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The physiological plasminogen activators (pPAs), such as urokinase (UK) and tissue plasminogen activator (tPA), can activate any mammalian PG molecule irrespective of its source organism, indicating that these protein-protein interactions are conserved due to the mutual co-evolution of interaction partners (Goh et al., 2000; Pazos et al., Giuliani et al., 2002; Gladysheva et al., 2003). In contrast, members of the pathogenic bacterial genera Streptococcus and Staphylococcus secrete co-factor proteins, such as streptokinase (SK) and staphylokinase (SAK), respectively, also known as non-physiological plasminogen activators (nPAs), which activate PG in a species-specific manner. These bacterial proteins promote pathogenesis by promoting the dissemination of the bacteria in their host. The members of nPAs do not have any intrinsic enzymatic activity but activate the host’s PG by indirect mechanism, wherein SK (or SAK) first combines with ‘partner’ plasminogen in an equimolar (1:1) manner to form tight, enzymatically active activator complexes. In case of SK, which is the main subject of the present treatise, the initial SK.HPG complex matures to a SK.HPN activator complex which then catalytically transforms ‘substrate’ HPG to HPN in large numbers. Despite several years of research, a compelling need exists to glean insights about the molecular mechanisms of substrate PG activation in the SK.HPN ‘system’ to unveil the ‘designed principles’ of nPAs so as to facilitate the design of more effective thrombolytic drugs.

An interesting insight into the molecular mechanism of substrate PG activation by SK.HPN activator complex came from studies from our laboratory which has been amongst the first to examine the enzyme-substrate molecular interactions wherein the activator enzyme interacts via a ‘ternary’ complex with substrate plasminogen i.e. [SK.HPN]-[HPG], and identified the role of a discrete micro-structure, namely the 250-loop (Dhar et al., 2002) residing in the β domain of SK in substrate recognition via the substrate kringle domains. The laboratory also reported the importance of kringle-mediated long-range interactions between the activator enzyme and its macromolecular substrate in the generation of the high catalytic efficiency associated with the SK.HPN enzymatic complex (Sundram et al., 2003). In the present investigation, in order to decipher the exact “nature” of participation in catalysis of different kringle domains of substrate PG in SK.HPN, or in other plasminogen activators, different partially truncated forms of PG were prepared via molecular cloning techniques. These included the isolated catalytic domain (CD), with and without the sequential addition of the five kringle
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domains at its proximity, like K5CD (miniPG), K4K5CD (midiPG), K3K4K5CD (K3PG), K2K3K4K5CD (K4PG), besides full-length PG (HPG) after expression in the methylotrophic yeast, Pichia pastoris. As a eukaryote, P. pastoris has many advantages such as protein processing, protein folding, and post-translational modification. These proteins were then purified to the level of >95 % using the standardized chromatographic procedures.

After formation of the binary complex with either SK.HPN or SAK.HPN, different recombinantly derived truncated forms of HPG were used as substrate. Steady-state kinetic studies were performed with the kringle-less derivative, μPG as substrate, showed that SK.HPN showed drastically compromised catalytic turnover (only 1-2 % of the native SK.HPN versus HPG) as well as 4-5 fold reduced affinity compared to that against full-length substrate HPG. Interestingly, the addition of only one kringle (in this case, K5-CD) onto the “μPG scaffold”/catalytic domain, increased the affinity of the substrate for the SK.HPN activator enzyme to near-native levels, and an increase in catalysis by 20-fold as compared to μPG as the substrate. These results revealed that the kringle-mediated long-range interactions between SK-HPN and HPG are ‘dual’ in nature i.e. one that is involved in imparting ‘substrate recognition’ ability to the activator enzyme and the other in ‘catalytic processing’ of the substrate. It can be thus reasoned that although a basal level of substrate HPG catalysis (1-2 %) is imparted through the interaction of the catalytic domain only, the presence of the 5th kringle next to the catalytic domain in substrate dramatically increases both the enzyme affinity as well as catalytic rates. In contrast, with the physiological activator, such as UK, activates the isolated catalytic domain, μPG, with almost similar catalytic rates as compared to full-length PG, indicating a fundamentally different mode of substrate interaction compared to streptokinase.

The addition of the 4th kringle onto the “miniPG scaffold” led to the amplification of catalytic turnover by 4-fold as compared to substrate miniPG (where only one kringle, K5 was attached to the catalytic domain, as described in the last paragraph), and brought the overall catalytic efficiency to almost that of full-length PG. The presence of K4 and K5, thus ‘lifted-up’ the catalytic potentiation in the macromolecular substrate from almost one percent (in μPG) to nearly cent percent (in midiPG, with two kringle domains), when compared to that with full-length PG. These studies have clearly demonstrated, for the first time, the special importance of the 4th and 5th kringles in “substrate assisted” catalytic potentiation in the SK.HPN system.
Although midiPG, when used as the substrate, generated near-native catalytic turnover rates compared to the Glu-PG (plasma) form of the substrate, but still a relatively minor deficiency or lag, of about 1.6-fold, was observed as compared to that of the conformationally open-form of HPG viz. Lys-PG as the substrate. The Lys-PG is formed by proteolysis of the Glu-PG form (removal of the N-terminal 77 residues by plasmin digestion) and converts the overall conformation from a ‘closed’ to ‘open’ form. The contributions of the rest of the three kringles (K1-3) were then elucidated for the compensation of the lag in the catalytic turnover between Lys-PG and Glu-PG, using higher molecular weight forms of plasminogen as substrates, namely r-K3PG, r-K4PG, and r-K5PG: LysPG expression obtained from Pichia. In these plasminogen derivatives, somewhat surprisingly, further amplification in catalytic turnover (as compared to ‘natural’ LysPG derived from plasma Glu-PG converted in vitro by controlled proteolysis) could not be obtained, but rather, a minor dip in catalytic rates was observed without affecting the enzyme-substrate affinity. The plausible explanation for this slight diminution in catalytic rates is the presence of ‘non-native’ type glycosylated moieties (high-mannose type) in the yeast-derived Lys-PG, which might be either interfering with the available interaction sites or altering the structure of the molecule, thereby decreasing its participation in the catalytic turnover with the SK.HPN activator complex.

Further, to see whether the exploitation of kringle during PG activation by non-physiological HPG activators is a ‘generalized’ phenomenon or it is a case-specific reaction, we examined if SAK, which is a single-domain molecule, exploits any/all of the five kringles during substrate HPG activation akin to that observed with SK. This comparison showed that the catalytic efficiency of SAK.HPN with substrate µPG was relatively higher than that observed with SK.HPN, thus implying a much higher dependency of SK on the kringle domains mediated long-range protein-protein interactions in catalysis. In case of miniPG, the substrate affinity was increased whereas its catalytic turnover increased by only 1.5-fold as compared to substrate µPG. However, midiPG, which contains the 4th and 5th kringles together, showed an increment of about 2-fold as compared to miniPG. Interestingly, when we used r-K3PG, r-K4PG, and r-K5PG: LysPG as the substrates, the catalytic rate was hardly affected which is contrary to SK.HPN. These results indicate that SAK, a single-domain molecule, in contrast to SK, has evolved enzyme-substrate interactions in such a manner so as to utilize a more limited molecular surface in the substrate (full-length human plasminogen) for attaining its full-blown activity.
Thus, the molecular mechanism by which SK exploits the substrate kringle for catalytic enhancement is broadly similar to SAK but their exploitation is at a much higher extent. The tri-domain SK consists of three independent domains, and once it forms an activator complex with plasmin, it utilizes long-range protein-protein interactions virtually in a “hierarchical-order”, to efficiently exploit almost the complete “molecular surface” of substrate plasminogen (consisting of all its five kringle domains) for its highly efficient catalysis. On the contrary, SAK, containing only a single domain, once complexed with plasmin, has to perforce utilize a much more limited molecular surface in substrate plasminogen, thereby restricting its molecular contacts, a phenomenon which tends to also explain the reason for its overall lowered catalytic power (about one-fourth) compared to the SK.HPN activator complex.

The direct physiological activators, such as UK, showed weak affinity with \( \mu \text{PG} \) that corresponded to 3-fold diminution as compared to that of full-length substrate Glu-PG. However, the maximal catalytic turnover with substrate \( \mu \text{PG} \) by UK was almost similar to that with full-length Glu-PG, indicating that kringle probably do not play a direct role in generating catalytic rates as seen with the nPAs. In comparison to the open-form of HPG \textit{viz.} LysPG, the catalytic turnover of substrate \( \mu \text{PG} \) was found to be diminished by nearly 3-fold, indicating that the overall conformation of the substrate might contribute towards catalytic rates. The addition of K4 and K5 onto the \( \mu \text{PG} \) scaffold compensated the minor lag of conformational dependent catalytic rate and brought the catalytic rates similar to plasma-purified and proteolytically processed native-LysPG. The plausible explanation for the relatively small increment in catalytic turnover might be the optimal positioning of substrate once conjugated through the catalytic domain as well as the kringle domains, and thus providing the stability to the activation loop carrying the scissile peptide bond. The higher molecular weight plasminogen derivatives showed little enhancement in catalysis in plasma due to the ‘non-native’ glycosylation in the \textit{Pichia}-derived proteins. Unlike SK.HPN, UK was not sensitive to the type of carbohydrate moieties attached onto the PG polypeptide backbone. These results reveal that UK, which is a member of the serine protease family, recognizes the catalytic domain preferentially and activates the isolated catalytic domain, \( \mu \text{PG} \), with a similar catalytic rate as compared to Glu-PG. In addition, irrespective of the species of PG, UK can activate it solely based on the features of the catalytic domain which are conserved across animal species. Consequently, the kringles remain free to participate in various physiological protein-protein interactions such as with fibrin and cellular receptors.
depending upon the physiological demands. However, the non-physiological plasminogen activators have evolved substrate recognition features that are centered on the highly conserved kringle domains so that they can "by- pass" the evolutionary drift in the target substrates catalytic domain through which the host may try to evade the pathogens at the molecular level.

Since addition of K4 and K5 onto the miniPG and µPG 'scaffolds' have shown the dramatic enhancement in catalytic rates in case of SK.HPN mediated substrate activation, therefore we used these 'simple' model proteins for further insights regarding the molecular mechanism of substrate-assisted catalysis. In order to further elucidate the kringle mediated interactions in SK.HPN system, different non-native chimeric variants of miniPG and midiPG viz. K1CD, K2CD, K4CD and K1K2CD (carrying the catalytic domain alongwith a non-natural configuration of the kringles) were constructed in P. pastoris, and purified to > 95% homogeneity, and then examined for substrate activation. The 'swapping' of Kringle-5 with other kringles with varying lysine binding strengths, individually, altered the relative enzyme-substrate affinities for SK.PN which is in direct proportion to the known "strength" of the LBS of the substituted kringle domain. Interestingly, despite the lowered substrate affinities in the various chimeric variants of miniPG, the catalytic turnover was similar to that seen with native miniPG. The secondary structure composition, its stability, and microenvironment of the catalytic domain of these 'artificial' substrates were authenticated by various means, such as CD spectroscopy, plasmin resistance, and UK-mediated activation, all of which proved these to be identical to the native proteins, namely miniPG and midiPG. These observations indicated that as far as the catalytic turnover is concerned, all the kringles are proficient enough (despite their primary structure dissimilarities) to assist in attainment of 'native like' catalytic rates when conjugated to µPG, perhaps because SK senses the specific "conserved conformational-trait" of the kringle domain. Furthermore, we established that as far as the substrate affinity is concerned, the 250-loop of SK-β domain senses the lysine binding strength of individual kringles when these are conjugated to the catalytic domain. Once the positive-charged lysine residues of the 250-loop were mutated, loss in the substrate affinity was observed in all the 'artificial' chimeric proteins along with the native miniPG, but without any significant changes in the maximal catalytic rates. These observations lead to the unmistakable conclusion that lysine binding strengths of the kringles govern the substrate affinity but the catalytic turnover rate was influenced likely by the kringle conformation in the SK.HPN system. To further explore this notion and the
possible site-specific interactions of discrete residues of the kringle-LBS during catalysis of substrate HPG by the SK.HPN activator complex, a series of alanine-scanning mutants of the LBS in miniPG and midiPG proteins were prepared. These results revealed a major contribution of a negatively-charged cluster in the 5th kringle domain of miniPG, e.g. DGD55,56,57AAA, which exhibited a nearly 10-fold reduction in catalytic turnover when used as the substrate of SK.HPN, without significant loss in substrate affinity as compared to native miniPG. In order to see if relatively subtle alterations in kringle conformation was a reason for catalytic potentiation, a few key, hydrophobic residues of the LBS pocket were also mutated, such as F36A, W62A and L71R which also showed lowering in catalytic rates by 3-fold, 20-fold and 4-fold, respectively, without any alteration in enzyme-substrate affinity in the SK.HPN system. In case of kringle-4, LBS residue mutations in midiPG protein, namely K4-D55A, K4-D57A, K4-W62A, K4-R71A, and W62A in kringle-5, reduced the catalytic rates by 2-fold, 4-fold, 1.5-fold, and 2-fold, respectively, without any change in affinity. Overall these results clearly indicated that the critical residues of the K5-LBS and K4-LBS seem to be essential for SK.HPN mediated substrate activation. Taken together, the present results highlights an evolutionary mechanism whereby SK.HPN-mediated activation of human plasminogen may have evolved to utilize specifically the kringle conformation for successful catalytic amplification, over and above that generated by interaction with the catalytic domain of substrate for a basal level of activity and specificity, thereby becoming virtually independent of the mutational drift that might occur in the catalytic domain of the macromolecular substrate *per se*. This is in fundamental contrast to the situation with physiological plasminogen activators, such as UK, which recognize (and catalytically utilize) predominantly the serine protease domain of the substrate for substrate recognition and turnover, where there is no need for a ‘tussle’ between enzyme and substrate to co-evolve differentially.

The molecular mechanism of *substrate-assisted catalysis* of SK, wherein exploitation of long-range ‘supra-catalytic’ centred based interactions between substrate and enzyme seems to be an underlying leitmotif, is also observed to some extent in SAK, is thus quite distinct from the physiological PG activators such as UK, which exhibit catalysis with almost similar rates irrespective of the presence or absence of kringle domains in their macromolecular substrate. Unlike UK, where short-range interactions involving the catalytic domain is primarily responsible for full-blown catalytic rates, long-range protein-protein interactions involving the key negatively charged LBS as well
the conformation in and around the LBS pocket of the kringle domains regions of the substrate HPG have been exploited by SK through evolutionary selection of a three-domain design to generate the highest degree of catalytic efficiency amongst all known HPG activators. Although further studies are warranted to acknowledge the exact epitopes involved in the molecular mechanism of substrate-assisted catalysis of SK, the present study has successfully deciphered some of the major causes of the significant contribution of the kringle domains in catalysis by the bacterial plasminogen activators. Hopefully, these studies will pave the way for a more detailed structure-function co-relationship amongst the existing thrombolytic proteins and lead to the design of useful site-specific proteases.