Regulation of Nonproteolytic Active Site Formation in Plasminogen[†]

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Received December 18, 2006; Revised Manuscript Received May 4, 2007

ABSTRACT: Streptokinase may be less effective at saving lives in patients with heart attacks because it explosively generates plasmin in the bloodstream at sites distant from fibrin clots. We hypothesized that this rapid plasmin generation is due to SK's singular capacity to nonproteolytically generate the active protease SK·Pg*, and we examined whether the kringle domains regulate this process. An SK mutant lacking Ile-1 (ΔIle1-SK) does not form SK·Pg*, although it will form complexes with plasmin that can activate plasminogen. When compared to SK, ΔIle1-SK diminished the generation of plasmin in plasma by more than 30-fold, demonstrating that the formation of SK·Pg* plays an important role in SK activity in the blood. The rate of SK·Pg* formation (measured by an active site titrant) was much slower in Glu-Pg, which contains five kringle domains, than in Pg forms containing one kringle (mini-Pg) or no kringles (micro-Pg). In a similar manner, *Streptococcus uberis* Pg activator (SUPA), an SK-like molecule, generated SUPA·Pg* much slower with bovine Pg than bovine micro-Pg. The velocity of SK·Pg* formation was regulated by agents that influence the conformation of Pg through interactions with the kringle domains. Chloride ions, which maintain the compact Pg conformation, hindered SK·Pg* formation. In contrast, ϵ-aminocaproic acid, fibrin, and fibrinogen, which induce an extended Pg conformation, accelerated the formation of SK·Pg*. In summary, the explosive generation of plasmin in blood or plasma, which diminishes SK's therapeutic effects, is attributable to the formation of SK·Pg*, and this process is governed by kringle domains.

Plasmin dissolves blood clots by cleaving fibrin strands. Plasminogen (Pg) is the inactive zymogen of plasmin. Pg activators generate plasmin by cutting the Arg561−Val peptide bond of the Pg (Figure 1A). Eukaryotic Pg activators (e.g., tissue Pg activator and urokinase) are serine proteases that directly cleave Pg to produce plasmin at the fibrin surface (Figure 1 A, path 1). In contrast, the bacterial plasminogen activators streptokinase (SK),[1] *Streptococcus uberis* plasminogen activator (SUPA, PauA, or SKuberis), and staphylokinase (SAK) have no intrinsic protease activity (*1*, *2*). These cofactors form stable complexes with plasmin, converting plasmin into a protease that can cleave Pg to plasmin (Figure 1A, path 2) (*1*, *3−9*). SK and SUPA, but not SAK, also form active protease complexes with Pg (SK·Pg* or SUPA·Pg*), and these complexes are also Pg activators (Figure 1A, path 3) (*6*, *9*, *10*). However, the generation of SK·Pg* may contribute to SK's ability to produce plasmin in the blood at sites distant from the fibrin clot; this appears to limit the clot-dissolving properties of SK in humans (*11−14*).

Recent studies have identified several important structural and functional elements in SK. The initial formation of an inactive SK·Pg complex is rapid, followed by a slow formation of the active SK·Pg* complex (*1*). There is strong evidence that SK·Pg* generation occurs through the "molecular sexuality hypothesis" whereby Ile1 of SK (α-domain) forms a salt bridge with Asp[740] of Pg to trigger a conformational change that nonproteolytically produces an active site in the Pg moiety of the SK·Pg complex (*1*, *2*, *15*). A similar process occurs in SUPA·Pg complexes to generate SUPA·Pg* (*4*, *5*, *16*). However, the factors in Pg that regulate the formation of SK·Pg* are poorly understood.

The physiologic forms of human (Glu-Pg) and bovine Pg contain an NH$_2$-terminal peptide, five kringle domains, and a protease domain. The isolated protease domain is termed micro-Pg while the form containing the protease domain and the last kringle domain is known as mini-Pg. Unfortunately, the available crystal structure of the SK·micro-plasmin complex only reveals the interaction of SK with the micro-plasmin; there is no information on SK·Pg* or kringles (*6*). The kringle domains participate in high-affinity binding interactions with SK (*17−20*) and also mediate the interactions of Pg with activators, substrates, inhibitors, fibrin, cell membranes, Cl ions (Cl$^-$), lysines, etc. These molecules stabilize either the compact (Cl$^-$) or extended (fibrin, lysines) conformations of Pg, but their effects on the formation of SK·Pg* are unclear (*11*, *17−19*, *21−35*).

1 Abbreviations: Glu-Pg, human Glu-plasminogen; SK, streptokinase; SUPA, *Streptococcus uberis* plasminogen activator; SAK, staphylokinase; α-domain, alpha domain; β-domain, beta domain; γ-domain, gamma domain; (DD)E, complex of fibrin D-dimer noncovalently associated with fragment E; EACA, ϵ-amino-*n*-caproic acid; S2251, H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide dihydrochloride; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; PAGE, polyacrylamide gel electrophoresis; Pg*, Pg converted to an active protease without cleavage to plasmin; P(g), Pg or plasmin.
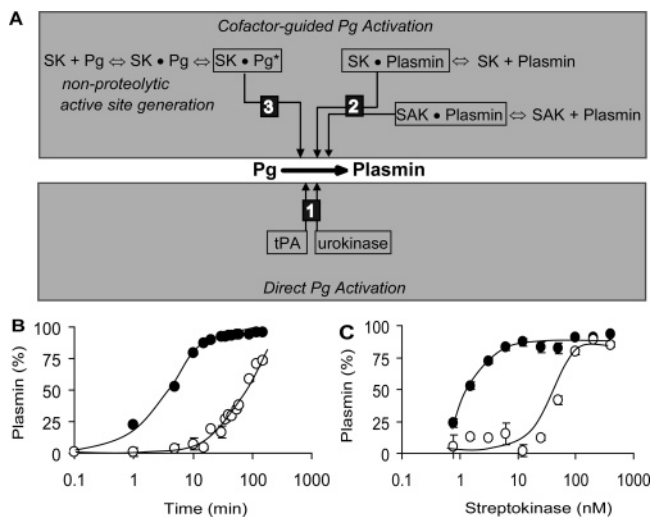
FIGURE 1: (A) Schematic mechanisms of Pg activation. Direct Pg activation: Tissue Pg activator and urinary type Pg activator directly cleave Pg to plasmin (pathway 1). Cofactor-guided Pg activation: Complexes of plasmin with SK or SAK directly cleave Pg to plasmin (pathway 2). Alternatively, SK nonproteolytically generates an active site in Pg (SK·Pg*). Then SK·Pg* cleaves Pg to plasmin (pathway 3). Note: SUPA can replace SK in this scheme. (B, C) Generation of plasmin in human plasma by SK (closed circles) or ΔIle1-SK (open circles). SK or ΔIle1-SK was added to human plasma and incubated at 37 °C. The plasmin generated in plasma was measured as described in Experimental Procedures. The mean (±SD) is shown. (B) Rate of plasmin formation. The reaction was performed with SK or ΔIle1-SK (25 nM). Plasma samples were taken at the indicated times. (C) Dose-related generation of plasmin in plasma. The reaction was performed with various concentrations SK or ΔIle1-SK (0−400 nM). Plasmin generation was determined after 30 min of incubation.

The formation of SK·Pg* has been challenging to assess experimentally because of the difficulty isolating this process from the formation of SK·plasmin or plasmin. Previous analyses have been limited by potential difficulties with plasmin contamination, distinguishing between the formation of the inactive SK·Pg complex and the active SK·Pg* complex, as well as by inappropriate substrate or enzyme conditions (*11*, *36*, *37*). Unfortunately, active site inhibited Pg is also of limited utility for analyzing the process of SK·Pg* formation because it already contains a productively rearranged active site (*36*, *37*). To overcome these problems, we used an active site titrant (fluorogenic *p*-guanidinobenzoyl substrate) that traps SK·Pg* (*9*, *10*, *38*, *39*).

This report investigates the hypothesis that the explosive generation of plasmin in plasma by SK and SK-like molecules (e.g., SUPA) is due to the generation of SK·Pg*, a process which itself may be regulated by Pg kringles and their ligands (e.g., Cl⁻, Lys analogues, and fibrin). We show for the first time that SK·Pg* generation regulates plasmin formation in plasma. Surprisingly, although the kringle domains are known to promote the formation of the initial inactive SK·Pg complex, the presence of kringle domains decreases the rate of formation of SK·Pg* and SUPA·Pg*. Molecules (e.g., fibrinogen) that interact with the kringle domains to stabilize the extended conformation of Pg accelerate the formation of the active complexes (*11*, *40*). These findings provide new insights into the explosive ability of SK to activate Pg in the circulation.

EXPERIMENTAL PROCEDURES

Protein and Reagents. Bovine Pg was prepared from fresh, citrated bovine plasma by affinity chromatography with lysine-substituted agarose (*41*). Bovine mini-Pg was prepared by limited digestion of bovine Pg with porcine pancreatic elastase (Sigma) coupled to agarose (*42*). Mini-Pg was purified by affinity chromatography on lysine−agarose. The purified bovine Pg and bovine mini-Pg were analyzed by 12% SDS−PAGE and by immunoblotting analysis using goat anti-bovine Pg antibody (American Diagnostica Inc.). SDS−PAGE and immunoblot analysis of bovine Pg and mini-Pg showed only one band of relative mass 72 and 38 kDa, respectively. Glu-Pg, mini-Pg, and micro-Pg preparations were pretreated with aprotinin−agarose (Sigma) for 4 h at 4 °C to remove trace amounts of plasmin. After treatment, no plasmin was detected by SDS−PAGE and immunoblotting under reducing conditions or by amidolytic kinetic analysis with a plasmin substrate S2251 (H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide dihydrochloride; Chromogenix).

Human or bovine (DD)E fragments were obtained from human/bovine fibrinogen (Chromogenix) by simultaneous digestion with plasmin (12 nM) and thrombin (1 IU/mL) at 37 °C for 12 h (*43*). The (DD)E preparations were pretreated with aprotinin−agarose to remove active plasmin.

Cloning, Expression, Purification, and Titration of Recombinant Proteins. Recombinant micro-Pg, mini-Pg SK, micro-Pg$_{R561A}$ mutant, ΔIle1-SK, SUPA, and individual SK domains, α-domain (residues 1−148), β-domain (residues 149−293), and γ-domain (residues 294−414), were cloned, expressed in bacteria, purified, and characterized as described (*2*, *5*, *14*, *44*, *45*).

Production of the Monoclonal Antibodies against Human Plasminogen. Six female Balb/C mice (Charles River, Wilmington, MA) were immunized intraperitoneally with 10 μg of micro-plasmin immobilized on aprotinin−Sepharose in complete Freund's adjuvant and 2 weeks later were given booster injections of 10 μg with incomplete Freund's adjuvant. The boost was repeated three times every 2 weeks. After 2 weeks the antibody titer was determined by solid-phase radioimmunoassay binding against human recombinant micro-Pg, micro-plasmin (after micro-Pg activation by urokinase), micro-Pg$_{R561A}$ mutant, and BSA. The two mice with highest titers were hyperimmunized with 50 μg of recombinant micro-plasmin in saline intraperitoneally 4 days prior to fusion. Somatic cell fusion was performed as described (*46*). The fusion frequency was 50%. Hybridoma supernatants were tested for the production of anti-micro-plasmin mAb by solid-phase RIA in a COBRA II gamma counter, using ¹²⁵I-labeled sheep anti-mouse antibody (American Diagnostica Inc.) as a secondary antibody. Antibody against micro-plasmin was identified in 3.2% of clones. These 32 hybridomas were then tested for the production of plasmin-specific antibody by immunoblotting using human plasmin (Sigma) as an antigen. Hybridomas were cloned by limiting dilution and serotyped (Zymed).

Detection of Plasminogen Concentration in Human Plasma. Fresh frozen human plasma (270 μL) was preincubated with 30 μL of SK or ΔIle1-SK (0−400 nM) at 37 °C. The residual concentration of plasma Pg was determined by amidolytic assay in the presence of SK as described (*47*). Briefly, plasma

aliquots (3 μL) were removed from the reaction and added to a microtiter plate containing SK (1 μM) in 100 μL of assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH7.4). After incubation for 10 min at room temperature, S2251 (500 μM) substrate was added. The generation of amidolytic activity was monitored at 405 nm, 25 °C, for 5 min in a microplate reader (Bio-Tek Instruments Inc.). The production of *p*-nitroanilide is linear under these conditions and corresponds to the rate of amidolytic activity of the formed SK·plasmin complex. The plasmin generated in these samples was estimated by reference to the values obtained with human pooled fresh frozen plasma as a standard.

Kinetic Assay. The activation of human or bovine Glu-Pg, mini-Pg, and micro-Pg by SK, SUPA, SK·plasmin, or SUPA·plasmin complexes was performed with a *p*-nitro-anilide substrate S2251 as described (*14*) in an assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4). The reaction was initiated by the addition of activators to assay buffer containing S2251 (500 μM) and human/bovine Pg (300 nM) (human Glu-Pg; Chromogenix). The generation of amidolytic activity (at 405 nm) was monitored at 37 °C for 10 min in a microplate reader under conditions where less than 10% of substrate was consumed during the course of the reaction. A similar activation experiment was performed in the presence of EACA (10 mM) or human/bovine (DD)E fibrin fragment (3 μM) preincubated with Pgs. Plasmins and mini-plasmins were generated from Glu-Pg or mini-Pg by uroki-nase−Sepharose (1:30, mol:mol ratio) at 37 °C for the times required to achieve maximal amounts of active enzyme (typically 40−60 min).

The development of amidolytic activity in the SUPA·bovine Pg (20 nM) complex was determined in the presence of EACA (0−100 mM) in assay buffer, pH 7.4, at 37 °C by monitoring the cleavage of S2251 (500 μM) at 405 nm for 10 min in a microplate reader as described (*11*).

Fluorescence Active Site Generation in Pg, Mini-Pg, and Micro-Pg (Human, Bovine) by SK or SUPA. Nonproteolytic active site generation by SK or SUPA in Pgs was examined with the fluorogenic *p*-guanidinobenzoyl substrate 4-methyl-umbelliferyl *p*-guanidinobenzoate (MUGB) (*1, 5, 9, 10, 14, 44, 45, 48−50*) in an F-2500 Hitachi fluorescence spectro-photometer under conditions where [SK] or [SUPA] > [Pg] and [MUGB] > [Pg] as described (*5*). Briefly, Pgs (150−400 nM) or nothing was added to a cuvette containing 2 μM MUGB in filtered assay buffer (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4) at 25 °C. After 5 min, SK or SUPA (250−500 nM), buffer alone (control), or preformed (on ice) complexes of SK·Pg or SUPA·Pg were added, and the development of fluorescence was monitored continuously with excitation at 365 nm and emission at 445 nm. The same experiment was performed in the presence of EACA (10 mM), human/bovine (DD)E fibrin fragment (3 μM), and SK α-, SK β-, and SK γ-domains (500 nM), as well as bovine serum albumin or maltose binding protein (3 μM). The agents were preincubated with assay buffer and MUGB during 20 min. The activation of Pgs (200 nM) by SAK or ΔIle1-SK (250 nM), which are incapable of Pg* formation, were examined as negative controls. Sample emission intensities were measured in the photon counting mode and are expressed as pulses per second (pps). From pps the concen-tration of active Pgs was determined by reference to known concentrations of active plasmin.

Scheme 1
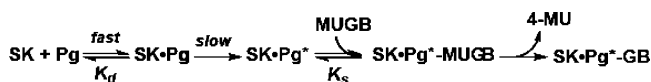
The formation of the inactive SK·Pg complex is a critical prerequisite for the formation of the active SK·Pg* complex. The formation of the SK·Pg complex is very fast; for Glu-Pg $k_{on} = 3 \times 10^7$ M^{-1} s^{-1} and $K_d = 0.03−0.23$ nM (*18, 44, 52*). SK also binds tightly to mini-Pg ($K_d = 0.26$ nM); the binding affinity to micro-Pg ($K_d = 17$ nM) is lower (*18, 44, 52*). Under conditions in which SK·Pg complex formation is not rate limiting, the formation of the active SK·Pg* complex (*10*) in the presence of MUGB can be considered as a first-order rate reaction and described by Scheme 1. In this scheme SK·Pg*-GB is the acyl-enzyme, 4-MU (4-methylumbelliferone) represents the product fluorophore, and K_s is the dissociation constant for the noncovalent SK·Pg*·MUGB complex formation. Under conditions of [SK] or [SUPA] > [Pg] $\gg K_d$ and [MUGB] $\gg$ [SK·Pg*], [MUGB] $\gg K_s$, the rate of 4-MU production reflects the generation of SK·Pg*. The generation of SK·Pg* is described by the equation:

$$[4\text{-MU}] = [\text{SK·Pg*}] = [\text{SK·Pg*}]_f(1 - e^{-k_{obs}t}) \quad (1)$$

where k_{obs} is the observed first-order rate constant for the conversion of SK·Pg to SK·Pg* and [SK·Pg*]$_f$ is the final concentration of SK·Pg* (*10, 38, 39*). It is important to note that the initial complex between SK and Pg does not contain the active site; therefore, this complex cannot interact with MUGB and compromise 4-MU production (*9, 10*).

Continuous Assay for Determination of the Rates of Active Site Generation in Pg Complexes with SK or SUPA. The formation of SK·Pg* or SUPA·Pg* was detected in a Cary 100-Bio spectrophotometer by appearance of amidolytic activity in the equimolar SK·Pg or SUPA·Pg complexes at 4 °C, a temperature at which S2251 substrate amidolysis occurs but Pg activation does not (*11*). Human or bovine Pg, mini-Pg, or micro-Pg (300 nM) was added to the cuvette in the presence of S2251 (500 μM) with or without EACA (10 mM). After incubation for 5 min at 4 °C, SK or SUPA (300 nM) was added, and the release of *p*-nitroanilide was monitored.

Western Blot Analysis. Nonproteolytic active site genera-tion in bovine mini-Pg by SUPA and human micro-Pg by SK was performed in the presence of MUGB as described above. Reactions were quenched by adding SDS sample buffer with 2-mercaptoethanol and boiling for 5 min. Proteins were separated under reducing conditions by SDS−PAGE (12% gels) and transferred to Immobilon-P transfer mem-brane (Millipore Corp., Billerica, MA). Bovine or human Pgs were detected by polyclonal goat anti-bovine Pg antibody (American Diagnostica Inc.) and monoclonal mouse anti-human Pg antibody. After being washed with TBS containing 0.1% (v/v) Tween 20, bound anti-Pg antibody was detected by secondary rabbit anti-goat (Zymed, San Francisco, CA) or rabbit anti-mouse antibody (Amersham Biosciences) conjugated to alkaline phosphatase.

Change of Intrinsic Fluorescence. Pgs (50 nM) were added to a quartz cuvette, containing 50 mM Tris-HCl, 100 mM NaCl, 20% glycerol, pH 7.4, buffer. The intrinsic fluores-

cence was monitored at excitation 282 nm and emission 340 nm until it was stable for 20 min at 25 °C. Then EACA (1−35 mM) was added incrementally while the fluorescence was monitored. The changes in fluorescence signal (ΔF, %) were calculated in comparison to the initial values.

RESULTS

Activation of Pg in Human Plasma by SK. To examine the hypothesis that the production of plasmin in plasma depends on the formation of SK·Pg*, we used a mutant lacking Ile1 of SK (ΔIle1-SK). ΔIle1-SK can form an activator complex with plasmin but not with Pg because the absence of the N-terminus prevents the conversion of the inactive SK·Pg complex to SK·Pg* (*1, 2, 15*). At roughly therapeutic concentrations (25 nM), SK converted 50% of plasma Pg to plasmin after 5 min (Figure 1B). In contrast, at the same dose, ΔIle1-SK converted 50% of plasma Pg to plasmin after 90 min (Figure 1B). Viewed differently, 30-fold more ΔIle1-SK mutant was required to activate 50% of plasma Pg in 30 min (Figure 1C). Thus the formation of SK·Pg* was a major determinant of plasmin generation in plasma.

Active Site Generation in Human Pg. To examine the formation of SK·Pg* independently from plasmin formation, experiments were performed under conditions that removed or inactivated trace amounts of plasmin in the Pg preparations (see Experimental Procedures). The absence of plasmin was confirmed by immunoblotting (e.g., Figure 2F,G; see below), and no activation was observed in the presence of ΔIle1-SK (Figure 2A,C; see below). Removal of the kringle domains decreases the affinity of SK for Pg; the values for the K_d are 0.03−0.23, 0.26, and 17 nM for Glu-Pg, mini-Pg, and micro-Pg, respectively (*18, 44, 52*). However, for both Glu-Pg and micro-Pg, formation of the inactive SK·Pg complex is rapid, while formation of the active SK·Pg* is slow (*1*). In all of the following experiments, the concentration of SK and Pgs was at least 10 times greater than their K_ds for complex formation (*18, 20, 44, 52*).

We first examined the formation of SK·micro-Pg* using a mutant (micro-Pg$_{R561A}$), which cannot be converted to micro-plasmin and therefore can only generate an active site through a nonproteolytic mechanism. As observed previously (*1, 2*), SK·micro-Pg$_{R561A}$* formation was complete in ~30 min; preincubation on ice did not change the kinetics of activation (Figure 2A). No active site formation was seen with micro-Pg$_{R561A}$ alone or with the ΔIle1-SK·micro-Pg$_{R561A}$ complex (Figure 2A). The reaction of SK with wild-type micro-Pg follows a similar time course (Figure 2B); no micro-plasmin is produced under these conditions (Figure 2F).

Surprisingly, the formation of both SK·Glu-Pg* and SK·mini-Pg* was much slower than SK·micro-Pg* even though both bind to SK with higher affinity (see above) (Figure 2B). In these experiments, SK and Pg were preincubated at 5 min to form SK·Pg (*2, 18*). In addition, increasing the concentration of SK did not change the rate of SK·Glu-Pg* formation (Figure 2C), which indicates that the rate-limiting step is not formation of the inactive SK·Pg complex. The generation of SK·Glu-Pg*, SK·mini-Pg*, and SK·micro-Pg* could be described by a first-order equation (eq 1) (Figure 2B, inset) (*9, 10, 38, 39*). The values of k_{obs} for the SK·micro-Pg*,

SK·mini-Pg*, and SK·Glu-Pg* are 0.19 ± 0.01, 0.04 ± 0.01, and 0.01 ± 0.01 min^{-1}, respectively (Figure 2B, inset). Given that Glu-Pg contains five kringle domains, mini-Pg contains one kringle domain, and micro-Pg contains no kringle domains, these observations suggest that the kringle domains inhibit the conversion of SK·Pg to SK·Pg*. This is in stark contrast to the well-established data that Pgs containing kringle domains bind more quickly and tightly to SK (*18, 20*).

Active Site Generation in Bovine Pg. We have previously shown that formation of the SUPA·Pg* complex proceeds more slowly than SK·Pg* and is proceeded by a short lag phase (*5*). We hypothesized that this brief lag represented the initial formation of the inactive SUPA·Pg complex; preincubation of SUPA and Pg on ice eliminated the lag phase (Figure 2D and inset 1). Nevertheless, the activation of bovine Pg by SUPA showed a similar pattern to SK: formation of SUPA·Pg* was much slower than SUPA·mini-Pg* formation (Figure 2D). The values of k_{obs} for SUPA·Pg* and the SUPA·mini-Pg* are 0.01 ± 0.01 and 0.37 ± 0.02 min^{-1}, respectively (Figure 2D, inset 2). The inhibitory effect of kringles on formation of SUPA·Pg* was also separately confirmed by the continuous amidolytic assay for active site formation described in Chibber et al. (*11*) and Wang et al. (*1*) (Figure 2E). This assay is conducted at 4 °C to block plasmin formation. Thus the velocity of active site generation by both SK and SUPA is inversely related to the number of kringles in the Pg molecule (micro-Pg > mini-Pg > Glu-Pg; Figure 2B,D,E).

Influence of EACA on Pg Conformation and Nonproteolytic Active Site Formation. The above reactions contained physiologic concentrations of Cl$^-$ (100 mM) which stabilize Glu-Pg in a compact conformation (*17, 32, 33*). Ligands which interact with the lysine binding sites on the kringles (e.g., EACA) induce an extended Glu-Pg conformation (*22, 32, 33*); this conformational change is associated with changes in the intrinsic fluorescence of the molecule (*30, 32*). High concentrations of EACA can inhibit SK·Pg formation and also compete with substrates for the protease active site (*18, 44*). Therefore, we designed experimental conditions under which EACA would selectively bind to the kringle domains (*18, 53−55*). EACA (10 mM) was sufficient to maximally unfold the Pg molecules as reflected by a change in the relative equilibrium fluorescence values (% ΔF, Figure 3 A) but had negligible effects on the activity of SK·Pg*.

If the compact conformation of Glu-Pg inhibits the formation of SK·Pg*, then EACA should accelerate the formation of SK·Pg*. This prediction was confirmed. EACA accelerated the formation of SK·Pg* generation by 2.7 ± 0.1-fold and SUPA·Pg* formation by 24 ± 1-fold (Figure 3). In contrast, EACA did not affect the formation of SK·micro-Pg* (Figure 3D) as expected given that EACA interacts with the kringles. Also as expected, staphylokinase, which cannot generate an active site, failed to activate Pg in the presence of EACA (Figure 3C).

Chloride Ions and Active Site Generation. The extended conformation of Pg is favored at low Cl$^-$ concentrations (*11, 27*). The formation of SK·Pg* was rapid in the absence of Cl$^-$ (Figure 4A). Increasing Cl$^-$ concentrations from 0 to 100 mM inhibited SK·Pg* in a concentration-dependent manner (Figure 4B). These observations support the hypoth-
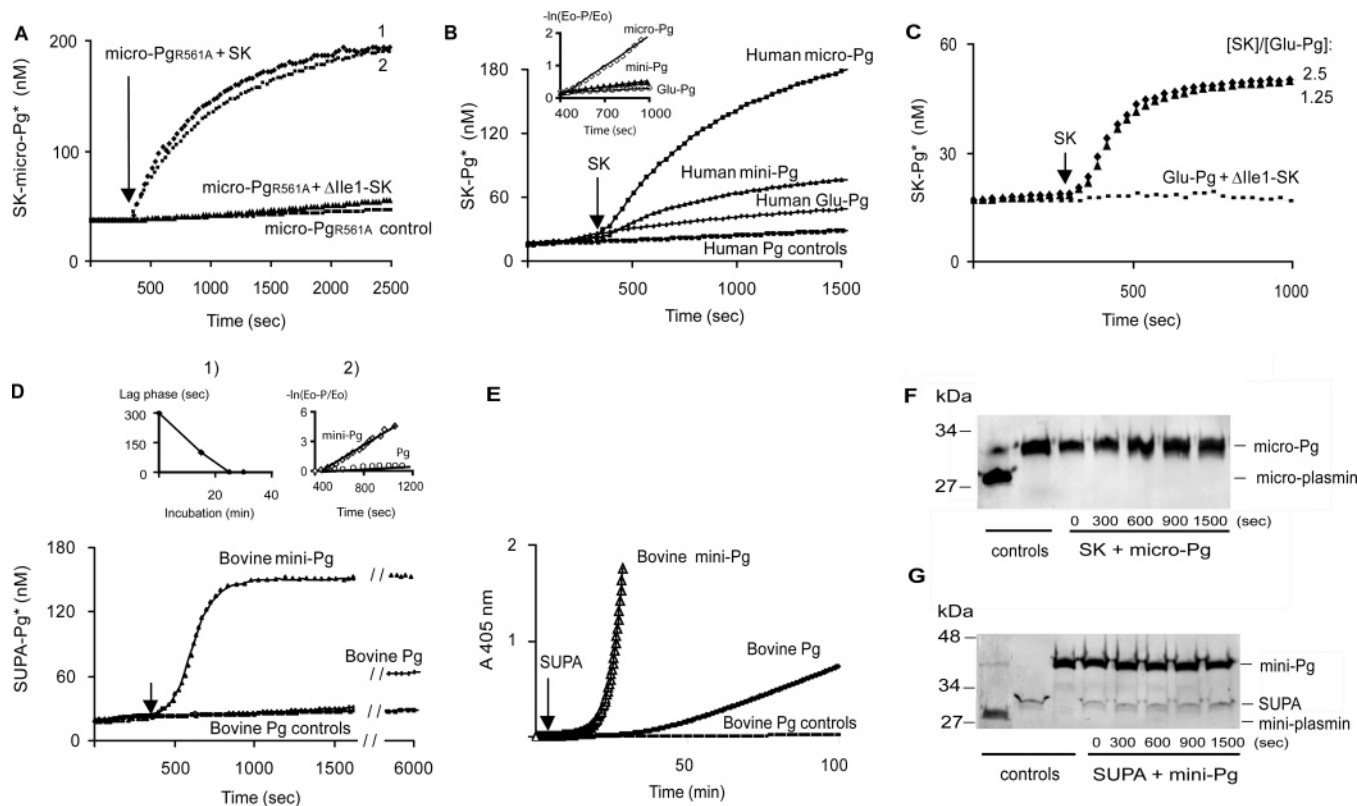
FIGURE 2: Generation of Pg* human or bovine Pg fragments by SK or SUPA. (A) Generation of SK·micro-Pg$_{R561A}$*. Micro-Pg$_{R561A}$ (400 nM) and SK (400 nM) were preincubated on ice for 0 (*1*) or 15 min (*2*), and the complex (200 nM final concentration) was added to a cuvette containing 2 μM MUGB in assay buffer. ΔIle1-SK (200 nM) was used as a control. (B) Generation of SK·Pg*. Pg, mini-Pg, or micro-Pg (200 nM active protein) was added to a cuvette containing 2 μM MUGB in assay buffer. After 300 s preincubation SK (250 nM), or buffer alone, SAK, or ΔIle1-SK (250 nM) (controls) was added to separate reactions. Inset: Representation of the reactions for micro-Pg, mini-Pg, and Glu-Pg fit to a first-order equation. (C) Generation of SK·Pg* at different [SK]/[Glu-Pg] ratios. Human Glu-Pg (200 nM) was added to a cuvette containing 2 μM MUGB in assay buffer. After 300 s preincubation SK (250 or 500 nM) or ΔIle1-SK (250 nM) was added to separate reactions. (D) Generation of SUPA·Pg*. Bovine Pg or mini-Pg (2.3 μM) and SUPA (2.5 μM) were preincubated on ice for 25 min, and the complex (200 nM) was added to a cuvette containing 2 μM MUGB in assay buffer. An arrow indicates the start of the reaction. Inset 1: Effect of time of formation of the SUPA·mini-Pg complex on the lag phase in formation of the SUPA·mini-Pg* complex. Bovine mini-Pg (2.3 μM) and SUPA (2.5 μM) were preincubated on ice for 0, 15, 25, or 30 min, and the complex (200 nM) was added to a cuvette containing 2 μM MUGB in assay buffer. Inset 2: Representation of the SUPA·mini-Pg* and SUPA·Pg* reaction fit to an equation describing a first-order reaction. (E) Amidolytic activity generated by SUPA·Pg* or SUPA·mini-Pg* was measured continuously by adding bovine Pg or mini-Pg (300 nM) and S2251 (500 μM) to assay buffer, to the final volume 300 μL, at 4 °C, followed by the addition of SUPA (300 nM) after 5 min to start the reaction. (F, G) Immunoblot analysis of active site generation reactions [(B) and (D) above] to detect plasmin contamination. Samples were taken after 0, 300, 600, 900, and 1500 s of incubation of SK·human micro-Pg* (F) and bovine SUPA·mini-Pg* (G) under the reaction conditions shown in (B) and (D) above. For comparison, bovine mini-plasmin or human micro-plasmin are shown in the left lanes. Samples were subjected to SDS−PAGE followed by immunoblotting with an anti-human Pg protease domain antibody (F) or a polyclonal anti-bovine Pg (G). Note there is mild cross-reactivity of the goat anti-bovine Pg antibody with SUPA in (G).

esis that the compact conformation of Glu-Pg inhibits the generation of the protease active site.

Influence of (DD)E Fibrin Fragment on Active Site Generation. Blood contains several other ligands that bind to the kringle domains and modulate the conformation of Glu-Pg molecule, thereby regulating the fibrinolytic system (*21, 30, 35*). For example, fibrin (DD)E fragments and Cl$^-$ ions have opposing effects in regulating Pg activation (*11, 26*). Therefore, we examined whether human or bovine fibrin (DD)E fragments also regulate the rate of nonproteolytic active site formation. Human (DD)E increased the velocity of SK·Pg* formation by 8.7 $\pm$ 0.7-fold (Figure 5A). In contrast, staphylokinase was incapable of generating an active site in the absence or presence of (DD)E as expected (Figure 5A). Neither bovine serum albumin nor maltose binding protein (not shown) affected the generation of the protease active site (Figure 5A,B). Bovine fibrin (but not human fibrin) (DD)E fragments also accelerated SUPA·bovine Pg*

formation by 2.2 $\pm$ 0.2-fold (Figure 5C). Similar results were also obtained for human fibrinogen at physiologic concentrations (3 μM, not shown). However, human (DD)E had significantly less effect on the formation of SK·micro-Pg*, as it increased the maximal velocity of this process by only 2.1 $\pm$ 0.1-fold (Figure 5 B). These observations indicate that the (DD)E fragment accelerates the formation of SK·Pg* via an interaction with the kringle domains.

Effect of SK Domains on Active Site Generation. The previous experiments indicated that the kringle domains modulate the conversion of SK·Pg to SK·Pg* by controlling the conformation of Glu-Pg. This process may be regulated by interactions between SK domains and the kringles. Therefore, we examined whether isolated SK α-, SK β-, and SK γ-domains, which are incapable of the active site generation by themselves, can accelerate the formation of SK·Glu-Pg*. The isolated domains markedly accelerated the formation of SK·Pg*, increasing the maximal velocity of this
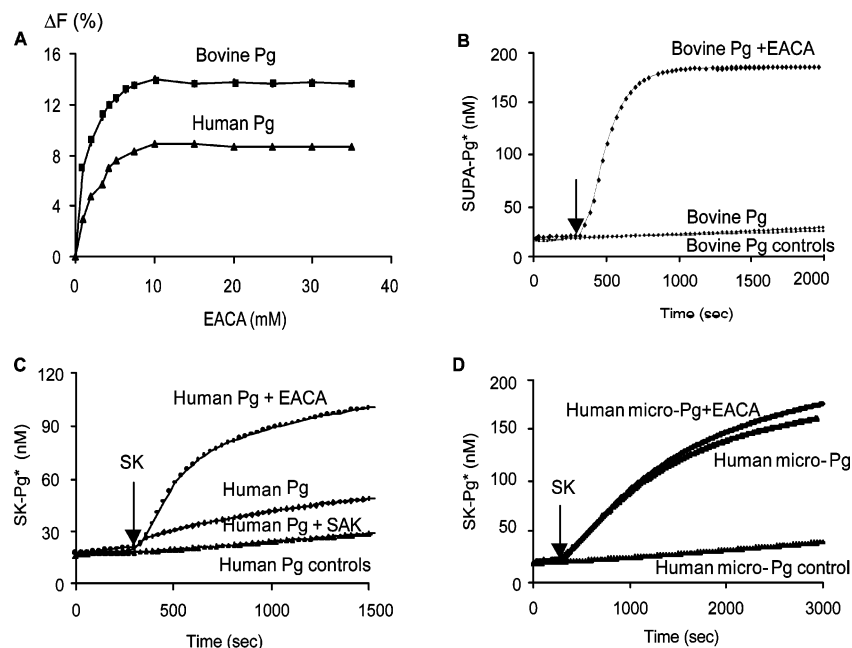
FIGURE 3: EACA alters the conformations of bovine and human Pg and the rate of Pg* formation. (A) Relative change in the intrinsic fluorescence (ΔF, %) of bovine and human Pg mediated by EACA. Pg (50 nM) was added to a quartz cuvette containing Tris buffer (50 mM Tris-HCl, 100 mM NaCl, 20% glycerol, pH 7.4). The intrinsic fluorescence was monitored until a stable background was achieved. Then EACA was added sequentially, and the change in fluorescence was monitored. (B−D) Influence of EACA on SK·Pg*, SK·micro-Pg*, or SUPA·Pg* generation. (B) Bovine Pg (200 nM) was preincubated on ice for 30 min with SUPA (250 nM) in the presence or absence of 10 mM EACA in filtered assay buffer and moved to a cuvette. MUGB (2 μM) was added to the cuvettes to start separate reactions as indicated. Human Glu-Pg (C) or human micro-Pg (D) (200 nM) was added to a cuvette containing 2 μM MUGB and 10 mM EACA in filtered assay buffer. After 5 min incubation, SK, SAK (250 nM), or buffer alone (control) was added to separate reactions as indicated.
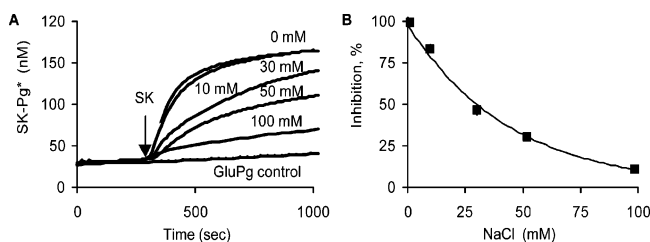

FIGURE 4: Influence of Cl$^-$ ions on SK·Pg* generation. Human Pg (200 nM) was added to a cuvette containing 2 μM MUGB and various amounts of NaCl (0−100 mM) in filtered 10 mM Hepes buffer, pH 7.4 at 25 °C. After 5 min preincubation SK (250 nM) or buffer alone (control) was added to separate reactions. (A) The development of fluorescence was monitored continuously with excitation at 365 nm and emission at 445 nm. (B) The dose−response inhibition of Cl ions on active site formation was determined by replotting the data in (A) above as a function of [NaCl].

process by 3.5−5.5-fold (Figure 6) while bovine serum albumin and maltose binding protein (not shown) had no effect. Given that SK domains bind to kringles with affinities that are 2−3 logs lower than the binding of SK to Pg (*52*), they [like DD(E)] may exert their effects via a conformational change in Pg which increases the rate of formation of SK·Glu-Pg*, rather than by promoting the initial formation of the SK·Glu-Pg complex.

Activation of Bovine or Human Pg, Mini-Pg, and Micro-Pg by SK or SUPA. Initial experiments (Figure 1B, C) suggested that SK·Pg* formation was an important determinant of plasmin formation in plasma. If this is true, then kringle domains should have a significant effect on Pg activation, i.e., the conversion of substrate Pg to plasmin. Consistent with this hypothesis, we found that Pg activation

rates directly paralleled the rate of SK·Pg* generation: micro-Pg activation was much faster than Glu-Pg activation (Figure 7B). The activation of Glu-Pg was accelerated by EACA (10 mM) (Figure 7B), which also enhanced the velocity of SK·Pg* generation (Figure 3C). However, EACA (10 mM) did not affect the activation of micro-Pg by SK (Figure 7B, inset). In a similar fashion, the activation of bovine mini-Pg by SUPA was faster than the activation of the bovine Pg molecule (Figure 7A), which was consistent with the faster generation of SUPA·mini-Pg* (Figure 2D,E). The activation of bovine Pg by SUPA was also markedly accelerated by EACA (Figure 7A), which paralleled the enhanced rate of SUPA·Pg* formation induced by EACA (Figure 3B).

DISCUSSION

Among Pg activators in current therapeutic use, SK is infamous for generating plasmin in human plasma in the absence of fibrin. This property has been attributed solely to the fact that the SK activator complex does not require fibrin as a cofactor for efficient Pg activation. However, the present experiments provide the first evidence that the rate of SK·Pg* formation is a major determinant of the plasmin formation in plasma. In turn, the rate of SK·Pg* or SUPA·Pg* formation is regulated by kringles and by kringle interactions with particular ligands. Indeed, kringle domains significantly inhibit the formation of the SK·Pg* or SUPA·Pg* moiety. SK·Pg* generation is faster in Pg fragments that completely lack kringles (micro-Pg), slower in mini-Pg, which lacks kringles 1−4, and slowest in Glu-Pg which contains all five kringles. A similar inhibitory effect of kringles is seen for the generation of bovine Pg* by SUPA.
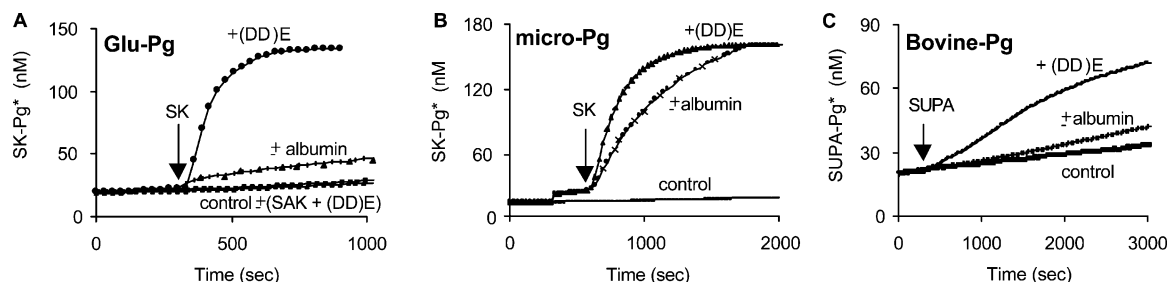
FIGURE 5: Influence of fibrin (DD)E fragments on SK·Pg* and SUPA·Pg* generation. Human or bovine (DD)E (3 μM) or bovine serum albumin (3 μM, a control) was preincubated for 20 min in assay buffer containing 2 μM MUGB. Then (A) human Glu-Pg, (B) human micro-Pg, or (C) bovine Pg (all 200 nM) was added to the cuvette. After an additional 5 min preincubation, SK, SUPA, SAK (250 nM), or assay buffer alone (control) was added as indicated.
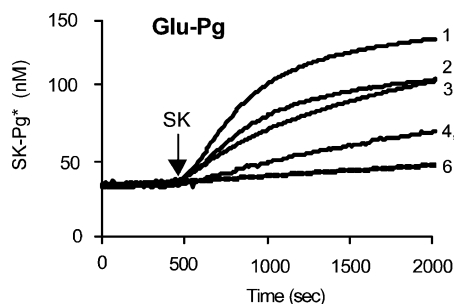

FIGURE 6: Influence of SK domains on SK·Pg* generation. Single SK domains (500 nM), (1) SK γ-domain, (2) SK β-domain, (3) SK α-domain, (4) bovine serum albumin (3 μM), or (5) buffer, were preincubated for 20 min in assay buffer containing 2 μM MUGB; then human Glu-Pg (200 nM) was added to the cuvette. After an additional 5 min preincubation, SK (250 nM) or assay buffer alone [control (6)] was added to the separate reaction.
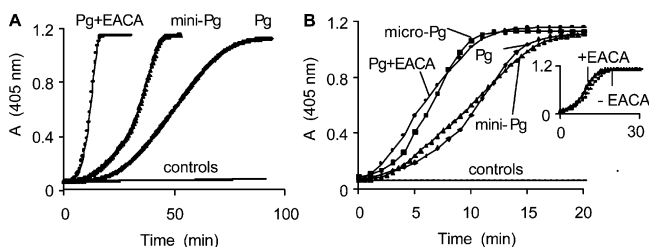

FIGURE 7: Pg activation by SUPA or SK. (A) Bovine Pg or mini-Pg (300 nM) was activated by SUPA (20 nM) or nothing (control) in assay buffer at 37 °C in the presence or absence of EACA (10 mM). (B) Human Pg, mini-Pg, and micro-Pg (300 nM) were activated by SK (20 nM) or nothing (control) in assay buffer at 37 °C in the presence or absence of EACA (10 mM). The production of plasmin was monitored using S2251 substrate (500 μM). Inset: Activation of human micro-Pg (300 nM) by SK (20 nM) in the presence or absence of EACA (10 mM).

The inhibitory effects of kringles appear to be mediated through lysine binding site interactions. SK·Pg* formation is significantly faster in nonphysiologic solutions lacking chloride ions, where lysine binding interactions are ablated and Glu-Pg is unfolded (*11, 26, 27*). Similarly, in solutions with physiologic concentrations of Cl⁻, SK·Pg* and SUPA· Pg* generation is accelerated by ligands such as EACA that interact with kringle lysine binding sites to diminish intramolecular interactions and unfold the Pg molecule. Nonproteolytic activation is also promoted by physiologic kringle ligands such as (DD)E fibrin fragments and fibrinogen. Taken together, these results indicate that nonproteolytic activation of Pg by the SK-like activators is regulated through the ligands that modulate kringle interactions.

In serum or in solutions containing physiologic concentrations of Cl ion there is evidence that kringle interactions keep

Glu-Pg in a compact, tightly folded form which is relatively resistant to SK and tissue Pg activator (*11, 40*). This may represent a safeguard mechanism to prevent uncontrolled plasmin generation (*17, 25, 29−31, 34, 35, 40, 59*). The lysine residue(s) in the NH$_2$-terminal peptide of Glu-Pg interact(s) with kringle 5, and the lysine residue(s) in kringle 3 interact(s) with kringle 4 to maintain Glu-Pg in a tight or closed conformation (*25, 30, 31, 34, 59*). In blood, Pg binds to cells and the fibrin surface through its lysine binding sites and converts to an extended "open" conformation that is activated more rapidly to form plasmin (*7, 60*). In physiologic solutions in vitro, EACA, at concentrations of $\geq$10 mM, overcomes intramolecular interactions among kringles and induces a full unfolding of the Pg molecule (*31*). The binding of SK also unfolds the Glu-Pg molecule (*45*), which suggests that one or more intermolecular interactions between kringle domain(s) and SK domain(s) modify the conformation of Glu-Pg to facilitate the process of nonproteolytic activation. Thus kringle interactions may regulate nonproteolytic active site formation by altering Pg conformation.

However, kringles may also regulate nonproteolytic activation through mechanisms independent of their effect on the conformation of Glu-Pg. Indeed, the interaction of the SK β-domain with Pg appears to be mediated almost exclusively through interaction with Pg kringles, because in the crystal structure of the SK·microplasmin (which lacks kringles) there are relatively few intermolecular contacts between the SK β-domain and the protease domain (*6*). Recent studies have shown that isolated kringles bind well to isolated SK domains (*45*) but more weakly to the protease domain. Kringles appear to contribute significantly to the binding interactions that occur between SK and Pg in the activator complex (*18*). Thus the first interactions of SK with Glu-Pg may be through the kringle domains as suggested by previous studies (*18, 44, 52, 61*). The initial binding of SK to the kringles may unfold Glu-Pg to permit subsequent interactions between SK and the protease domain. Only then may the activator pocket of Pg become accessible for interactions with Ile1 of the N-terminus of SK, which triggers nonproteolytic Pg active site formation and sparks the Pg activation process (*1, 2*). Additional interactions between the γ-domain of SK and the protease domain may act to accelerate the process of nonproteolytic activation (*5, 20*).

The kringle domains also play a crucial role in regulating the ability of SK to efficiently catalyze Pg activation. It has been established that the kringle-less SK·micro-plasmin complex is a significantly more efficient Pg activator than SK·plasmin (*18, 50, 62*). Conversely, Pg variants lacking

kringles are activated significantly less efficiently than Pg substrates containing five kringles (*50, 63*). At the same time, the role of fibrin and other physiologic kringle ligands in regulating nonproteolytic activation of kringle-containing Pgs has not been fully appreciated, perhaps because SK has been thought to be a fibrin(ogen)-independent Pg activator (*64*). However, the present experiments show that fibrinogen accelerates the formation of SK·Pg* which activates substrate Pg to plasmin in the blood. Similarly, fibrin enhances the formation of an active SK·Pg* complex, thereby accelerating Pg activation at the site of a thrombus. Kringle ligands appear to have an even more dramatic effect on Pg activation by SUPA given the marked acceleration in active site generation produced by EACA (Figure 7A). In addition to accelerating the process of nonproteolytic active site generation (described in this report), previous studies have established that fibrin-(ogen) can increase the rate of Pg activation by enhancing the catalytic efficiency of the active SK·P(g) complex (*26, 65−69*). The fact that fibrin (DD)E fragments accelerated the rate of nonproteolytic active site formation in human micro-Pg by SK also suggests that interactions of fibrin-(ogen) and other ligands with the protease domain of the enzyme may also have a physiologic role in modulating the fibrinolytic system. Thus there are now abundant data that kringle interactions with physiologic ligands such as fibrin-(ogen) play a central role in modulating key steps in Pg activation by the SK-like agents, as well as other Pg activators.

REFERENCES

1. Wang, S., Reed, G. L., and Hedstrom, L. (1999) Deletion of Ile1 changes the mechanism of streptokinase: evidence for the molecular sexuality hypothesis, *Biochemistry 38*, 5232−5240.
2. Wang, S., Reed, G. L., and Hedstrom, L. (2000) Zymogen activation in the streptokinase-plasminogen complex. Ile1 is required for the formation of a functional active site, *Eur. J. Biochem. 267*, 3994−4001.
3. Leigh, J. A. (1993) Activation of bovine plasminogen by *Streptococcus uberis*, *FEMS Microbiol. Lett. 114*, 67−71.
4. Johnsen, L. B., Rasmussen, L. K., Petersen, T. E., Etzerodt, M., and Fedosov, S. N. (2000) Kinetic and structural characterization of a two-domain streptokinase: dissection of domain functionality, *Biochemistry 39*, 6440−6448.
5. Sazonova, I. Y., Houng, A. K., Chowdhry, S. A., Robinson, B. R., Hedstrom, L., and Reed, G. L. (2001) The mechanism of a bacterial plasminogen activator intermediate between streptokinase and staphylokinase, *J. Biol. Chem. 276*, 12609−12613.
6. Wang, X., Lin, X., Loy, J. A., Tang, J., and Zhang, X. C. (1998) Crystal structure of the catalytic domain of human plasmin complexed with streptokinase, *Science 281*, 1662−1665.
7. Parry, M. A., Zhang, X. C., and Bode, I. (2000) Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues, *Trends Biochem. Sci. 25*, 53−59.
8. Lijnen, H. R., and Collen, D. (1995) Mechanisms of physiological fibrinolysis, *Bailliere's Clin. Haematol. 8*, 277−290.
9. Reddy, K. N., and Markus, G. (1972) Mechanism of activation of human plasminogen by streptokinase. Presence of active center in streptokinase-plasminogen complex, *J. Biol. Chem. 247*, 1683−1691.
10. McClintock, D. K., and Bell, P. H. (1971) The mechanism of activation of human plasminogen by streptokinase, *Biochem. Biophys. Res. Commun. 43*, 694−702.
11. Chibber, B. A., Radek, J. T., Morris, J. P., and Castellino, F. J. (1986) Rapid formation of an anion-sensitive active site in stoichiometric complexes of streptokinase and human [Glu1]-plasminogen, *Proc. Natl. Acad. Sci. U.S.A. 83*, 1237−1241.
12. Torr, S. R., Nachowiak, D. A., Fujii, S., and Sobel, B. E. (1992) "Plasminogen steal" and clot lysis, *J. Am. Coll. Cardiol. 19*, 1085−1090.
13. Onundarson, P. T., Haraldsson, H. M., Bergmann, L., Francis, C. W., and Marder, V. J. (1993) Plasminogen depletion during streptokinase treatment or two-chain urokinase incubation correlates with decreased clot lysability ex vivo and in vitro, *Thromb. Haemostasis 70*, 998−1004.
14. Reed, G. L., Houng, A. K., Liu, L., Parhami-Seren, B., Matsueda, L. H., Wang, S., and Hedstrom, L. (1999) A catalytic switch and the conversion of streptokinase to a fibrin-targeted plasminogen activator, *Proc. Natl. Acad. Sci. U.S.A. 96*, 8879−8883.
15. Mundada, L. V., Prorok, M., DeFord, M. E., Figuera, M., Castellino, F. J., and Fay, W. P. (2003) Structure-function analysis of the streptokinase amino terminus (residues 1−59), *J. Biol. Chem. 278*, 24421−24427.
16. Ward, P. N., F. T., Rosey, E. L., Abu-Median, A. B., Lincoln, R. A., and Leigh, J. A. (2004) Complex interactions between bovine plasminogen and streptococcal plasminogen activator PauA, *J. Mol. Biol. 342*, 1101−1114.
17. Castellino, F. J., and McCance, S. G. (1997) The kringle domains of human plasminogen, *Ciba Found. Symp. 212*, 46−60.
18. Lin, L. F., Houng, A., and Reed, G. L. (2000) Epsilon amino caproic acid inhibits streptokinase-plasminogen activator complex formation and substrate binding through kringle-dependent mechanisms, *Biochemistry 39*, 4740−4745.
19. Young, K. C., Shi, G. Y., Wu, D. H., Chang, L. C., Chang, B. I., Ou, C. P., and Wu, H. L. (1998) Plasminogen activation by streptokinase via a unique mechanism, *J. Biol. Chem. 273*, 3110−3116.
20. Loy, J. A., Lin, X., Schenone, M., Castellino, F. J., Zhang, X. C., and Tang, J. (2001) Domain interactions between streptokinase and human plasminogen, *Biochemistry 40*, 14686−14695.
21. Wiman, B., Boman, L., and Collen, D. (1978) On the kinetics of the reaction between human antiplasmin and a low-molecular-weight form of plasmin, *Eur. J. Biochem. 87*, 143−146.
22. Lerch, P. G., Rickli, E. E., Lergier, W., and Gillessen, D. (1980) Localization of individual lysine-binding regions in human plasminogen and investigations on their complex-forming properties, *Eur. J. Biochem. 107*, 7−13.
23. Vali, Z., and Patthy, L. (1982) Location of the intermediate and high affinity omega-aminocarboxylic acid-binding sites in human plasminogen, *J. Biol. Chem. 257*, 2104−2110.
24. Lucas, M. A., Straight, D. L., Fretto, L. J., and McKee, P. A. (1983) The effects of fibrinogen and its cleavage products on the kinetics of plasminogen activation by urokinase and subsequent plasmin activity, *J. Biol. Chem. 258*, 12171−12177.
25. Banyai, L., and Patthy, L. (1984) Importance of intramolecular interactions in the control of the fibrin affinity and activation of human plasminogen, *J. Biol. Chem. 259*, 6466−6471.
26. Chibber, B. A., and Castellino, F. J. (1986) Regulation of the streptokinase-mediated activation of human plasminogen by fibrinogen and chloride ions, *J. Biol. Chem. 261*, 5289−5295.
27. Urano, T., Chibber, B. A., and Castellino, F. J. (1987) The reciprocal effects of epsilon-aminohexanoic acid and chloride ion on the activation of human [Glu1]plasminogen by human urokinase, *Proc. Natl. Acad. Sci. U.S.A. 84*, 4031−4034.
28. Thewes, T., Constantine, K., Byeon, I. J., and Llinas, M. (1990) Ligand interactions with the kringle 5 domain of plasminogen. A study by ^{1}H NMR spectroscopy, *J. Biol. Chem. 265*, 3906−3915.
29. Ramakrishnan, V., Patthy, L., and Mangel, W. F. (1991) Conformation of Lys-plasminogen and the kringle 1-3 fragment of plasminogen analyzed by small-angle neutron scattering, *Biochemistry 30*, 3963−3969.
30. Christensen, U., and Molgaard, L. (1992) Positive co-operative binding at two weak lysine-binding sites governs the Glu-plasminogen conformational change, *Biochem. J. 285*, 419−425.
31. Marshall, J. M., Brown, A. J., and Ponting, C. P. (1994) Conformational studies of human plasminogen and plasminogen fragments: evidence for a novel third conformation of plasminogen, *Biochemistry 33*, 3599−3606.
32. Mangel, W. F., Lin, B. H., and Ramakrishnan, V. (1990) Characterization of an extremely large, ligand-induced conformational change in plasminogen, *Science 248*, 69−73.
33. McCance, S. G., and Castellino, F. J. (1995) Contributions of individual kringle domains toward maintenance of the chloride-induced tight conformation of human glutamic acid-1 plasminogen, *Biochemistry 34*, 9581−9586.
34. Cockell, C. S., Marshall, J. M., Dawson, K. M., Cederholm-Williams, S. A., and Ponting, C. P. (1998) Evidence that the conformation of unliganded human plasminogen is maintained via

an intramolecular interaction between the lysine-binding site of kringle 5 and the N-terminal peptide, *Biochem. J. 333*, 99−105.

35. Jones, A. L., Hulett, M. D., Altin, J. G., Hogg, P., and Parish, C. R. (2004) Plasminogen is tethered with high affinity to the cell surface by the plasma protein, histidine-rich glycoprotein, *J. Biol. Chem. 279*, 38267−38276.

36. Boxrud, P. D., and Bock, P. E. (2004) Coupling of conformational and proteolytic activation in the kinetic mechanism of plasminogen activation by streptokinase, *J. Biol. Chem. 279*, 36642−36649.

37. Boxrud, P. D., Verhamme, I. M., and Bock, P. E. (2004) Resolution of conformational activation in the kinetic mechanism of plasminogen activation by streptokinase, *J. Biol. Chem. 279*, 36633−36641.

38. Bender, M. L., Kezdy, F. J., and Wedler, F. C. (1967) Alpha-chymotrypsin: enzyme concentration and kinetics, *J. Chem. Educ. 44*, 84−88.

39. Chase, T., Jr., and Shaw, E. (1969) Comparison of the esterase activities of trypsin, plasmin, and thrombin on guanidinobenzoate esters. Titration of the enzymes, *Biochemistry 8*, 2212−2224.

40. Gaffney, P. J., Urano, T., de Serrano, V. S., Mahmoud-Alexandroni, M., Metzger, A. R., and Castellino, F. J. (1988) Roles for chloride ion and fibrinogen in the activation of [Glu1]plasminogen in human plasma, *Proc. Natl. Acad. Sci. U.S.A. 85*, 3595−3598.

41. Deutsch, D. G., and Mertz, E. T. (1970) Plasminogen: purification from human plasma by affinity chromatography, *Science 170*, 1095−1096.

42. Moroz, L. A. (1981) Mini-plasminogen: a mechanism for leukocyte modulation of plasminogen activation by urokinase, *Blood 58*, 97−104.

43. Olexa, S. A., and Budzynski, A. Z. (1979) Binding phenomena of isolated unique plasmic degradation products of human cross-linked fibrin, *J. Biol. Chem. 254*, 4925−4932.

44. Reed, G. L., Lin, L. F., Parhami, S. B., and Kussie, P. (1995) Identification of a plasminogen binding region in streptokinase that is necessary for the creation of a functional streptokinase-plasminogen activator complex, *Biochemistry 34*, 10266−10271.

45. Sazonova, I. Y., Robinson, B. R., Gladysheva, I. P., Castellino, F. J., and Reed, G. L. (2004) Alpha domain deletion converts streptokinase into a fibrin-dependent plasminogen activator through mechanisms akin to staphylokinase and tissue plasminogen activator, *J. Biol. Chem. 279*, 24994−25001.

46. Reed, G. L. D., Matsueda, G. R., and Haber, E. (1990) Synergistic fibrinolysis: combined effects of plasminogen activators and an antibody that inhibits alpha 2-antiplasmin, *Proc. Natl. Acad. Sci. U.S.A. 87*, 1114−1118.

47. Friberger, P., Knos, M., Gustavsson, S., Aurell, L., and Claeson, G. (1978) Methods for determination of plasmin, antiplasmin and plasminogen by means of substrate S-2251, *Haemostasis 7*, 138−145.

48. Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S., and Elmore, D. T. (1973) Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration, *Biochem. J. 131*, 107−117.

49. Lin, L. F., Oeun, S., Houng, A., and Reed, G. L. (1996) Mutation of lysines in a plasminogen binding region of streptokinase identifies residues important for generating a functional activator complex, *Biochemistry 35*, 16879−16885.

50. Gladysheva, I. P., Sazonova, I. Y., Chowdhry, S. A., Liu, L., Turner, R. B., and Reed, G. L. (2002) Chimerism reveals a role for the streptokinase beta-domain in nonproteolytic active site formation, substrate, and inhibitor interactions, *J. Biol. Chem. 277*, 26846−26851.

51. Collen, D. (1980) On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture, *Thromb. Haemostasis 43*, 77−89.

52. Conejero-Lara, F., Parrado, J., Azuaga, A. I., Dobson, C. M., and Ponting, C. P. (1998) Analysis of the interactions between streptokinase domains and human plasminogen, *Protein Sci. 7*, 2190−2199.

53. Alkjaersig, N., Fletcher, A. P., and S., S. (1959) ε-Aminocaproic acid: an inhibitor of plasminogen activation, *J. Biol. Chem. 234*, 832−837.

54. Iwamoto, M., Abiko, Y., and Shimizu, M. (1968) Plasminogen-plasmin system. 3. Kinetics of plasminogen activation and inhibition of plasminogen-plasmin system by some synthetic inhibitors, *J. Biochem. (Tokyo) 64*, 759−767.

55. Menhart, N., Hoover, G. J., McCance, S. G., and Castellino, F. J. (1995) Roles of individual kringle domains in the functioning of positive and negative effectors of human plasminogen activation, *Biochemistry 34*, 1482−1488.

56. Gladysheva, I. P., Turner, R. B., Sazonova, I. Y., Liu, L., and Reed, G. L. (2003) Co-evolutionary patterns in plasminogen activation, *Proc. Natl. Acad. Sci. U.S.A. 100*, 9168−9172.

57. Barlow, G. H., Summaria, L., and Robbins, K. C. (1984) Hydrodynamic studies on the streptokinase complexes of human plasminogen, Val442-plasminogen, plasmin, and the plasmin-derived light (B) chain, *Biochemistry 23*, 2384−2387.

58. Boxrud, P. D., and Bock, P. E. (2000) Streptokinase binds preferentially to the extended conformation of plasminogen through lysine binding site and catalytic domain interactions, *Biochemistry 39*, 13974−13981.

59. Padmanabhan, K., Wu, T. P., Ravichandran, K. G., and Tulinsky, A. (1994) Kringle-kringle interactions in multimer kringle structures, *Protein Sci. 3*, 898−910.

60. Borza, D. B., and Morgan, W. T. (1997) Acceleration of plasminogen activation by tissue plasminogen activator on surface-bound histidine-proline-rich glycoprotein, *J. Biol. Chem. 272*, 5718−5726.

61. Rodriguez, P., Fuentes, P., Barro, M., Alvarez, J. G., Munoz, E., Collen, D., and Lijnen, H. R. (1995) Structural domains of streptokinase involved in the interaction with plasminogen, *Eur. J. Biochem. 229*, 83−90.

62. Shi, G. Y., Change, B. I., Wu, D. H., Ha, Y. M., and Wu, H. L. (1990) Activation of human and bovine plasminogens by the microplasmin and streptokinase complex, *Thromb. Res. 58*, 317−329.

63. Wohl, R. C., Summaria, L., and Robbins, K. C. (1980) Kinetics of activation of human plasminogen by different activator species at pH 7.4 and 37 °C, *J. Biol. Chem. 255*, 2005−2013.

64. Marder, V. J., and Sherry, S. (1988) Thrombolytic therapy: current status (1), *N. Engl. J. Med. 318*, 1512−1520.

65. Camiolo, S. M., Markus, G., Evers, J. L., and Hobika, G. H. (1980) Augmentation of streptokinase activator activity by fibrinogen or fibrin, *Thromb. Res. 17*, 697−706.

66. English, P. D., Smith, R. A., Dupe, R. J., Green, J., and Hibbs, M. J. (1981) The thrombolytic activity of streptokinase in the rabbit, *Thromb. Haemostasis 46*, 535−537.

67. Strickland, D. K., Morris, J. P., and Castellino, F. J. (1982) Enhancement of the streptokinase-catalyzed activation of human plasminogen by human fibrinogen and its plasminolysis products, *Biochemistry 21*, 721−728.

68. Fears, R., Hibbs, M. J., and Smith, R. A. (1985) Kinetic studies on the interaction of streptokinase and other plasminogen activators with plasminogen and fibrin, *Biochem. J. 229*, 555−558.

69. Takada, Y., and Takada, A. (1989) Evidence for the formation of a trimolecular complex between streptokinase, plasminogen and fibrinogen, *Thromb. Res. 53*, 409−415.