm heavy chain, yielding Lys77-Pm. Either Glu1-Pm or Lys77-Pm can catalyze cleavage of the Lys76-Lys77 peptide bond in any Glu1-Pg not yet activated, forming Lys77-Pg, which is readily activated to Lys77-Pm (Claeys and Vermylen, 1974, Thorsen and Mullertz, 1974).

In addition to high molecular weight form of uPA, the low molecular weight (LMW) form of uPA is generated by Pm or by uPA autocatalytically cleaving the Lys135–Lys136 peptide bond. Thus LMW uPA does not have the EGF-like and kringle domains, and the former A-chain consists only of a minichain of 22 amino acids linked to the catalytic domain by a single disulfide bridge. This is the major form found in urine.

The three dimensional structure of the double domain of uPA i.e. ‘EGF-like+Kringle’ was determined by NMR spectroscopy (1URK) (Hansen et al., 1994) (Fig. 1.8a). The kringle domain resembles the kringles in other kringle-containing proteins, e.g. human Pg and the EGF-like module has the same structural fold as the corresponding domain in
Figure 1.8: A) 3D structure of the double domain module (EGF-like and kringle) of human uPA (1URK). The disulfide bridges are shown in green sticks. B) 3D structure of human LMW uPA (1GJA). The residues of catalytic triad are shown in green sticks.
tPA (Fig. 1.7a). The crystal structure of LMW uPA (1GJA) has been determined which exhibits trypsin like fold (Katz et al., 2001) (Fig. 1.8b).

1.3.3.1.3 Pla from Yersinia pestis (Y. pestis)

In addition to above described mammalian direct Pg activators, which are actually serine proteases, there is another Pg activator of prokaryotic origin named as Pla from Yersinia pestis which can directly cleave the Arg561-Val562 bond of Pg to activate it. This is a surface protease of omptin family encoded by Y. pestis specific pPCP1 plasmid which acts as a virulence factor (Suomalainen et al., 2007). The role of Pla in the establishment of bubonic plague has been demonstrated through in vivo studies. Pla triggers uncontrolled plasmin proteolysis targeted at tissue barriers for bacterial invasion and spreading. It helps in adherence of bacterium to eukaryotic cells like endothelial cells (Lahteenmaki et al., 2001). As for its mode of action, it inactivates α2-antiplasmin to promote uncontrolled proteolysis through Pm (Kukkonen et al., 2001).

1.3.3.1.4 Vampire Bat Pg activator (Bat-PA)

A human Pg activator of eukaryotic origin was identified by Gardell and coworkers in the saliva of vampire bats (Desmodus rotundus) which helps these haematophagous animals in feeding (Gardell et al., 1989). This vampire bat Pg activator (Bat-PA) is a 33 kDa protein and shows 80% homology with tPA. Similar to tPA, Bat-PA contains an EGF-like domain named as F, a kringle and a proteinase domain (Gardell et al., 1989). The only kringle of this Pg activator resembles to K2 of tPA that does not contain LBS (lysine binding site). However there are two striking differences between tPA and Bat-PA. Unlike to tPA, BatPA is a single chain molecule and is not cleaved to two chain derivative due to absence of a plasmin sensitive site. Secondly, BatPA is strictly dependent on fibrin for its activity (Bergum and Gardell, 1992, Gardell et al., 1990). The crystal structure of Bat-PA has already been solved in an attempt to explain the paradigm for proteolysis of activation without cleavage (Renatus et al., 1997b).

1.3.3.2 Indirect Pg activators

As previously described, the indirect Pg activators do not have the serine protease activity of their own, but they form a complex with Pg/Pm and change its
substrate specificity to activate Pg. The typical examples of such type of bacterial Pg activators include SK, SAK, PauA, PadA etc.

1.3.3.2.1 Streptokinase (SK)

Streptokinase (SK) was reported first time from the culture supernatant of certain strains of β-hemolytic streptococcus in 1933 (Tillett and Garner, 1933). Later on, Milstone in 1941 demonstrated that SK achieved its fibrinolytic effect through activation of a plasma protein. Initially, SK was designated as fibrinolysin by Tillett and Milstone until it was found that this bacterial extract induce fibrinolysis indirectly through activation of a plasma protein. The term streptokinase was coined by Christensen (1945) to describe this bacterial extract. SK is a single chain protein of molecular weight 45-50 kDa (Brockway and Castellino, 1974). Although addition of catalytic levels of SK to human Pg produces the usual two-chain Pm molecule, SK is not, in itself, a protease. SK and human Pg bind in a 1:1 stoichiometry that after a series of steps, results in a SK-Pg* complex, generating an active site within the Pg moiety (Schick and Castellino, 1974). This complex is not stable and undergoes intramolecular cleavage at the Arg561-Val562 peptide bond in the Pg moiety (Bajaj and Castellino, 1977, Kosow, 1975) resulting in the formation of SK-Pm activator complex that can convert Pg to Pm and finally this Pm degrades the fibrin clots.

1.3.3.2.2 Staphylokinase (SAK)

Staphylokinase (SAK), a protein with a molecular weight of 15.5 kDa, is produced by Staphylococcus aureus and has been shown to have thrombolytic properties for more than 50 years (Lack, 1948, Lewis and Ferguson, 1949, Lewis and Ferguson, 1951, Sako and Tsuchida, 1983, Behnke and Gerlach, 1987). Since recombinant techniques made production of SAK possible in larger quantities (Sako, 1985), renewed interest was generated to more closely examine its thrombolytic efficacy in vitro and in animal models. Like SK, SAK is not an enzyme, but forms a stoichiometric complex with Pm that acts as an activator of Pg. In the absence of fibrin, this activator complex is rapidly neutralized by α2-antiplasmin (Lijnen et al., 1991b). SAK appears to be more fibrin specific than SK in in vitro experiments and animal models (Lijnen et al., 1991a, Lijnen et al., 1991b).
1.3.3.2.3 *Streptococcal Pg activators with different specificity*

In addition to these human Pg activators, some other activators from different species have been identified which have their own specificity for efficient activation of host Pg system. These activators have been of great interest for researchers because of their importance in understanding the complicated mechanism of Pg activation and evolution of Pg activators. Recently a number of Pg activators have been identified from streptococcal species which show considerable diversity. Esk and Psk were obtained from the isolates of equine and porcine isolates of *Streptococcus equisimilis* (Caballero et al., 1999). PadA, was identified in bovine isolates of *Streptococcus dysgalactiae* while PauA (also known as SUPA i.e. *Streptococcus uberis Pg activator*) and PauB were identified in bovine isolates of *Streptococcus uberis* (Leigh, 1994, Johnsen et al., 1999). PadA is a 16 kDa protein that displays specificity for bovine, ovine and caprine Pg but not human Pg (Ward et al., 2008). Similarly, PauA or SUPA which is a two domain protein can activate bovine Pg but not human Pg. Like SK, PadA and SUPA can form an active complex with Pg.

1.3.4 Plasminogen activators of bacterial origin: Streptokinase and Staphylokinase

There are different types of Pg activators ranging from mammalian physiological to bacterial invasive system. Pg-Pm system, which is an important part of haemostasis inside the body, can be exploited smartly by bacteria for its survival. Invasive human pathogens have evolved Pg activators, such as SK and SAK, which are not enzymes themselves but form 1:1 complexes with Pg/Pm. As compared to Pm alone, which has broad substrate specificity for substrates like fibrin(ogen), these 1:1 complexes acquire a remarkable specificity and efficiency against ‘narrower’ substrates, such as the activation loop of Pg (Parry et al., 1998, Wang et al., 1998). In the past years, tPA and SK have been widely used as thrombolytics but from industrial point of view, microbial Pg activators provide a much cheaper therapeutic choice as compared to tPA. Because of their clinical use bacterial Pg activators have been the subjects of intensive research.

1.3.4.1 *Streptokinase (SK)*

Streptokinase is a catabolic byproduct of β-hemolytic streptococcus and can catalyze the activation of Pg in a species specific fashion (McKee et al., 1971, Sodetz
and Castellino, 1972). In streptococcal infections, the bacterial protein SK has traditionally been regarded as a potential virulence factor that increases the invasiveness of the pathogens due to broad substrate specificity of the Pm generated due to action of SK. An important aspect has been added to this notion by detection of plasmin(ogen) receptors on the streptococcal surface (Lottenberg et al., 1992b, Kuusela et al., 1992, Berge and Sjobring, 1993) which serve to capture Pm and presumably, provide the organisms with a cell associated mechanism for the destruction of tissue barriers when SK is produced (Lottenberg et al., 1992a, Malke et al., 1994).

1.3.4.1.1 Physio-chemical properties of streptokinase

Molecular weight of SK was reported to be 47.6 kDa as determined by equilibrium sedimentation (De Renzo et al., 1967). Cystine and cysteine were absent and the molecule was assumed to be a single polypeptide chain with no subunits and without any phosphorus, carbohydrates and lipids. The complete amino acid sequence of SK was determined by Jackson and Tang in 1982. The nucleotide sequence of sk gene was determined by Malke et al., (Malke et al., 1985) from Streptococcus equisimilis H46A. Its isoelectric point was found to be about pH 4.7. SK can interact with Pg from few species like human, monkey, baboon, chimpanzee, cat, dog and rabbit. However, only the SK-human Pg complex has been shown to possess the ability to activate Pg from several other species such as sheep and bovine, which cannot be directly activated by SK (Markus and Werkheiser, 1964, Wulf and Mertz, 1969).

1.3.4.1.2 Mechanism of Pg activation by SK

Unlike other Pg activators such as tPA and uPA, (Lijnen and Collen, 1986) SK does not display any proteolytic activity of its own but functions as a ‘protein cofactor’ to Pg/Pm with which it binds in equimolar ratio and then changes its substrate specificity. According to the classical mechanism of trypsinogen (Bode, 1979, Bode et al., 1978) or chymotrypsinogen (Kerr et al., 1976) studies, insertion of the newly formed val562 amino terminus of Pg into the specific binding cleft brings about the conformational changes to produce the active enzyme, Pm. The most interesting fact about SK mediated Pg activation is that it does not require the proteolytic cleavage of Arg561-Val562 bond in SK-Pg complex but triggers the conformational changes in such
a way that the binary complex attains its activation potential (McClintock and Bell, 1971, Reddy and Markus, 1972).

A number of elegant biochemical and biophysical studies from different laboratories have provided insight into the fundamental steps involved in Pg activation by SK. The sequence of Pg activation by SK can be summarized as follows-

The first step is the formation of 1:1 stochiometric complex between SK and Pg. This was physically demonstrated by following analysis of equimolar SK-Pg complexes by electrophoresis in which the complexes migrated as a single band (Summaria et al., 1974). Complex formation between SK and Pg is immediately followed by structural rearrangements within this complex to form SK-Pg* (active conformation), the virgin enzyme, which has amidolytic activity (McClintock and Bell, 1971, Reddy and Markus, 1972). Since Val562-Asp740 salt bridge cannot form in SK-Pg* due to the absence of free Val562 at the amino terminus, another residue is likely to provide a connection for Asp740.

Two hypotheses have been proposed to explain the SK mediated Pg activation. First, the amino terminus of SKIle1, may form the crucial salt-bridge. This mechanism was termed ‘molecular sexuality’ by Bode and Huber and ‘amino terminal insertion hypotheses’ by Jackson and Tang (Jackson and Tang, 1978, Bode and Huber, 1976). Deletion of Ile1 of SK prevents the formation of SK-Pg* complex and was correlated with loss of function in conformational activation, which provides support for the molecular sexuality hypothesis (Wang et al., 1999). This hypothesis was further supported by fluorescence and kinetic studies of SK-Pg interactions with SK mutant lacking Ile1 (Boxrud et al., 2001). This study also elucidated the role of sequence specific binding of SK N-terminal region (first ten amino acids) with the binding cleft of Pg to induce a transition towards active conformation. According to an alternative hypothesis, Lys698 residue of Pg may occupy its amino terminal binding cleft in place of Val562 to form the salt-bridge with Asp740 (Wang et al., 1998). It has been suggested that the binding of γ domain to the autolysis loop of Pg induces the formation of critical intramolecular salt bridge between Lys698 and Asp740 of Pg that productively structures the activation domain (Wang et al., 1998). This proposal of “γ domain contact activation” is based on the observation that the analogous residue, Lys156, provides the counter-ion in the zymogen of tPA (Renatus et al., 1997a). But in
comparison to mutation of Ile1, mutation of Lys698 resulted in less severe impairment in the formation of SK-Pg* (Wang et al., 2000a). These results provided evidence still in favor of the molecular sexuality hypothesis. SK binds preferentially to the extended conformation of Pg through lysine binding sites to trigger conformational activation of Pg (Boxrud et al., 2000, Boxrud et al., 2001, Boxrud and Bock, 2000). SK can form functional binary activator complex with Pg as well as Pm and binds to Pm with much higher affinity (Boxrud et al., 2000). SK-Pg* complex is not stable and undergoes intramolecular cleavage at Arg561-Val562 peptide bond in Pg moiety (Kosow, 1975, Bajaj and Castellino, 1977), yielding an equimolar complex of SK-Pm in which the SK moiety has also undergone proteolytic transformation to a low molecular weight species (Brockway and Castellino, 1974).

In the final step SK-Pm complex activates ‘substrate’ Pg to Pm by selective cleavage of Arg561-Val562 peptide bond (Markus and Werkheiser, 1964, McClintock and Bell, 1971, Reddy and Markus, 1972). Whereas Pm also doesn’t possess the catalytic ability to activate Pg, the SK-Pm and SK-Pg complexes do have this capacity. Moreover, the main inhibitor of Pm i.e. α2-antiplasmin cannot inhibit SK-Pg/Pm complex.

1.3.4.1.3 Crystal structure of SK in complex with microplasmin (μPm)

The crystal structure of SK in complex with catalytic domain of human Pm (μPm or microplasmin) was solved at 2.9 Å by Wang et al. (Wang et al., 1998) (Fig. 1.9). To reduce the autolysis of the complex, the active-site residue Ser741 in Pm was replaced with an alanine. The crystal structure revealed that SK contains three sequential domains of roughly equal size, namely α, β and γ from amino- to carboxy termini, linked by two flexible loops. These three domains surround μPm to form a concave-shaped complex with the Pm active site located at the bottom of the concavity.

SK α domain (residues 1-146) binds to μPm mainly through interaction between αβ1 and αβ2 strands of SK and a loop region (residues 713-721) of μPm, in which μPm Arg719 forms a salt bridge with Glu39 of SK. The SK α domain also interacts with μPm near the active-site residues His603 and Asp646. The central domain of SK (SKβ domain, residues 147-290) contributes substantially to the overall shape of the putative
Figure 1.9: Ribbon diagram representation of SK-μPm complex (1BML). α, β and γ domains of SK are shown in blue ribbons and μPm in green.
substrate binding concave of the complex, suggesting that SKβ may also be involved in the recognition of substrate Pg.

In the crystal structure of SK-μPm complex, the SK γ domain (residues 291-414) binds to μPm near the region of activation bond, implying its function in the contact activation of Pg by SK. The "calcium-binding loop" (residues 622-628) and the "autolysis loop" (residues 692-695) of μPm have extensive electrostatic and hydrophobic interactions with major coiled coil region and the strands γβ1 and γβ2 of SK γ domain. The participation of the "calcium-binding loop" in this interaction suggested that the substantial sequence difference observed in this region between human and bovine Pg might contribute to the inability of SK to activate the later.

1.3.4.2 Staphylokinase (SAK)

Staphylokinase (SAK) is a 15.5 kDa protein secreted by certain strains of *Staphylococcus aureus* that has an immense therapeutic potential as a thrombolytic drug. The *sak* gene encodes a protein of 163 amino acids which is processed to a mature protein of 136 amino acids consisting of single polypeptide chain, devoid of any disulfide bridge (Behnke and Gerlach, 1987, Sako and Tsuchida, 1983). The fibrinolytic properties of SAK were shown more than 6 decades ago. The term ‘staphylokinase’ was coined in 1948 (Lack, 1948) in order to describe the product responsible for fibrinolytic activity of staphylococcus strains analogues to ‘streptokinase’ designation. As for the biological function of SAK, due to its Pg binding and activation properties, it has been used as a virulence factor by *S. aureus* in invasion and infection into the host by utilizing the proteolytic activity of Pm (Bokarewa *et al.*, 2006). Pg binding sites are also expressed by Staphylococci on their surface enolase and ribonucleotide reductase subunit 2 (Molkanen *et al.*, 2002, Christner and Boyle, 1996). SAK also increases bacterial resistance by interacting with short cationic peptides released by human neutrophils (HNPs) which are having antiviral and antibacterial properties (Jin *et al.*, 2004).

The fibrinolytic properties of SAK were studied in 1950s and it was reported in 1960 that SAK is a potent activator of Pg-Pm system (Davidson, 1960, Lewis and Ferguson, 1951). Natural SAK has been purified from *S. aureus* which were transformed
with bacteriophages containing sak gene (Glanville, 1963). Initial animal studies of SAK were conducted in dogs by Lewis (Lewis and Ferguson, 1951, Lewis and Ferguson, 1949) and the results were highly discouraging due to severe hemorrhage and toxicity which discontinued the research interest in SAK. The SAK thrombolytic properties were again studied in dogs by Kanae in 1986 in which he found that SAK leads to decomposition of fibrinogen and fibrin to a very high level (Kanae, 1986). These studies appear to have misleading conclusions as the dog is unusually sensitive to fibrinolytic activation with SAK probably due to poor α2-antiplasmin activity. But with the development of high expression system of SAK in E. coli through recombinant technology and in vivo studies in other animals in 1980s and 1990s proved the thrombolytic potential of this therapeutically important molecule (Collen and Lijnen, 1991, Sako, 1985). SAK has been demonstrated to induce fibrin specific thrombolysis in both human plasma (Collen, 1998) and in limited clinical trials (Vanderschueren et al., 1996a, Vanderschueren et al., 1995b, Vanderschueren et al., 1995a, Collen and Van de Werf, 1993). Studies on the thrombolytic potential of SAK in achieving perfusion in myocardial infarction and in the dissolution of platelet-rich clot (Vanderschueren et al., 1996a, Vanderschueren et al., 1995b, Collen and Van de Werf, 1993) have clearly established its immense utility in clinical medicine as a thrombolytic agent and suggested that it can be developed as a potent clot-dissolving drug.

1.3.4.2.1 Physio-chemical properties of SAK

The scientific interest in SAK resurfaced in 1980s again after early disappointing results in dog models and attempts were made for the biochemical and biophysical characterization of SAK only after well developed recombinant expression system for overproduction of SAK was established. The determination of complete nucleotide sequence of sak and its overexpression and overproduction in E. coli was done by Sako et al (Sako, 1985, Sako and Tsuchida, 1983). Three genes encoding wild-type variants of SAK (sak$c, sak$42D and sak$STAR) have been characterized (Behnke and Gerlach, 1987, Sako and Tsuchida, 1983). These natural variants of SAK differ at three amino acid position, 34, 36 and 43 of polypeptide chain. Wild-type SAK has sequence GKRNE LSPR; [Arg36Gly, Arg43His] SAK has sequence GKGNE LSPH; [Gly34Ser, Arg36Gly, Arg43His] SAK has sequence SKGNE LSPH (position 34, 36 and 43 are underlined). These SAK variants are stable at ambient temperature but have
different sensitivities to thermal inactivation (Schlott et al., 1994a). [Gly34Ser, Arg36Gly, Arg43His] SAK, (sakSTAR), the most resistant natural variant, has stability approaching the minimal requirement for pasteurization (Gase et al., 1994).

The solution structure of SAK was studied by circular dichroism (CD), fluorescence spectroscopy and dynamic light scattering (Gase et al., 1994, Damaschun et al., 1993). Analysis of far-UV CD spectrum of SAK yielded 20% α-helix, 30% β-sheet and 20% turns. By Fourier transform infrared spectroscopic study, a very high fraction of ~41% of β-sheet was obtained (Pribic et al., 1993). The three-dimensional NMR solution structure of SAK was determined by multi dimensional heteronuclear NMR spectroscopy (Ohlenschlager et al., 1998) in which SAK showed a strong asymmetry of hydrophilic and hydrophobic surface. The amino terminus, folds back onto protein core, thereby shielding amino acids with functional importance in Pg activation process. Finally, the crystal structure of SAK and its ternary complex with microplasmin was solved in late 90s (Parry et al., 1998, Rabijns et al., 1997) which provided the insights into mechanism of Pg activation.

1.3.4.2.2 Mechanism of Pg activation by SAK

SAK, a 136 amino acid protein, has no proteolytic activity of its own like SK, but it forms a 1:1 stoichiometric complex with Pm, which in turn activates other Pg molecules with high specificity (Parry et al., 1998, Collen et al., 1993b). The mechanism of Pg activation by SAK can be summarized as-

In the first step, SAK makes a 1:1 stoichiometric complex with Pm. Collen et al., observed a long initial lag phase before appearance of Pm activity and this lag phase was dependent on the initial concentration of contaminating Pm in the Pg preparation (Collen et al., 1993b). The active site acylation rate, determined by the titration with NPGB (p-nitrophenyl-p'-guanidinobenzoate), was dependent more on concentration of substrate Pg than that of SAK in activation mixture. In Pm free preparation of Pg through Aprotinin-agarose Chromatography, SAK was not able to induce the active site exposure of SAK-Pg complex and then subsequent activation of the substrate Pg. These studies suggested that SAK cannot form a functional activator complex with Pg and at least trace amount of Pm is required to form the binary Pg activator complex (Grella and Castellino, 1997).
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Generation of Pg activation potential in SAK proceeds via plasmin-mediated removal of ten amino terminal amino acids with exposure of Lys11 as the new amino terminus (Rajamohan and Dikshit, 2000, Schlott et al., 1998, Schlott et al., 1997). The presence of lysine residue within amino terminal region of SAK improves the capability of the enzyme complex, formed between SAK-Pm, to interact with substrate Pg (Rajamohan and Dikshit, 2000). Interestingly in SAK-Pm complex, SAK binds with Pm in the vicinity of its active site and provides the new subsites by making crucial contacts due to which the active site becomes more restricted, narrow and specific for activation loop of Pg. This bimolecular complex creates new exosites for the proper orientation and presentation of the substrate to active site (Parry et al., 1998).

In the final step, SAK-Pm binary activation complex cleaves Arg561-Val562 peptide bond of the substrate to produce Pm.

1.3.4.2.3 Site directed mutagenesis of SAK

The site directed mutagenesis studies have been carried out in the past in a continuous effort in various laboratories to decipher the underlying complicated mechanism for the formation of SAK-Pm binary complex and then the activation of the substrate. 'Clustered charged alanine scanning' was done by Silence et al. (Silence et al., 1995) in which two or more adjacent charged residues were mutated simultaneously to alanine to find out their role in Pg binding and activation. This study identified the role of crucial regions of SAK comprising amino acids 11-14 (SAK Lys11Ala, Asp13Ala, Asp14Ala), 46-50 (SAK Glu46Ala, Lys50Ala) and 65-69 (SAK Glu65Ala, Asp69Ala) in Pg activation process. The proteolytic removal of the first ten amino terminal residues of SAK by the cleavage of Lys10-Lys11 bond was demonstrated as a crucial requirement for the Pg activation potential of SAK by site directed mutagenesis of these two residues (Schlott et al., 1997). SAK molecule retained its Pg activation potential on substitution of Lys10 or Lys11 with Arg but lost this ability upon substitution of Lys10 or Lys11 with His. These results indicated that Pm-mediated removal of the 10 amino terminal amino acids with exposure of a charged amino terminal amino acid is prerequisite to generate SAK derivative with Pg activating potential. Further deletion and substitution mutagenesis studies of amino terminal segment (Lys11-Ser16), suggested that this region of SAK is not required for binding to Pg/Pm, but the
positively charged residues of this flexible region participate in reconfiguration of the active site of the Pm molecule to endow it with Pg-activating potential (Rajamohan and Dikshit, 2000, Schlott et al., 1998). The crucial role of Met26 of SAK in Pg activation was identified by mutating it to Cys or Leu (Schlott et al., 1994b). Three different nonoverlapping immunodominant epitopes were identified on SAK by Collen et al through site directed mutagenesis and antigenic studies (Collen et al., 1996b, Collen et al., 1996a). The role of 90 loop of SAK in interaction with kringle 5 of substrate Pg for its activation was proved by deletion and mutagenesis studies (Rajamohan et al., 2002). Most of these studies have been well supported and explained by the crystal structure of ternary complex of SAK with $\mu$Pm (Parry et al., 1998) but still mechanism is not fully understood in the absence of full length structure of Pg.

1.3.4.2.4 Crystal structure of SAK

Although a number of structural and functional studies were done to characterize SAK molecule as a Pg activator, but the structural details about the composition of SAK were provided in 1997 through X-ray crystallography. The crystal structure of SAK was solved to a resolution of 1.8 Å (Rabijns et al., 1997) (Fig. 1.10) which is composed of a mixed 5-stranded $\beta$-sheet, packed on a single 12-residue $\alpha$-helix. Strands 3, 4 and 5 are adjacent and antiparallel, as are strands 1 and 2 but the strands 1 and 5 lay adjacent and parallel to each other. SAK folds into a compact, flattened and somewhat elongated structure corresponding to an ellipsoid. Residues 16-20 extend from the compact ellipsoid of the molecule pointing towards a large intermolecular cavity in which the rest of the N-terminus (residues 1-15) seems to be disordered. The first 10 amino acids were missing and had been proteolytically degraded. Alanine scanning studies have identified particular regions in SAK (amino acid 46 and 50; 65 and 69) playing crucial role for complex formation with Pg and for induction of active-site exposure in Pg respectively. Interestingly, in the crystal structure, these regions were mapped to the same side of SAK structure. Moreover, the amino terminus, which has previously been shown to be necessary for activity of SAK (Silence et al., 1995), is also situated on this side of the molecule (although it was disordered). This may imply that complex formation with Pg occurs through this side of molecule and it seems likely that its solvent exposed part plays important role in complex formation. From crystal structure it is suspected that amino terminus does not
Figure 1.10: 3D structure of SAK (2SAK).
fold back onto molecule, but it rather acts like a flexible arm that can capture molecules. Three non-overlapping immunodominant epitopes were recognized: amino acid 74, 75 and 77 were found to constitute epitope 1 of SAK, while epitope 3 is composed of amino acids 35, 38, 80 and 82. The residue constituting epitope 2 are not yet known (Collen et al., 1996a).

To elucidate the underlying mechanism of Pg activation by SAK, catalytic domain of human Pm was crystallized in a 2:1 ternary complex with SAK and resolved at 2.65 Å (Parry et al., 1998) (Fig. 1.11). This unique macromolecular assembly reflected a Pg-activating complex of SAK-μPm, binding a second μPm molecule in a substrate-like manner and is the first experimental structure of a proteinase-cofactor complex with its substrate. It showed that core of both μPm monomers was folded into two six-stranded β-barrels connected by various loops, two helical turns and one carboxy terminal α-helix. The upper left rim of the active site of the enzyme was involved in SAK binding. This enzyme-cofactor interaction has a strong electrostatic component, whereby an overall negative potential on the SAK surface is compensated by positively charged residues on the adjacent μPm surface. Residues Lys224-SAKGlu19-Arg175-SAKGlu46 participated in an extended network of salt bridges and charged hydrogen bonds, which is almost fully shielded from the bulk solvent through hydrophobic patch formed by SAKMet26, SAKTyr24 and SAKTyr44. A salt bridge between Arg94 of μPm and SAKGlu75 provides another rigidifying anchor. SAKTyr73 moves about 15 Å as compared to its position in free SAK. Upon Pm binding, the β1-β2 sheets around SAKPro42 open to prevent a collision between SAKPro42 and the Pm surface. Gln177 of μPm intrudes between both strands to form a new extended, charged hydrogen bond network between residues SAKGlu38-Lys101-SAKSer41-Gln177-SAKAsn28 adopting both surfaces for interaction. As a result of this rearrangement, residues SAKHis43 and SAKTyr44 protrude into the active-site cleft of μPm, where they restrict the S2 and S3/S4 pockets of enzyme, making it more suitable for the binding of Pg activation loop of substrate Pg. The interaction surface that the cleaved substrate shares with SAK, in particular with its 'front' side is much extended and complementary. The clustered side chains of His23, Pro24, His25 slot into a hydrophobic groove formed by the side chains of SAKTyr62, SAKTyr66, SAKAla70 and SAKTyr73. Moreover residues SAKGlu46-SAKPro48 juxtagpose, in an antiparallel
Figure 1.11: Crystal structure of SAK with μPm in ternary complex (1BUI). SAK is shown in pink ribbons, μPm enzyme (partner) is shown in blue and μPm substrate is shown in green ribbons.
manner, segment Gln5-Glu7 of A-chain of cleaved substrate. The observed importance of Glu46 of SAK for Pg activation found in clustered charged residue-to-alanine mutagenesis scan (Silence et al., 1995) is well explained in terms of its strong electrostatic interaction with Arg175. SAK does not affect the active-site geometry of the Pm, but instead modifies subsite specificity by providing additional docking site for enhanced presentation of substrate Pg to active site of Pm.

1.3.4.2.5 Regulation of SAK mediated Pg activation by α2-antiplasmin and fibrin

The remarkable feature of SAK is its capability to induce highly fibrin-specific thrombolysis both in human plasma in vitro (Collen et al., 1993c, Lijnen et al., 1992, Lijnen et al., 1991b, Matsuo et al., 1990) and in clinical trials in vivo (Collen and Van de Werf, 1993, Collen et al., 1993a). Unlike to SK-Pg complex, SAK-Pg complex is not able to induce generation of the functional active site in Pg moiety. Secondly, SAK-Pm complex is inhibited by a2-antiplasmin in plasma in the absence of fibrin and SAK molecule is dissociated from this complex to recycle back to other Pg/Pm molecules. So the clot specific Pg activation by SAK is attributed to the complex interactions between SAK, Pg/Pm and fibrin molecules (Lijnen et al., 1992). But in case of SK, the functional binary activator complex cannot be inhibited by α2-antiplasmin and causes systemic Pg activation irrespective of clot specific site leading to bleeding complications. Fibrin specificity of SAK-Pm complex is attributed to the lysine binding sites in the Pg/Pm moiety. It has been demonstrated that SAK discriminates between free and fibrin bound Pg and activation of Pg by SAK-Pm gets enhanced on the partially degraded fibrin clot due to exposure of much more lysine residues (Sakharov et al., 1996). Moreover, SAK-Pm complex and Pm bound to fibrin are protected from inhibition by α2-antiplasmin.

1.3.4.2.6 Role of kringle domains in Pg activation by SAK

The crucial role of kringle domains of Pg/Pm in Pg activation by SAK-Pm complex was demonstrated by Arai et al., (Arai et al., 1998) in the presence of ε-amino-caproic acid (EACA), a lysine analogue which binds with Pg through LBS (lysine binding site) in a kringle structure. Activation of Pg by SAK-Pm complex increased initially, but then decreased in an EACA concentration-dependent manner. This decrease was explained by more than 10-fold higher $K_m$ for activation of Pg with a catalytic amount of SAK-Pm complex in presence of EACA. $K_m$ for activation of miniPg
(K5+μPm), which lacks first four kringle structures, by SAK-Pm complex, was at least 3.5 fold higher than that for activation of Pg by SAK-Pm complex. Furthermore, EACA showed a negligible inhibitory effect on the activation of miniPg. Since EACA binds to Pg via LBSs in the kringle structure, it was suggested that the LBSs in K1+2+3+4 domain play a role in the activation of Pg by SAK-Pm complex and in binding of Pg to SAK.

1.3.4.2.6 Structural-functional comparison of staphylokinase with streptokinase

SAK and SK, form 1:1 stoichiometric complex with Pg/Pm to initiate fibrinolysis in humans by converting Pg into Pm (Buck et al., 1968, Collen et al., 1993b). In this complex SAK or SK serves as a cofactor that redirects the substrate specificity of Pm from the cleavage of fibrin to the cleavage of Pg. The unique feature of SK is its capability to generate a catalytic active site in Pg molecule (Pg*) after structural rearrangements without any proteolytic cleavage (Reddy and Markus, 1972). Pg* is an active zymogen which subsequently converts itself intramolecularly to Pm. In contrast to SK, SAK cannot induce the active site generation in Pg rather it requires Pm initially to make a functional binary activator complex and subsequently catalyses the conversion of Pg into Pm (Grella and Castellino, 1997). Moreover, SK has a higher binding affinity for Pg/Pm as compared to SAK. A second difference in specificity between the complexes is that the SAK-μPm complex is sensitive to α2-antiplasmin inhibition whereas the SK-μPm complex is not. SK appears to block access of serine protease inhibitor to the active site of Pm more effectively than SAK. The functional differences between these two cofactors can be explained to some extent on the basis of structure. SK contains three domains (α, β and γ) (Wang et al., 1998), whereas SAK contains only one domain (Rabijns et al., 1997). Consequently, the interactions of SK with Pg and Pm are extensive and complex. For example SK has 4100 Å² of intermolecular contact with μPm (Wang et al., 1998), whereas SAK has only 1750 Å² of contact (Parry et al., 1998). Thus, SK forms a tighter and more stable complex with Pg than SAK (Okada et al., 2001b). Comparison of the structures suggests that SK essentially surround the catalytic domain of Pm whereas SAK contacts only one side. The contacts of μPm with SAK are similar to those made by the α domain of SK (Parry et al., 1998, Wang et al., 1998). Active site of Pm is nestled below the plane of SK, possibly restricting access of some substrates and inhibitors including α2-antiplasmin.
Substrate access appears less restricted in SAK-μPm complex (Esmon and Mather, 1998).

One physiologically relevant difference between SAK and SK is that, unlike SK, SAK exhibits considerable specificity for Pg activation at the site of blood clot both \textit{in vitro} and \textit{in vivo}. Pg binding to fibrin degradation products dramatically enhances SAK binding and Pg activation. SAK needs to be proteolytically processed to release an amino terminal peptide to become a functional Pg activator (Schlott \textit{et al.}, 1998). As mentioned by Parry \textit{et al.}, (1998), modeling studies suggest that it is possible for amino terminus of SAK to contact kringle 5 of Pg (Parry \textit{et al.}, 1998). If the fibrin binding altered the conformation of kringle domains, this type of interaction could conceivably contribute to enhance SAK binding.

\textbf{1.3.4.2.7 Clinical trials of SAK in patients}

The thrombolytic potential of SAK has been evaluated in-patients with acute myocardial infarction and with peripheral arterial occlusion (Vanderschueren \textit{et al.}, 1996a, Vanderschueren and Collen, 1996, Vanderschueren \textit{et al.}, 1995b, Vanderschueren \textit{et al.}, 1994, Collen and Van de Werf, 1993). In two small pilot studies, 10 patients with coronary artery occlusion were treated with SAK in conjugation with aspirin and heparin (Collen and Van de Werf, 1993). Complete arterial recanalization was obtained in eight patients and partial recanalization in one patient. Plasma levels of fibrinogen, Pg and α2-antiplasmin remained unchanged, confirming the high fibrin specificity of SAK in human. In an open randomized multicenter trial, SAK was compared to accelerated weight-adjusted rtPA in 100 patients with myocardial infarction (Vanderschueren \textit{et al.}, 1995b, Vanderschueren \textit{et al.}, 1995a). rtPA caused a 36% drop in fibrinogen and 60% decrease in Pg and α2-antiplasmin. No strokes, allergic reactions or other side effects were recorded in SAK but the majority of the patients developed neutralizing IgG after two weeks (Vanderschueren \textit{et al.}, 1997b, Vanderschueren \textit{et al.}, 1996b). Thirty patients with peripheral arterial occlusion were treated with intra-arterial SAK. Recanalization was completed in 25 patients (83%), partial in 2 and absent in 3 patients. Two major hemorrhagic complications occurred, including one fatal hemorrhagic stroke. No severe allergic reactions were observed. These pilot clinical studies suggested that intravenous/intra-arterial SAK is a potent rapidly acting and highly fibrin-selective thrombolytic agent in-patients with acute myocardial infarction and with peripheral arterial occlusion.
1.4 Scope of the present study

Acute myocardial infarction due to coronary artery occlusion caused by a thrombus formation leads to cardiovascular heart disease (CHD), the most common cause of mortality in various countries. The various arterial and venous thromboembolic events including myocardial infarction, ischemic stroke, and peripheral artery thrombosis characterize the potentially life-, organ- and limb threatening vascular diseases. The use of thrombolytic drugs for such complications has been an attractive immediate therapy for the treatment of patients and it has shown a great reduction in mortality. However, the thrombolytics have limited success in achieving the 'open artery principle' of myocardial infarction treatment (Braunwald, 1993, Morais, 1995), *i.e.* restoration of complete perfusion in the obstructed artery as soon as possible and maintaining the vascular patency. The thrombolytic molecules approved for clinical usage include tPA, uPA, SK, anisolyted Pg-SK activator complex (APSAC) and recombinant tPA. Accelerated tPA and SK are currently best available thrombolytics and are widely used for thrombolytic therapy. tPA is more potent than SK as it achieves complete perfusion in 54% of patients as compared to 30% in case of SK. Moreover being fibrin specific, tPA leads to lesser systemic breakdown of plasma proteins such as Pg, α2antiplasmin etc. The Pg activation by SK in a non fibrin-specific manner causes a real problem of haemorrhage due to bleeding complications (1993a, , 1993b ). Moreover, being a bacterial protein, there is always a risk of anaphylactic response including death and high production of anti-SK antibodies in the circulatory system restricts multiple treatments with streptokinase (Lee *et al.*, 1993a, Lee *et al.*, 1993b). The major advantage of SK is its better half life (30 min) as compared to tPA (5 min). So, tPA, no doubt has better thrombolytic properties as compared to SK, but SK has been a preferred choice of thrombolytic therapy because of its cost effectiveness (microbial source) and better half life as compared to tPA. Accordingly, the development of novel agents with enhanced fibrinolytic efficacy and specificity with a favourable cost-benefit ratio is a therapeutic priority.

The latest addition to this clot buster’s list is SAK, another Pg activator of bacterial origin. Pg activation by SAK is highly fibrin specific due to rapid inhibition of generated SAK-Pm complex by α2-antiplasmin in plasma. This inhibition is reduced 100 fold at the fibrin surface, which allows preferential Pg activation at fibrin clot
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The clinical trials have established the thrombolytic potential of SAK in patients with acute myocardial infarction and with peripheral arterial occlusion. Randomized double bolus patency trial of SAK in comparison to tPA showed that the SAK molecule is as much effective as tPA in rate of coronary artery patency but without any fibrinogen or plasma proteins breakdown as compared to tPA. Comparison of these results with SAK in this study with those obtained with SK in GUSTO-I (Global Utilization of Streptokinase and Tissue Plasminogen Activator to Treat Occluded Arteries) trial suggested that SAK would outperform SK in terms of optimal coronary recanalization (Vanderschueren et al., 1997a). In comparison to SK, SAK has shown better thrombolytic potential in terms of its fibrin specificity and thrombolytic efficacy in animal models (Collen et al., 1993a). So, being an efficient molecule as a Pg activator and a strong candidate as a thrombolytic, SAK provides an ideal system to explore about its structure function aspects which can be helpful in its improvement as a clot buster. It is therefore required to conduct studies which could educate on all the aspects of its structure and function.

The crystal structure of ternary complex of SAK with two molecules of μPm has provided a lot of information on the intermolecular contacts and their role in Pg binding and activation process. However, the complete understanding of Pg activation by SAK is still lacking as the ternary structure of SAK with μPm does not show the participation of the kringle domains of Pm. The molecular mechanism, by which these kringle domains modulate binary complex formation and overall processing of Pg by SAK, is not very well known at present. SAK binds with one μPm molecule in the vicinity of its active site and alters the geometry of the active site by creating new subsites making the active site highly specific for Pg activation. This binary SAK-μPm complex provides new exosites for the proper docking of the substrate Pg for its catalytic processing. So there are two fundamental aspects related to functionality of SAK for Pg activation.

1. Understanding of the molecular mechanism by which SAK interacts with Pm to change its broad substrate specificity to highly precise Pg activation, is of utmost importance.

2. Activation of substrate Pg through cleavage of its activation loop by SAK-Pm binary activator complex and then release of the cleaved substrate, constitute interesting events to explore fully for better understanding of SAK mediated Pg activation.
The formation of SAK-Pm binary activator complex requires a number of intermolecular contacts between the two molecules. The interface region of SAK with \( \mu \)Pm spanning from Gly36-Glu46 generates a network of salt-bridges and hydrogen bonding (e.g. SAKGlu38-PmLys101; SAKSer41-PmGln177; SAKGlu46-PmArg175) for making a functional binary activator complex. The crucial role of Arg175 of Pm in making binary complex with SAK and SK shows the similar type of interaction. Structural comparison of the SAK-\( \mu \)Pm and SK\( \alpha-\mu \)Pm (in SK, PDBID: 1BML) shows the presence of similar type of residues participating in the intermolecular interactions at the interface. This indicates that these residues have been well conserved in the evolutionary process for their crucial role in Pg binding and activation. The interface of the enzyme complex formed by these two activator molecules also displays existence of two closely spaced pair of basic and aromatic residues (His43-Tyr44 in SAK and Lys36-Phe37 in SK) that protrude into the active site cleft of the partner Pm. However, ternary structure of \( \mu \)Pm-SAK-\( \mu \)Pm complex does not show direct interaction of His43 and Tyr44 residues of SAK with the partner Pm and lacks a clear picture of their role in modulation of the specificity switch. So site directed mutagenesis of these residues can provide their crucial role in intermolecular interactions and specificity switch. In addition to this, there are other residues participating in the interface which do not show the direct contacts at the interface but may be participating in the structural integrity of the interface in order to generate various interface contacts. Site directed mutagenesis studies of interface residues can provide structure function aspects of this region for making bimolecular complex with Pm.

Like SAK, SK also forms a 1:1 complex with Pg/Pm, and acts a cofactor in the activation of Pg substrate. Crystal structure of streptokinase in complex with catalytic domain of \( \mu \)Pm has indicated the participation of all three (\( \alpha \), \( \beta \), \( \gamma \)) domain of SK in this process. Although three domains of SK show no sequence similarity with each other or SAK, remarkable structural similarity is displayed among these domains alongwith SAK. Contacts of SAK with \( \mu \)Pm are similar to those made by \( \alpha \) domain of SK. But unlike SAK, individual domains of SK posses negligible plasminogen activation property. It has been observed that single domain SAK binds with lower affinity to Pm and SAK-Pm activator complex possesses a relatively lower catalytic efficiency as compared to SK-Pg/Pm complex. Additionally, the SAK-Pm complex, but not the SK-Pm complex is inactivated by \( \alpha 2 \)-antiplasmin. Unlike SAK, Pg activation activity of
individual SK α domain is not significant (Loy et al., 2001). The cooperative function of these domains for Pg binding and full activation potential of SK is already well established through various studies. Thus, chimeric studies of SAK with these SK domains can provide new insights into the mechanistic differences in the function of these two activators.

The exploration of above mentioned aspects might give some novel insights into the unique mechanism of Pg activation by SAK that could eventually be utilized to design a better Pg activator molecule.

The clinical trials on SAK have already established its strong candidature as a thrombolytic drug over the existing clot busters in market; however various clinical trials and animal studies have highlighted two major disadvantages of SAK. SAK has a very short plasma half life and its administration induces neutralizing antibody formation in majority of patients (Vanderschueren et al., 1997a). Various strategies like chimera formation or chemical conjugation etc. were used in the past to overcome these common problems associated with therapeutics. It is well known that the plasma clearance and the immunogenicity of heterologous proteins may be reduced by derivatization with poly (ethylene glycol) (PEG) (Abuchowski et al., 1977a, Abuchowski et al., 1977b). Usually, PEG (Veronese, 2001, Veronese et al., 2001) molecules are covalently attached to proteins via lysine residue side chains. SAK contains as many as 20 lysine residues, some of which are essential for its activity, precluding derivatization at lysine residues as an option. However, the SAK molecule does not contain cysteine, which provides the opportunity to place a cysteine at virtually any nonessential position in the molecule by site-directed mutagenesis, creating a specific target for site-directed PEGylation. So the therapeutic potential of SAK can be improved by site-specific PEGylation.

Thus, being the smallest and a single domain Pg activator, SAK offers an ideal system to develop it as a better thrombolytic molecule either by its covalent modification or through protein engineering by integrating with other molecules having desired features for improving its therapeutic potential.