

## Kinetic and Structural Characterization of a Two-domain Streptokinase: Dissection of Domain Functionality<sup>†</sup>

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*Received September 27, 1999; Revised Manuscript Received February 8, 2000*

**ABSTRACT:** The mammalian protease plasminogen can be activated by bacterial activators, the three-domain ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) streptokinases and the one-domain ( $\alpha$ ) staphylokinases. These activators act as plasmin(ogen) cofactors, and the resulting complexes initiate proteolytic activity of host plasminogen which facilitates bacterial colonization of the host organism. We have investigated the kinetic mechanism of the plasminogen activation mediated by a novel two-domain ( $\alpha$ ,  $\beta$ ) streptokinase isolated from *Streptococcus uberis* (Sk<sup>U</sup>) with specificity toward bovine plasminogen. The interaction between Sk<sup>U</sup> and plasminogen occurred in two steps: (1) rapid association of the proteins and (2) slow transition to the active complex Sk<sup>U</sup>–PgA. The complex Sk<sup>U</sup>–PgA converted plasminogen to plasmin with the following parameters:  $K_m \leq 1.5 \mu\text{M}$  and  $k_{\text{cat}} = 0.55 \text{ s}^{-1}$ . The ability of proteolytic fragments of Sk<sup>U</sup> to activate plasminogen was investigated. Only two C-terminal segments (97–261 and 123–261), which both contain the  $\beta$ -domain (126–261), were shown to be active. They initiated plasminogen activation in complex with plasmin, but not with plasminogen, and thereby exhibited functional similarity to the staphylokinase. The fusion protein His<sub>6</sub>–Sk<sup>U</sup> (i.e., Sk<sup>U</sup> with a small N-terminal tag) acted exclusively in complex with plasmin as well. These observations demonstrate that (1) the N-terminal  $\alpha$ -domain, including a native N-terminus, was necessary for “virgin” activation of the associated plasminogen in the Sk<sup>U</sup>–PgA complex and (2) the C-terminal  $\beta$ -domain of Sk<sup>U</sup> is important for recognition of the substrate in the Sk<sup>U</sup>–PgA complex.

Plasmin (Pn)<sup>1</sup> is a potent mammalian proteinase which has several important physiological functions such as blood clot dissolution (fibrinolysis), cellular migration, and cancer metastasis. The zymogen of Pn, called plasminogen (Pg), is synthesized in the liver and found in blood and extracellular fluids. Two specific proteins, tissue-type and urokinase-type Pg activators (tPA and uPA, respectively), convert Pg into Pn. In addition to these two physiological Pg activators, several microbial pathogens synthesize their own activators with specificity toward host Pg (1, 2). Generation of Pn assists the pathogenic microorganism in proteolysis of fibrin and other proteins in the extracellular matrix. This, in turn,

facilitates the bacterial penetration of normal tissue barriers and enables bacterial colonization of deep tissue sites (1). The best known activators are the streptokinases (Sk<sup>E</sup>) isolated from *S. equisimilis* and the staphylokinases (Sak) from *S. aureus*. These proteins have been studied extensively because of their high fibrinolytic potential and specificity toward human Pg. Sk<sup>E</sup> is currently used as a drug in thrombolytic therapy.

The activators from different sources are characterized by different features of their Pg activation mechanisms. The physiological activators (tPA and uPA) convert Pg to Pn by hydrolysis of the Arg<sub>561</sub>–Val<sub>562</sub> peptide bond. Subsequent formation of a salt bridge between the newly formed N-terminal amino group of Val<sub>562</sub> and the carboxyl group of Asp<sub>740</sub> induces the active conformation of the enzyme. This scheme is analogous to the well-studied mechanism employed by other chymotrypsin-like proteases. The bacterial activators Sk<sup>E</sup> and Sak are not proteases but act as cofactors in Pg–Sk<sup>E</sup> or Pn–Sak complexes that dramatically alter the specificity of the associated plasmin(ogen) from that of a nonplasminogen activator into that of a highly efficient one. Binding of Sk<sup>E</sup> to Pg changes the conformation of the serine proteinase domain in the associated Pg. This causes exposure of the active site of the proteinase without its proteolytic cleavage, thereby providing the Sk<sup>E</sup>–Pg complex with “virgin” enzymatic activity supplemented by affinity toward free Pg (3). Sak cannot induce the corresponding transformation, and the complex Sak–Pg is proteolytically inactive.

<sup>†</sup> This work is part of the FØTEK program supported by the Danish Dairy Research Foundation (Danish Dairy Board) and the Danish Government.

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<sup>1</sup> Abbreviations: [Asp<sup>1</sup>]plasminogen, native form of bovine plasminogen with an aspartic acid at the N-terminus;  $\alpha_2$ -AP,  $\alpha_2$ -antiplasmin; bPg, bovine plasminogen; Fb, fibrinogen; [Glu<sup>1</sup>]plasminogen, native form of human plasminogen with a glutamic acid at the N-terminus; hPg, human plasminogen; His<sub>6</sub>–Sk<sup>U</sup>, the fusion streptokinase with an N-terminal His-tag and factor Xa site; Pg, plasminogen; Pn, plasmin; Sak, staphylokinase from *Staphylococcus aureus*; SE-HPLC, size-exclusion HPLC.; Sk<sup>E</sup>, traditional streptokinase isolated from various human isolates of hemolytic *Streptococcus equisimilis* and *Streptococcus pyogenes* strains; Sk<sup>U</sup>, streptokinase from *Streptococcus uberis*; Sk<sup>U</sup>–Pg, inactive complex; Sk<sup>U</sup>–PgA, active complex; Sk<sup>N</sup>, a proteolytic fragment of Sk<sup>U</sup>; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

Only complex formation between Sak and the active protease Pn produces a construction which combines the proteolytic ability with the specificity toward Pg (4, 5).

The identification of the regions in Sk<sup>E</sup> and Sak responsible for virgins activation of Pg as well as activation of substrate Pg has become a controversial issue. The structures of the ternary microplasmin–Sak–microplasmin complex and the microplasmin–Sk<sup>E</sup> complex were recently solved (6, 7). They revealed a striking similarity of the single  $\alpha$ -domain of Sak to the N-terminal  $\alpha$ -domain and the central  $\beta$ -domain of Sk<sup>E</sup>, but less so to the C-terminal  $\gamma$ -domain of Sk<sup>E</sup>. The structure of the ternary enzyme–substrate complex, microplasmin–Sak–microplasmin, revealed detailed structural information on the protein–protein interactions resulting in activator complex formation and substrate recognition (6). However, the mechanism whereby Sk<sup>E</sup> induces virgins activity in the Sk<sup>E</sup>–Pg complex was not evident from the microplasmin–Sk<sup>E</sup> structure (7). Still, interactions between the  $\gamma$ -domain of Sk<sup>E</sup> and the autolysis loop of Pg were proposed to trigger the activation (7, 8). In contrast, arguments based on functional studies of a Sk<sup>E</sup> mutant lacking the N-terminal Ile residue have claimed that Sk<sup>E</sup> activates Pg according to the molecular sexuality hypothesis, where the N-terminal Ile of Sk<sup>E</sup> mimics the function of the plasmin Val<sub>562</sub> N-terminus generated by activation cleavage with tPA or uPA (9). Also on the basis of the crystal structure of the microplasmin–Sk<sup>E</sup> complex, the  $\alpha$ -domain was proposed to enhance substrate recognition of the activator complex. Furthermore, other functional studies have indicated that several regions in both the  $\alpha$ - and  $\beta$ -domains are involved in substrate recognition, and regions in the  $\beta$ -domain have likewise been suggested to be essential for activator complex formation (10–12).

We have recently described the purification and cloning of a two-domain ( $\alpha$ ,  $\beta$ ) streptokinase from *S. uberis* (Sk<sup>U</sup>) with specificity toward bovine Pg (13). In this paper, we characterize the Pg activation mediated by recombinant Sk<sup>U</sup>, the Sk<sup>U</sup> fragments, and the N-terminal-modified fusion protein. Kinetic data indicated that Sk<sup>U</sup> (despite the absence of the  $\gamma$ -domain) could induce virgins enzymatic activity in bovine Pg. The isolated C-terminal  $\beta$ -domain of Sk<sup>U</sup> (as well as Sk<sup>U</sup> with the N-terminal tag) was incapable of virgins activation of Pg, but formed an activation complex with Pn.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Super Taq polymerase was from HT Biotechnology (Cambridge, United Kingdom), nucleotide triphosphates were from Amersham Pharmacia Biotech (Uppsala, Sweden), oligonucleotides were from DNA technology (Aarhus, Denmark), and all other enzymes were from New England Biolabs (Hitchin, United Kingdom). PCR was performed in a Hybaid ABACUS thermal cycler (Middlesex, United Kingdom). Sequencing was performed with a dye terminator cycle sequencing kit from Perkin-Elmer (Foster City, CA). Sequencing, ligation, transformation of *Escherichia coli*, DNA preparation, PCR, and other DNA-modifying processes were carried out according to the manufacturers' recommendations or standard laboratory procedures. Yeast extract and tryptone were from DIFCO, and rifampicin was from ICN Biomedicals (Aurora, OH). BL21 cells were purchased from Stratagene (La Jolla, CA). Factor Xa protease

immobilized on the column was from Protein Engineering Technology (Aarhus, Denmark), and trypsin, elastase, and chymotrypsin were from Worthington (Freehold, NJ). S-2251 (H-D-Val-Leu-Lys-pNA) was obtained from Chromogenix (Mölnådal, Sweden). Bovine [Asp<sup>1</sup>]plasminogen (bPg), human [Glu<sup>1</sup>]plasminogen (hPg), human  $\alpha_2$ -antiplasmin, and streptokinase secreted from a  $\beta$ -hemolytic streptococcus (Sk<sup>E</sup>) were from American Diagnostica (Greenwich, CT). Bovine plasmin was from Boehringer Mannheim (Mannheim, Germany). All plasminogens, free of 6-aminohexanoic acid, used in the species-specific experiment were obtained from Sigma (St. Louis, MO.). Bovine CNBr–fibrinogen fragments were prepared as described previously (14). The plasminogen used for zymography was prepared from intravenously collected bovine plasma and purified by chromatography on lysine–Sephacrose followed by gel filtration in the presence of the serine protease inhibitors phenylmethylsulfonyl fluoride, benzamidine, and soybean trypsin inhibitor obtained from Sigma. Pg-depleted bovine fibrinogen (Fb) was purchased from Enzyme Research Laboratories (South Bend, IN).

**Expression of Sk<sup>U</sup>.** The full-length native Sk<sup>U</sup> sequence was amplified by PCR using a previously isolated genomic fragment as template (13) and the corresponding primers: the forward primer 5'-CGCGGATCCATCCAGGGTCG-TATAACCGTTATGATTCCG-3' containing a *Bam*HI restriction site and a factor Xa-encoding cleavage site (IQGR<sup>1</sup>); the reverse primer 5'-CGCGGATCCTTAAGGTT-TATAACTTTTTTTGG-3' also containing a *Bam*HI restriction site. The amplified fragment was digested with *Bam*HI and subcloned into the expression vector pT7H6gs (15) derived from the plasmid pBR328. The resulting expression plasmid pT7H6gs–Sk<sup>U</sup> had the ability to express the native Sk<sup>U</sup> with an N-terminal His-tag followed by a factor Xa cleavage site. The identity of Sk<sup>U</sup> was confirmed by sequencing on both strands. Expression was performed in 1 L portions of 2 xYT medium supplemented with 0.1 g of ampicillin, 10 mM MgSO<sub>4</sub>, and 15 mL of a culture of pT7H6gs–Sk<sup>U</sup> recombinant BL21 cells. Expression was induced at OD<sub>600</sub> = 0.8 by adding bacteriophage  $\lambda$ -CE-6 (16). After 50 min of incubation, rifampicin (100 mg/L) was added, and expression was continued for 3.5 h followed by harvesting at 3000g for 5 min.

**Purification and Cleavage with Factor Xa.** Cells from 1 L of the medium were resuspended in 20 mL of 100 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 2 mM EDTA, and 20 mM dithiothreitol. Afterward, 100 mL of phenol, pH 8.0, was added, and the suspension was hand-shaken and left at room temperature for 10 min. The lysed cells were then sonicated for 15 min and centrifuged (15 min, 10000g), and the phenol phase was collected. Proteins were precipitated from the phenol phase by 2.5 vol of 96% ethanol, centrifuged (20 min, 3000g), and dissolved in 10 mL of 6 M guanidinium–HCl, 50 mM Tris–HCl, pH 8.0. The solubilized mixture was centrifuged (15 min, 10000g), and the recombinant protein was adsorbed on Ni–NTA resin by batch incubation. The matrix with bound His<sub>6</sub>–Sk<sup>U</sup> was packed in a 40 mL column, washed with 1 vol of 6 M guanidinium–HCl, 50 mM Tris–HCl, pH 8.0, followed by several volumes of 8 M urea, 0.5 M NaCl, 50 mM Tris–HCl, pH 8.0, until A<sub>280</sub> became stable. Afterward, the protein was refolded in the column by changing the buffer to 150 mM NaCl, 50 mM Tris–HCl, pH 8.0. Elution was performed in the same buffer supple-

mented with 10 mM EDTA. The eluate was concentrated on an Amicon-10 membrane, and the N-terminal His<sub>6</sub> peptide was cleaved in a column with the coupled factor Xa at 4 °C until approximately half of the sites were cleaved. The native streptokinase was separated from the remaining fusion protein by passage through Ni-NTA resin in 500 mM NaCl, 50 mM Tris-HCl, pH 8.0. The recombinant protein was eluted in the unbound fraction and dialyzed against 20 mM NH<sub>4</sub>HCO<sub>3</sub>. The sample was freeze-dried, resolubilized in 50 mM Tris-HCl, pH 7.5, at a concentration of 3 mg/mL, and stored at -20 °C.

**Limited Proteolysis.** Elastase, trypsin, and chymotrypsin were incubated with Sk<sup>U</sup> at a ratio of 1:400 (w/w) at 37 °C. Samples were taken at time intervals and analyzed by SDS-PAGE. Sk<sup>U</sup> fragments were purified by reversed-phase HPLC on a Vydac C<sub>18</sub> column (Hesperia, CA). Identification of the fragments was carried out by sequencing on an ABI 477/120A protein sequencer (PE Applied Biosystems, Foster City, CA).

**Determination of Molecular Masses by SE-HPLC.** Gel filtration of Sk<sup>U</sup> and Sk<sup>E</sup> was performed on a SMART HPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with a Superdex 75 PC 3.2/30 column equilibrated with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl at room temperature. The following mixture of proteins was used for calibration: cytochrome *c* (12.4 kDa), β-lactoglobulin (35 kDa), bovine serum albumin (67 kDa), and IgG (160 kDa). Formation of Sk<sup>U</sup>-bPg and Sk<sup>E</sup>-hPg complexes was carried out at approximately equimolar protein concentrations (2 μM) at room temperature for 15 min in the running buffer supplemented with 0.05 mM protease inhibitor *p*-nitrophenyl *p*-guanidinobenzoate (17). The complexes were detected by gel filtration on a Superose 12 PC 3.2/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.2 M sodium phosphate, pH 6.8, 20 mM 6-aminohexanoic acid.

**Zymography.** Zymography was performed as described previously by Andreassen et al. (18) using standard SDS-PAGE conditions and agarose gels containing bovine fibrinogen (1.7 mg/mL) and bovine Pg (8 μg/mL). Fibrin polymerization was initiated by human thrombin (0.03 U/mL).

**Plasminogen Activation Assays.** All experiments (both here and below) have been performed in the following medium: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.15% Tween 20, and 0.5 mM S-2251. Determination of *v<sub>a</sub>* and *k<sub>s</sub>* (see the Kinetic Analysis of Plasminogen Activation) was carried out at 37 °C in a total volume of 0.3 mL with final concentrations of Sk<sup>U</sup> at 1 nM and Pg at 0.02–3.0 μM. The reaction was initiated by addition of 0.1 mL of Sk<sup>U</sup> + S-2251 to 0.2 mL of Pg and monitored at 405 nm in a Camspec M350 double beam UV-Vis spectrophotometer (Camspec Ltd., United Kingdom). Other assays were performed in microtiter plates at room temperature, and changes of absorbency were followed in a Bio-Tek EL 340 Biokinetics Reader (Bio-Tek Instruments Inc., Winooski, VT).

**Fibrinogen or CNBr-Fibrinogen Stimulation.** Pg (0.1 μM) in the reaction mixture was preincubated for 5 min with various amounts of Fb or CNBr-Fb, and the reactions were initiated by addition of 0.1 nM Sk<sup>U</sup>.

**Plasminogen Activation Mediated by the Sk<sup>U</sup> Fragment (97–261).** In one setup, consumption of S-2251 was followed in time at 0.1 μM Pg and various amounts of Sk<sup>U,97–261</sup>

(0.1–0.8 μM) at 20 °C. In another experiment, the reaction was monitored at fixed amounts of Sk<sup>U,97–261</sup> (0.25 μM) and Pg (0.1 μM) on the background of different concentrations of Pn (0–2500 pM). In assays where suppression of endogenous Pn was required, Pg was preincubated with α<sub>2</sub>-AP at the ratio 100:1 for 3 h before initiation of the reaction.

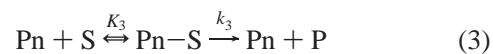
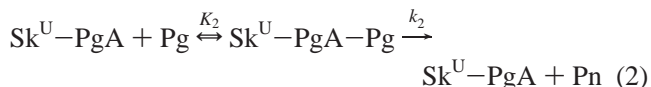
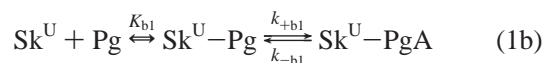
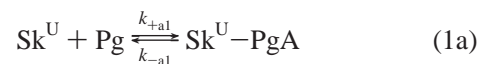
**Inhibition of the Sk<sup>U</sup>-Pg Complex Activity by α<sub>2</sub>-Antiplasmin or Sk<sup>U</sup> Fragments.** Suppression of the amidase activity of Sk<sup>U</sup>-Pg toward S-2251 was monitored when 0.1 μM Pg and 0.3 μM α<sub>2</sub>-AP were preincubated for 1 min in the reaction mixture (20 °C) followed by addition of 0.2 μM Sk<sup>U</sup>. Inhibition of the proteinase activity of Sk<sup>U</sup>-Pg toward free Pg was measured when the mixture 10 nM Sk<sup>U</sup> + 10 nM Pg + 30 nM α<sub>2</sub>-AP was preincubated for 1 h and then added to the reaction medium with 0.1 μM Pg (final concentration of Sk<sup>U</sup> 0.5 nM). Inhibition of Sk<sup>U</sup>-mediated Pg activation by Sk<sup>U</sup> fragments was registered after 1 min of incubation of 0.1 μM Pg with 0.2 μM fragment in the reaction medium followed by addition of 1 nM Sk<sup>U</sup>.

**Plasminogen Activation Mediated by His<sub>6</sub>-Sk<sup>U</sup>.** Catalytic (1 nM) or saturating amounts (0.2 μM) of His<sub>6</sub>-Sk<sup>U</sup> were added to the reaction medium with 0.1 μM Pg, and the proteinase or amidase activity was followed in time. In assays where suppression of endogenous Pn was required, Pg was preincubated for 3 h with α<sub>2</sub>-AP at a ratio of 100:1, and the reaction was initiated by addition of His<sub>6</sub>-Sk<sup>U</sup>.

**Specificity of Sk<sup>U</sup> toward Plasminogen from Different Species.** In one setup, 0.01 U of Pg from bovine, equine, human, porcine, rabbit, and caprine species was mixed with 1 nM Sk<sup>U</sup> in the reaction medium. In another setup, a mixture of 0.1 μM Sk<sup>U</sup> and 0.1 μM bPg was incubated for 5 min followed by addition of 1 nM complex to the reaction medium with 0.01 U of Pg from different species.

**Kinetic Analysis of Plasminogen Activation Mediated by Sk<sup>U</sup>.** The process was followed in the coupled reaction assay described by the following reactions:

Model 1



Reactions 1a and 1b represent two alternative mechanisms of interaction between Sk<sup>U</sup> and Pg: (1a) with direct formation of the catalytically active complex Sk<sup>U</sup>-PgA; (1b) with formation of an inactive intermediate, Sk<sup>U</sup>-Pg, followed by transition to the active complex Sk<sup>U</sup>-PgA. *k<sub>+a1</sub>* and *k<sub>-a1</sub>* are the rate constants of Sk<sup>U</sup>-PgA formation according to reaction 1a, *K<sub>b1</sub>* is the dissociation constant of Sk<sup>U</sup>-Pg according to reaction 1b, *k<sub>+b1</sub>* and *k<sub>-b1</sub>* are the rate constants of transition between Sk<sup>U</sup>-Pg and Sk<sup>U</sup>-PgA according to reaction 1b, *K<sub>2</sub>*, *K<sub>3</sub>*, and *K<sub>4</sub>* are the Michaelis constants (*K<sub>m</sub>*) of the corresponding enzymes, and *k<sub>2</sub>*, and *k<sub>3</sub>*, *k<sub>4</sub>* are the



catalytic constants ( $k_{\text{cat}}$ ) of the corresponding enzymes. The values of  $K_4$  and  $k_4$  are expected to be close to  $K_3$  and  $k_3$  as follows from the data of Whol et al. (19). The process was followed at different Pg concentrations by liberation of *p*-nitroaniline (P),  $\epsilon_{405} = 10\,000\text{ M}^{-1}\text{ cm}^{-1}$ .

We have made one assumption to simplify the mathematical expression of the above model. Generation of the product (P) was exclusively ascribed to reaction 3 because  $[\text{Pn}] \gg [\text{Sk}^{\text{U}}\text{-PgA}]$  almost from the very beginning of the process. At the same time, the derived equations considered the participation of  $\text{Sk}^{\text{U}}\text{-PgA}$  in complex formation with S (reaction 4) which, to some extent, affects the apparent affinity of the activating complex to Pg (reaction 2). The time of the reaction was relatively short (4 min), and the appearance of the  $\text{Sk}^{\text{U}}\text{-Pn}$  complex, as well as proteolytic degradation of  $\text{Sk}^{\text{U}}$ , was ignored.

A general description of an analogous model has been given in our previous publication (14), and only the key steps of the analysis are mentioned here. The raw data ( $[\text{P}]$  versus time) were replotted in the coordinates  $y$  versus  $t^2$  (see below eqs I and II below), as such a transformation is necessary for an accurate analysis of the prestationary kinetics (14). The known parameters:  $[\text{S}]_0 = 500\ \mu\text{M}$ ,  $K_3 = 200\ \mu\text{M}$ ,  $k_3 = 1000\ \text{min}^{-1}$  (20) were used for conversion of  $[\text{P}]$  values to  $y$  values by eq II. The prestationary phase (either reaction 1a or reaction 1b) would reveal itself in coordinates  $y$  versus  $t_2$  as an initial lag phase described by the observed rate coefficient  $k_*$ . The lag phase is expected to be followed by the linear steady-state phase characterized by the slope coefficient  $v_a$  (the Pg activation velocity). Both  $k_*$  and  $v_a$  can be calculated by nonlinear regression analysis of the experimental points according to (14)

$$y = y_0 + v_a t^2 - \frac{2v_a}{k_*} t + \frac{2v_a}{k_*^2} (1 - e^{-k_* t}) \quad (\text{I})$$

$$y_0 \approx 0 \text{ at } t = 0$$

$$y = 2 \frac{K_3}{k_3} \ln \left( \frac{[\text{S}]_0 e^{[P]/K_3}}{[\text{S}]_0 - [\text{P}]} \right) \quad (\text{II})$$

$$v_a = \frac{k_2 [\text{Sk}^{\text{U}}\text{-PgA}]_{\text{total}} [\text{Pg}]}{K_2 \left( 1 + \frac{[\text{S}]}{K_4} \right) + [\text{Pg}]} \quad (\text{III})$$

$$k_* = k_{+a1} [\text{Pg}] + k_{-a1} \quad (\text{IVa})$$

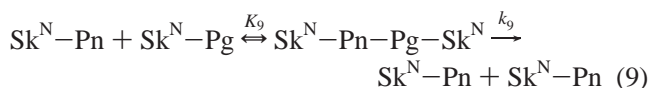
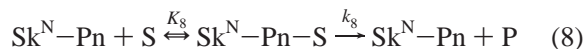
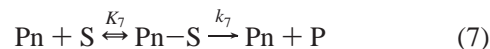
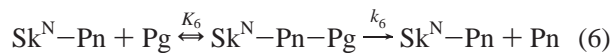
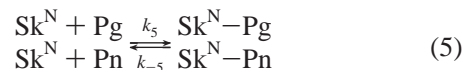
$$k_* = \frac{k_{+b1} [\text{Pg}]}{K_{b1} + [\text{Pg}]} + k_{-b1} \quad (\text{IVb})$$

The parameters of eq I ( $k_*$  and  $v_a$ ) were calculated for different Pg concentrations, which provided their dependences on  $[\text{Pg}]$ . The chart  $k_*$  versus  $[\text{Pg}]$  would be either linear (eq IVa) or hyperbolic (eq IVb), depending on the model of Pg binding to  $\text{Sk}^{\text{U}}$  (reaction 1a or 1b, respectively). The chart  $v_a$  versus  $[\text{Pg}]$  was fitted according to eq III to calculate coefficients of the  $\text{Sk}^{\text{U}}\text{-PgA}$ -catalyzed reaction:  $K_2^{\text{app}} = K_2(1 + [\text{S}]/K_4)$  and  $k_2$ .

*Kinetic Analysis of Plasminogen Activation Mediated by  $\text{Sk}^{\text{U}}$  Fragments.* The activity of the  $\text{Sk}^{\text{U}}$  fragments generated by limited proteolysis could potentially follow the mechanism

either identical to that of the full-length protein (model 1) or similar to that of staphylokinase. In the last case, the  $\text{Sk}^{\text{U}}$  fragment of *N*-residues ( $\text{Sk}^{\text{N}}$ ) binds to both Pg and Pn, but only the  $\text{Sk}^{\text{N}}\text{-Pn}$  complex possesses Pg-specific proteolytic activity. High concentration of  $\text{Sk}^{\text{N}}$  in the reaction medium introduced several new elements when compared with model 1. The process was described by a set of chemical reactions shown in the model below:

#### Model 2



Reaction 5 depicts two binding reactions with identical parameters: association of  $\text{Sk}^{\text{N}}$  with either Pg or Pn. Reaction 7 is, actually, identical to reaction 3 in model 1, except for the temperature of the reaction (20 °C), and  $K_7 = 200\ \mu\text{M}$  and  $k_7 = 400\ \text{min}^{-1}$ . The amidase activity of the  $\text{Sk}^{\text{N}}\text{-Pn}$  complex is shown in reaction 8, and  $d[\text{P}]/dt$  in this reaction has to be taken into account because of the high  $\text{Sk}^{\text{N}}$  concentration under the conditions of the experiment. One can expect  $K_8 \approx K_7$  and  $k_8 \approx k_7$ . The potential activity of  $\text{Sk}^{\text{N}}\text{-Pn}$  toward  $\text{Sk}^{\text{N}}\text{-Pg}$  (reaction 9) should be considered under conditions of  $[\text{Sk}^{\text{N}}] \gg [\text{Pg}]$ .

The designed model has no algebraic solution resulting in the equation  $[\text{P}] = f(t)$ . Instead, the appearance of P was simulated on a computer using the program Gepasi (21) with the assigned set of initial concentrations and the kinetic parameters either determined in a separate reaction (line 7) or chosen by trial and error during fitting of the simulated curves to the experimental points; see the Results.

## RESULTS

*Expression and Structural Characterization of  $\text{Sk}^{\text{U}}$  and the  $\text{Sk}^{\text{U}}\text{-Pg}$  Complex.* The recombinant  $\text{Sk}^{\text{U}}$  was expressed as a fusion protein with an N-terminal His<sub>6</sub>-tag followed by a factor Xa cleavage site. The yield of recombinant His<sub>6</sub>- $\text{Sk}^{\text{U}}$  was 30 mg/L culture after purification. Treatment with factor Xa at 37 °C at room temperature indicated a substantial nonspecific degradation of the protein. Digestion at 4 °C improved the specificity, and only minor degradation was observed under these conditions (Figure 1A).

Molecular masses of  $\text{Sk}^{\text{U}}$  and  $\text{Sk}^{\text{E}}$  (used as a reference) were estimated by gel filtration as 34 and 54 kDa, respectively, which agreed with the calculated values of 30.7 and 47 kDa (Figure 2A). The monomeric nature of  $\text{Sk}^{\text{U}}$  was confirmed by chemical cross-linking (data not shown). Formation of  $\text{Sk}^{\text{U}}\text{-bPg}$  and  $\text{Sk}^{\text{E}}\text{-hPg}$  complexes was demonstrated by SE-HPLC (Figure 2B,C). Thus, the elution peaks of the corresponding mixtures were shifted to higher molecular masses when compared with bPg/hPg and  $\text{Sk}^{\text{U}}/\text{Sk}^{\text{E}}$  (taken separately). SDS-PAGE analysis of a cross-

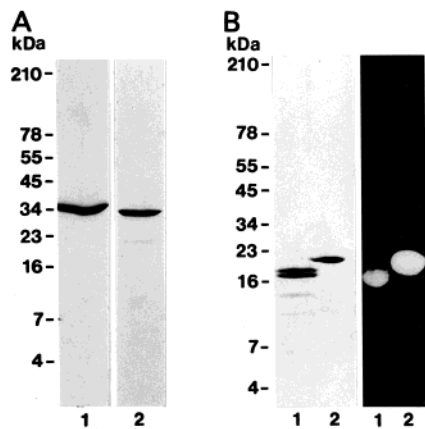


FIGURE 1: SDS-PAGE and zymography analysis of  $Sk^U$  and enzymatic derived fragments. (A) SDS-PAGE of the recombinant protein  $His_6-Sk^U$  (lane 1) and the same protein after treatment with factor Xa (lane 2). (B) SDS-PAGE (left) and zymography analysis (right) of the purified chymotryptic fragments  $Sk^{U.123-261}$  (lane 1) and  $Sk^{U.97-261}$  (lane 2). The fragments were identified by the N-terminal sequence. Molecular mass markers are indicated to the left in each panel.

linked preparation ( $Sk^U$  plus bPg) as well as native PAGE of the same mixture also indicated formation of the  $Sk^U$ -bPg complex (data not shown). We could not detect binding of  $Sk^U$  and  $Sk^E$  to the "wrong" plasminogens (human and bovine, respectively) under the experimental conditions.

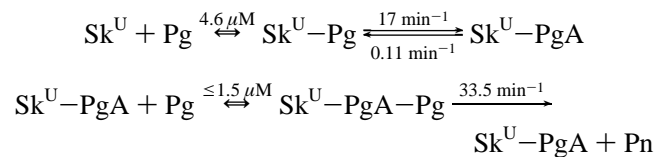
**Kinetic Characterization of the  $Sk^U$ -PgA-Catalyzed Plasminogen Activation.**  $Sk^U$  was able to react with bovine Pg and to a lower extent with equine or ovine Pg. The complex of  $Sk^U$  with bovine Pg was able to activate Pgs from all tested sources (bovine, ovine, pig, equine, rabbit, and human), and the system  $Sk^U$  plus bovine Pg was used in the following experiments. The rate coefficient ( $k_*$ ) of the prestationary phase and the activity ( $v_a$ ) of  $Sk^U$ -PgA were calculated from the charts  $y$  versus  $t^2$  (Figure 3A) at different Pg concentrations according to model 1. The appropriate fit of the chosen mechanism.

Analysis of the prestationary phase showed a Michaelis-like dependence of  $k_*$  on [Pg] (Figure 3B), which corresponded to the mechanism with an inactive intermediate (reaction 1b and eq IVb). The dissociation constant of the

inactive complex  $Sk^U$ -Pg was calculated as  $K_{b1} = 4.6 \mu M$ . Transition between the inactive intermediate  $Sk^U$ -Pg and the active complex  $Sk^U$ -PgA was described by the rate coefficients  $k_{+b1} = 17 \text{ min}^{-1}$  (Figure 3B) and  $k_{-b1} = 0.11 \text{ min}^{-1}$  (Figure 3C). The dissociation constant ( $K_d$ ) of the activation complex  $Sk^U$ -PgA is then given by the equation  $K_d = K_{b1}(k_{-b1}/k_{+b1}) = 30 \text{ nM}$ .

The Pg-activating velocity (chart  $v_a$  versus [Pg] in Figure 3D) showed a Michaelis-like dependence with  $K_m^{app} = K_2(1 + [S]/K_4) = 1.5 \mu M$  and  $V_{max} = 0.033 \mu M \text{ min}^{-1}$  ( $k_{cat} = 33 \text{ min}^{-1}$ ). The real  $K_m$  value for  $Sk^U$ -PgA-catalyzed Pg conversion (i.e.,  $K_2$ ) can be estimated from  $K_m^{app}$  as  $K_2 \approx 0.4 \mu M$  considering  $K_4 \approx K_3 = 200 \mu M$  (19, 20) and  $[S] = 500 \mu M$ .

The mechanism, comprising both prestationary and stationary phases of  $Sk^U$  action, can be summarized as follows:



Short preincubation of  $Sk^U$  and Pg ( $[Sk^U] > [Pg]$ ) abolished the lag phase (Table 1). Both amidase and proteinase activity of the  $Sk^U$ -PgA complex could be suppressed by  $\alpha_2$ -AP (Table 1) in contrast to the  $\alpha_2$ -AP-insensitive  $Sk^E$ . Restoration of the proteinase activity after a 15 min lag was observed, when a mixture of  $Sk^U + Pg + \alpha_2$ -AP was added to the reaction medium with Pg. The effect can be ascribed to gradual dissociation of the inactive  $Sk^U$ -Pg- $\alpha_2$ -AP complexes and rebinding of  $Sk^U$  to fresh Pg. The presence of Fb or CNBr-Fb fragments stimulated the activity of  $Sk^U$ , but only Fb made the initial lag phase disappear (Table 1).

**Limited Proteolysis of  $Sk^U$ .** Limited proteolysis was carried out to confirm the anticipated domain structure of  $Sk^U$  and, at the same time, generate fragments for functional analysis. Digestion with proteases generated stable N-terminal fragments of similar size, 11~120 (elastase), 12~148 (trypsin), and 10~141 (chymotrypsin), and less stable C-terminal fragments 139~261, 158~261, 158~200, and 201~261

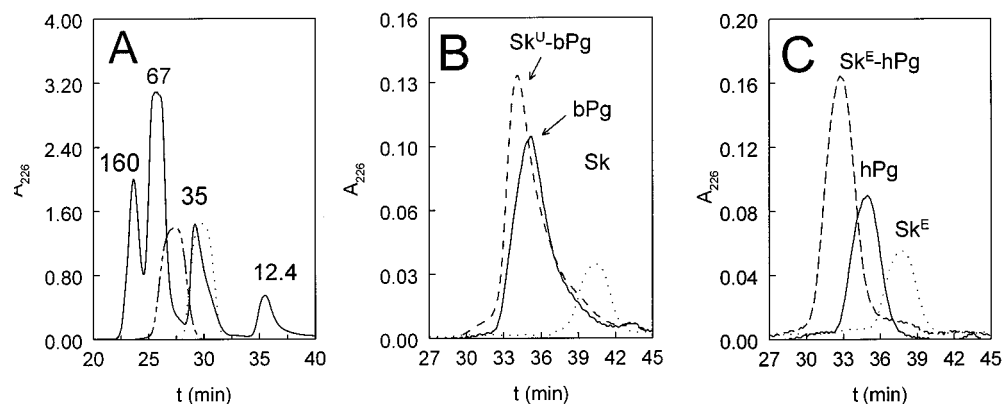


FIGURE 2: Estimation of molecular masses of the recombinant  $Sk^U$  with a normal N-terminus and the native  $Sk^E$ . Detection of  $Sk^U$ -bPg and  $Sk^E$ -hPg complexes. (A) The elution profile of the calibrating proteins is shown as a solid line, and the positions of  $Sk^U$  and  $Sk^E$  are indicated by dotted and broken lines, respectively. The molecular masses of the markers are shown in kilodaltons. (B) The elution profiles of the mixture  $Sk^U$  plus bPg (broken line), and the same proteins taken separately: bPg (solid line),  $Sk^U$  (dotted line). (C) The elution profiles of the mixture  $Sk^E$  plus hPg (broken line), and the same proteins taken separately: hPg (solid line),  $Sk^E$  (dotted line).

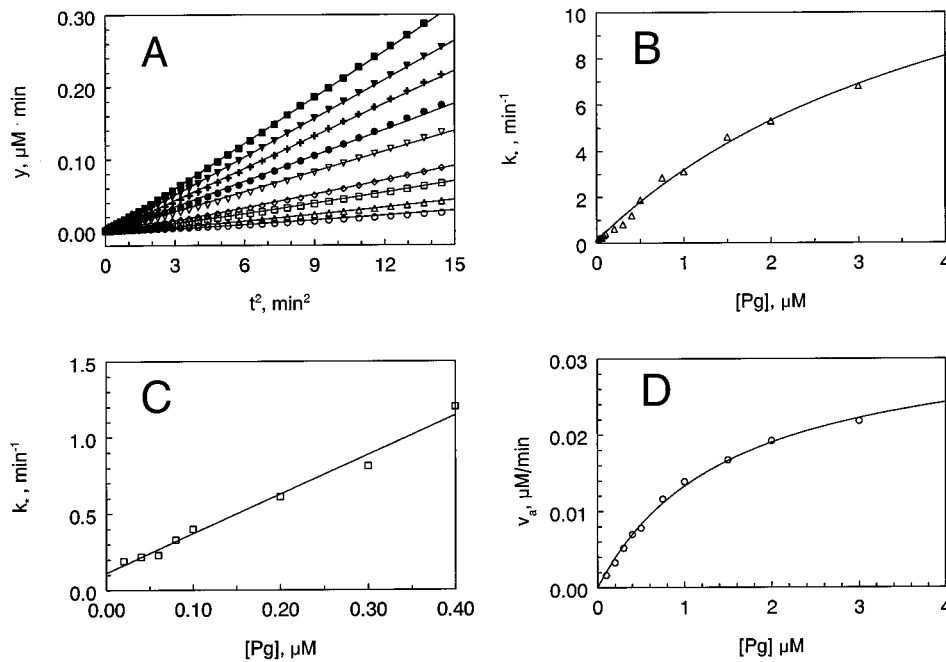


FIGURE 3:  $\text{Sk}^{\text{U}}$ -PgA-mediated plasminogen activation (37 °C). (A) The reaction at 1 nM  $\text{Sk}^{\text{U}}$  and 0.02–3.0  $\mu\text{M}$  Pg in the transformed coordinates  $y$  versus  $t^2$  (see eq II). The symbols  $\circ$ ,  $\triangle$ ,  $\square$ ,  $\diamond$ ,  $\nabla$ ,  $\bullet$ ,  $+$ ,  $\blacktriangledown$ , and  $\blacksquare$  correspond to  $[\text{Pg}]$  ( $\mu\text{M}$ ): 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.5, 2.0, and 3.0, respectively. Solid lines show the best fit according to eq I and model 1. (B) Analysis of the rate coefficient  $k_*$  of the prestationary phase. The  $k_*$  values at different concentrations of Pg were calculated from the curves in panel A. The dependence of  $k_*$  on  $[\text{Pg}]$  was fitted by eq IVb with  $K_{b1} = 4.6 \mu\text{M}$  and  $k_{+b1} = 17 \text{ min}^{-1}$ . (C) Estimation of  $k_{-b1}$  in eq IVb by linear regression analysis of  $k_*$  values at low  $[\text{Pg}]$  (0.02–0.4  $\mu\text{M}$ ),  $k_{-b1} = 0.11 \text{ min}^{-1}$ . (D) Analysis of the Pg-activating velocity ( $v_a$ ) in the stationary phase of the reaction. The  $v_a$  values were calculated from the curves in panel A. The dependence of  $v_a$  on  $[\text{Pg}]$  was fitted by eq III (Michaelis equation) with  $K_m^{\text{app}} = 1.5 \mu\text{M}$  and  $V_{\text{max}} = 0.033 \mu\text{M min}^{-1}$ .

Table 1: Influence of Different Effectors on the Amidase and Pg Activating Reactions (20 °C) Mediated by  $\text{Sk}^{\text{U}}$ <sup>a</sup>

reagents (preincubated reagents are underlined)	ratio, nM (amidase activity)	ratio, nM (Pg activation)	amidase activity		Pg activation	
			lag, min	s. slope, <sup>b</sup> %	lag, min	s. slope, %
$\text{Sk}^{\text{U}} + \text{Pg}$	200:100	1:100	5	100	5	100
$\text{Sk}^{\text{U}} + \text{Pg}$	200:100	1:100	0	100	0	100
$\text{Sk}^{\text{U}} + \text{Pg} + \alpha_2\text{-AP}$	200:100:300	na <sup>c</sup>	0	2	na	na
$\text{Pg} + \text{Sk}^{\text{U}} + \text{Pg} + \alpha_2\text{-AP}$	na	100:0.5:0.5:1.5	na	na	15	90
$\text{Sk}^{\text{U}} + \text{Pg} + \alpha_2\text{-AP}$	200:100:1	1:100:1	5	100	5	100
$\text{Sk}^{\text{U}} + \text{Pg} + \text{Fb}$	nd <sup>d</sup>	1:100:200	nd	nd	0	300
$\text{Sk}^{\text{U}} + \text{Pg} + \text{CNBr Fb}$	nd	1:100:200	nd	nd	5	600
$\text{Sk}^{\text{U}} + \text{Pg} + \text{Sk}^{\text{U},\sim 1\sim 140}$	nd	1:100:200	nd	nd	5	100
$\text{Sk}^{\text{U}} + \text{Pg} + \text{Sk}^{\text{U},\sim 140\sim 261}$	nd	1:100:200	nd	nd	5	100
$\text{Sk}^{\text{U}} + \text{Pg} + \text{Sk}^{\text{U},123\sim 261}$	nd	1:100:200	nd	nd	40	60
$\text{Sk}^{\text{U}} + \text{Pg} + \text{Sk}^{\text{U},97\sim 261}$	nd	1:100:200	nd	nd	60	30

<sup>a</sup> The amidase reaction was analyzed in the coordinates  $[\text{P}]$  (concentration of *p*-nitroaniline) versus  $t$ , and the lag time was determined as the intersection of the linear phase with the  $t$ -axis. The Pg activating reaction was analyzed in coordinates  $y$  (see eq II) versus  $t^2$ , and the lag was determined as the square root of the intersection of the linear phase with the  $t^2$ -axis. (See the Materials and Methods for further experimental details.) <sup>b</sup> s. slope = stationary slope. <sup>c</sup> na = not applicable. <sup>d</sup> nd = not determined.

(elastase) and 97–261 and 123–261 (chymotrypsin). These data are in agreement with the proposed structure of  $\text{Sk}^{\text{U}}$  (13) comprising one N-terminal domain (1–125) and one C-terminal domain (126–261) corresponding to  $\alpha$ - and  $\beta$ -domains of  $\text{Sk}^{\text{E}}$  (7, 22).

**Kinetic Characterization of the Activity of  $\text{Sk}^{\text{U}}$  Fragments.** Zymographic and kinetic analysis of several fragments generated by limited proteolysis indicated that only  $\text{Sk}^{\text{U},97\sim 261}$  and  $\text{Sk}^{\text{U},123\sim 261}$  had the ability to activate Pg (Figure 1B). None of the inactive fragments interfered with Pg activation as followed from the absence of any effect on the concomitant  $\text{Sk}^{\text{U}}$ -mediated Pg activation (Table 1). Only  $\text{Sk}^{\text{U},97\sim 261}$  and  $\text{Sk}^{\text{U},123\sim 261}$  were able to inhibit the reaction, presumably due to competition between these fragments and  $\text{Sk}^{\text{U}}$  for the

same binding site on Pg (Table 1). The inhibiting effect was expressed as a 40–60 min lag before gradual restoration of some catalytic activity. A mixture of the inactive fragments that generally reconstituted the full-length sequence of  $\text{Sk}^{\text{U}}$  had no activity.

Preliminary kinetic experiments in the coupled reaction mixture with the C-terminal fragments ( $\text{Sk}^{\text{U},97\sim 261}$  or  $\text{Sk}^{\text{U},123\sim 261}$ ) showed a qualitatively similar behavior, but with  $\text{Sk}^{\text{U},97\sim 261}$  being 2–3 times more effective. Therefore, the subsequent experiments were carried out with the more active moiety (Figure 4). As the first step, the process of Pg activation and accompanying liberation of *p*-nitroaniline was followed at different  $\text{Sk}^{\text{U},97\sim 261}$  concentrations (0–0.8  $\mu\text{M}$ ) (Figure 4A). Initial fit according to model 1 and eq I revealed an

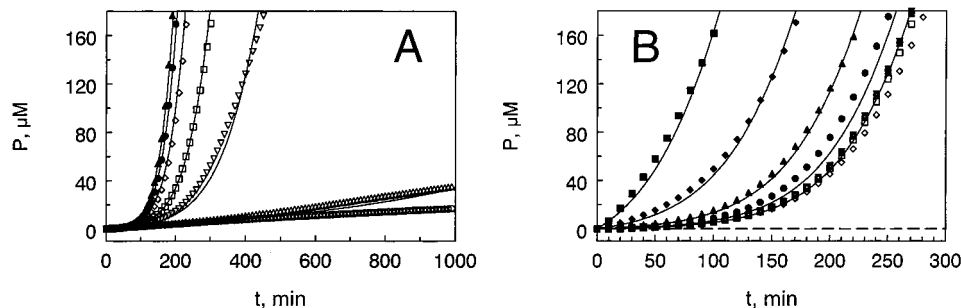


FIGURE 4:  $\text{Sk}^{\text{U},97-261}$ -Pn-mediated plasminogen activation (20 °C). Release of *p*-nitroaniline (P) was followed in time in the reaction medium containing  $\text{Sk}^{\text{U},97-261}$ , Pn (endogenous or added), and Pg. Solid lines show the best fit according to model 2. (A) The reaction was carried out with 0.1  $\mu\text{M}$  Pg (containing background Pn) and various concentrations of  $\text{Sk}^{\text{U},97-261}$ . The symbols  $\circ$ ,  $\triangle$ ,  $\nabla$ ,  $\square$ ,  $\diamond$ ,  $\bullet$ , and  $\blacksquare$  correspond to  $[\text{Sk}^{\text{U},97-261}]$  ( $\mu\text{M}$ ): 0, 0.01, 0.1, 0.2, 0.4, 0.6, and 0.8, respectively. (B) The reaction was carried out with 0.1  $\mu\text{M}$  Pg, 0.25  $\mu\text{M}$   $\text{Sk}^{\text{U},97-261}$  and various concentrations of added Pn. The symbols  $\circ$ ,  $\triangle$ ,  $\nabla$ ,  $\square$ ,  $\diamond$ ,  $\bullet$ ,  $\blacktriangle$ ,  $\blacklozenge$ , and  $\blacksquare$  correspond to the added [Pn] (pM): 0, 0.02, 0.1, 0.5, 3, 16, 80, 400, and 2000, respectively (background [Pn] = 50 pM). The dashed line corresponds to the reaction of  $\text{Sk}^{\text{U},97-261}$  with Pn-depleted preparation of Pg (Pg +  $\alpha_2$ -AP, 100:1).

unsatisfactory description of the experimental points (not shown).

An analogous experiment was carried out at constant concentrations of  $\text{Sk}^{\text{U},97-261}$  and Pg, but with different amounts of added Pn (0–2 nM) (Figure 4B). The effect became visible at [Pn] > 10 pM, which suggested that the concentration of endogenous Pn in the Pg preparation was 50–100 pM. When the activity of endogenous Pn in the Pg preparation was suppressed by preincubation with  $\alpha_2$ -AP, the fragment was incapable of Pg activation. The best fit according to model 1 provided an inadequate description of the experimental points (not shown).

The resulting simulations of the  $\text{Sk}^{\text{U},97-261}$ -catalyzed Pg activation, depicted as charts [P] versus  $t$ , were performed on the basis of model 2 (Figure 4, solid lines). The concentrations of  $\text{Sk}^{\text{U},97-261}$ , Pg, and S were assumed to be close to the initial values during the reaction time. The endogenous Pn concentration was assigned to 50 pM as estimated in the above paragraph. The reactions were presented as simple bimolecular processes with the apparent rate coefficients  $k_{\text{app}}$  chosen by trial and error except for the measured constants of Pn-catalyzed consumption of S.

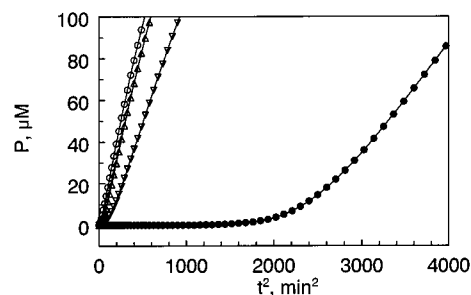
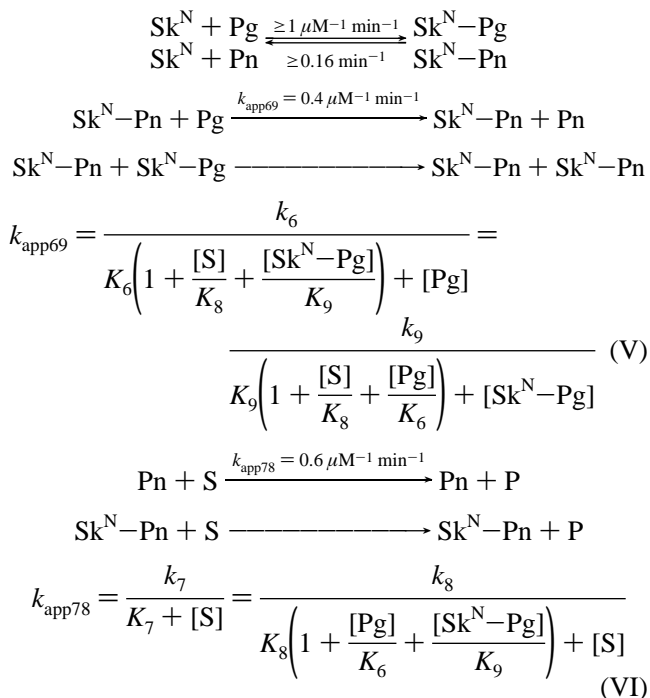


FIGURE 5:  $\text{His}_6$ - $\text{Sk}^{\text{U}}$ -mediated plasminogen activation (20 °C). The concentration of the liberated *p*-nitroaniline is depicted as a function of  $t^2$ . The reactions were carried out with 0.1  $\mu\text{M}$  Pg (containing background Pn) or a Pn-depleted preparation of Pg (Pg +  $\alpha_2$ -AP, 100:1) and 1 nM  $\text{His}_6$ - $\text{Sk}^{\text{U}}$  or 1 nM  $\text{Sk}^{\text{U}}$ . Components of the mixture: ( $\circ$ ) Pg and  $\text{Sk}^{\text{U}}$ , ( $\triangle$ ) Pg- $\alpha_2$ -AP and  $\text{Sk}^{\text{U}}$ , ( $\nabla$ ) Pg and  $\text{His}_6$ - $\text{Sk}^{\text{U}}$ , ( $\bullet$ ) Pg- $\alpha_2$ -AP and  $\text{His}_6$ - $\text{Sk}^{\text{U}}$ .

The first reaction defines equilibrium dissociation constants of the complexes  $\text{Sk}^{\text{U},97-261}$ -Pg and  $\text{Sk}^{\text{U},97-261}$ -Pn as  $K_d = 0.16 \mu\text{M}$  where any rate constants above the shown limits are suitable if they correspond to the established ratio. The individual constants of the three catalytic reactions involving  $\text{Sk}^{\text{U},97-261}$ -Pn were not determined; still, some reasonable assumptions could be drawn from the equivalence of the shown  $k_{\text{app}}$  in eqs V and VI related to the reactions 6 and 9 and 7 and 8 in model 2, respectively. Thus, the equivalence of  $K_6 = K_9$  and  $k_6 = k_9$  follows from eq V. Equal catalytic efficiencies of Pn and  $\text{Sk}^{\text{U},97-261}$ -Pn toward S testify for  $K_6, K_9 \gg [\text{Pg}]_{\text{total}} = 0.1 \mu\text{M}$ ,  $K_8 = K_7 = 200 \mu\text{M}$ , and  $k_8 = k_7 = 400 \text{min}^{-1}$  (eq VI). Under these circumstances,  $k_{\text{app}}$  of  $\text{Sk}^{\text{U},97-261}$ -Pn-catalyzed conversion of Pg (or  $\text{Sk}^{\text{U},97-261}$ -Pg) becomes equal to  $k_6/(3.5K_6) = 0.4 \mu\text{M}^{-1} \text{min}^{-1}$  and  $k_6/K_6 = 1.4 \mu\text{M}^{-1} \text{min}^{-1}$  (20 °C). The analogous parameter for the catalytic complex with the full-length activator ( $\text{Sk}^{\text{U}}$ -PgA) was estimated as  $k_2/K_2 \approx 25 \mu\text{M}^{-1} \text{min}$  (20 °C).

*Catalytic Activity of  $\text{His}_6$ - $\text{Sk}^{\text{U}}$ .* The reaction with  $\text{His}_6$ - $\text{Sk}^{\text{U}}$  was characterized by a prolonged lag phase which became particularly pronounced when the Pg preparation was depleted of endogenous Pn activity by preincubation with  $\alpha_2$ -AP at the ratio Pg: $\alpha_2$  = 100:1 (Figure 5). Gradual restoration of the activity after  $\alpha_2$ -AP treatment is likely to be caused by some small amounts of active Pn, sufficient to ignite the “chain reaction” after a substantial delay. In agreement with this observation, we found that addition of Pn shortened the lag phase (data not shown). The presence of  $\alpha_2$ -AP had no effect on the reaction mediated by  $\text{Sk}^{\text{U}}$  under



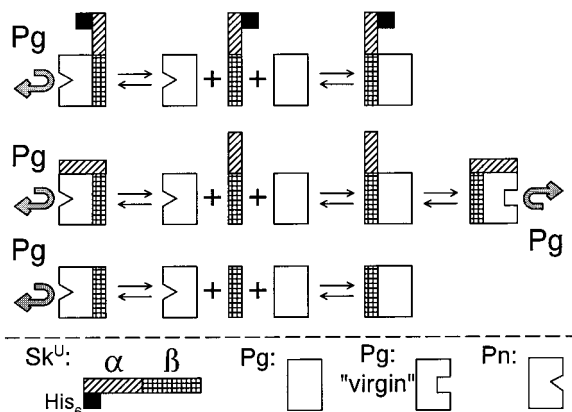


FIGURE 6: Schematic presentation of the Pg activating reaction mediated by native  $SK^U$ ,  $His_6-SK^U$ , and  $SK^{U,97-261}$ . Only native  $SK^U$  is able to catalyze the conformational change in the serine protease domain of Pg, resulting in the active  $SK^U-Pg$  activator complex.  $His_6-SK^U$  and  $SK^{U,97-261}$  only form activator complexes with Pn. The twisted arrows indicate the ability of the formed activator complexes to perform Pg activation.

analogous conditions. These data argue for the N-terminal-modified protein ( $His_6-SK^U$ ) being active only in complex with Pn, while the zymogen complex  $His_6-SK^U-Pg$  either is completely inert or has a very low activity.

## DISCUSSION

In the present work we have investigated Pg-activating reactions catalyzed by the full-length recombinant  $SK^U$  ( $\alpha,\beta$ -domains), the C-terminal fragment  $SK^{U,97-261}$  ( $\beta$ -domain), or the protein with an N-terminal tag,  $His_6-SK^U$  (modified  $\alpha,\beta$ ).

$SK^U$ -induced activation of bovine Pg was characterized by the presence of a prestationary lag phase. Kinetic analysis revealed a two-step mechanism of binding between  $SK^U$  and Pg. Formation of an initially inactive  $SK^U-Pg$  complex with the dissociation constant  $K_{b1} = 4.6 \mu M$  was followed by transition to the catalytic complex  $SK^U-PgA$ :  $k_{+b1} = 17 \text{ min}^{-1}$ ,  $k_{-b1} = 0.11 \text{ min}^{-1}$  (see model 1). A similar two-step mechanism has also been observed for  $SK^E$ -mediated Pg activation, where interaction between  $SK^E$  and hPg showed a temperature-dependent transition from an inactive complex to the active one (23). The dissociation constant of the catalytically potent complex  $SK^U-PgA$  was equal to  $K_d = K_{b1}(k_{+b1}/k_{-b1}) = 30 \text{ nM}$ , and this value is higher than the constants reported for  $SK^E$  and Sak bound to Pg/Pn:  $K_d = 1-2 \text{ nM}$  (24, 25). Lower affinity of  $SK^U$  toward Pg in comparison with  $SK^E$  may potentially be ascribed to lack of the  $\gamma$ -domain in the  $SK^U$  protein (13).

The parameters of the  $SK^U-PgA$ -catalyzed reaction ( $Pg \rightarrow Pn$ ,  $37^\circ C$ ) were estimated as  $K_m \leq 1.5 \mu M$  (presumably,  $K_m \approx 0.4 \mu M$  because  $K_4 \approx K_3$ , model 1),  $k_{cat} = 33 \text{ min}^{-1}$ , and  $k_{cat}/K_m \approx 80 \mu M^{-1} \text{ min}^{-1}$  ( $k_{cat}/K_m \approx 25 \mu M^{-1} \text{ min}^{-1}$  at  $20^\circ C$ ). The earlier obtained parameters of the  $SK^E$ -mediated reaction (19, 26, 27) varied in the range  $K_m = 0.12-0.4 \mu M$  and  $k_{cat} = 7-140 \text{ min}^{-1}$ . The same constants of Sak-Pn catalysis have been reported to be  $K_m = 2.4-6.1 \mu M$  and  $k_{cat} = 6-110 \text{ min}^{-1}$  (28, 29). The absence of the  $\gamma$ -domain in  $SK^U$  did not seem to alter the catalytic properties of its active complex with Pg when compared with the three- $\alpha,\beta,\gamma$ -domain  $SK^E$ .

$SK^U$  showed specificity toward bovine, equine, and ovine Pg in agreement with earlier observations (30), and the  $SK^U-$

Pg complex was able to activate all tested Pgs. This indicates that  $SK^U$  is highly specific during formation of the activator complex, but relatively nonspecific in activation of Pgs from different sources. Analogous observations have been made for different Pg complexes with  $SK^E$  (31). The excess of the physiological inhibitor of Pn ( $\alpha_2$ -AP) inactivated the  $SK^U-Pg$  complex (this work) as well as Sak-Pn (32). On the contrary, no effect of  $\alpha_2$ -AP on the activity of  $SK^E-Pg$  has been reported (33).

A two-domain structure of  $SK^U$  comprising an  $\alpha$ -domain from 1 to 125 (1-146 in  $SK^E$ ) and a  $\beta$ -domain from 126 to 261 (147-283 in  $SK^E$ ) was suggested from the data of limited proteolysis (this work) and from alignment with  $SK^E$  (13). When the proteolytic fragments of  $SK^U$  (containing either the N-terminal  $\alpha$ -domain or the C-terminal  $\beta$ -domain) were tested for potential activity, only two C-terminal fragments,  $SK^{U,97-261}$  and  $SK^{U,123-261}$ , revealed Pg-activating ability of similar character.

The catalytic process mediated by the more potent fragment  $SK^{U,97-261}$  was investigated in detail. The absence of 96 N-terminal residues did not inflict critical damage on either complex formation of  $SK^{U,97-261}-Pn/Pg$  or catalytic properties of  $SK^{U,97-261}-Pn$  when compared to  $SK^U-PgA$ : (1) the affinity of the fragment to Pn/Pg decreased 5 times ( $K_d = 160 \text{ nM}$ ); (2) the catalytic coefficient of the complex ( $k_{cat}/K_m = 1.4 \mu M^{-1} \text{ min}^{-1}$  at  $20^\circ C$ ) decreased 15-20 times, which can be interpreted, for instance, as 4-fold increased  $K_m$  supplemented by 4-fold decreased  $k_{cat}$ . The crucial effect of the N-terminal truncation was, however, expressed as abolished virgin activity of the  $SK^{U,97-261}-Pg$  complex. The fragment was completely incapable of rendering Pg activation in the absence of some trace amounts of Pn, which was proven by use of an  $\alpha_2$ -AP-treated preparation of Pg. On the other hand, even negligible amounts of  $SK^{U,97-261}-Pn$  stimulated not only conversion of Pg to Pn but also the proteolytic activation of the inert complex  $SK^{U,97-261}-Pg$  as was shown by the effective reaction at  $[SK^{U,97-261}] \gg [Pg]$ . The global fit of the experimental points according to the discussed mechanism (model 2) gave satisfactory results (Figure 4).

The possible participation of the native N-terminus of  $SK^U$  in the process of virgin activation of Pg was assessed by analyzing the kinetic behavior of the fusion protein  $His_6-SK^U$  (full-length  $SK^U$  modified by a small N-terminal tag). This  $\alpha$ -domain modified activator was unable to produce the active complex with Pg as noticed when the Pn-depleted preparation of Pg was used (Figure 5). At the same time,  $SK^U$  without tag-activated  $\alpha_2$ -AP treated Pg as good as the original preparation. These facts testify for an important role of the  $\alpha$ -domain and particularly its N-terminus during the virgin activation of Pg in accordance with the molecular sexuality hypothesis (34). Taken together, these data indicate that the induction of virgin activity in Pg is a consequence of a constraint, imposed on the serine protease molecule by the two-domain  $SK^U$  or the three-domain  $SK^E$ , that facilitates the transition of the protease domain into an active configuration upon insertion of the streptokinase N-terminus.

The performed work allowed the differential roles of the two domains in  $SK^U$  to be elucidated. The N-terminal domain of  $SK^U$  (corresponding to the  $\alpha$ -domain of  $SK^E$ ) was essential for transformation of the inactive complex  $SK^U-Pg$  to the active  $SK^U-PgA$ . The C-terminal domain of  $SK^U$  (corre-



sponding to the  $\beta$ -domain of Sk<sup>E</sup>) rendered binding to Pg/Pn and participated in the catalytic function of the Sk<sup>U</sup>-PgA complex. A similar function of the  $\beta$ -domain was suggested from experiments with  $\beta$ -domain-mutated Sk<sup>E</sup> (24). These findings are schematically outlined in Figure 6. In the top reaction, the inability/ability of His<sub>6</sub>-Sk<sup>U</sup> to form an activator complex with Pg/Pn is illustrated. Likewise, in the middle reaction, the ability of native Sk<sup>U</sup> to form an activator complex with Pg as well as Pn is shown. Finally, in the bottom reaction, the inability/ability of the  $\beta$ -domain to form an activator complex with Pg/Pn is indicated.

The absence of the  $\gamma$ -domain in Sk<sup>U</sup> (as compared to the three-domain Sk<sup>E</sup>) might be a reason the Sk<sup>U</sup>-PgA complex is less firm and sensitive to inhibition by  $\alpha_2$ -AP. Also the interactions of the  $\gamma$ -domain of Sk<sup>E</sup> with Pg could explain the faster generation of an active Sk<sup>E</sup>-Pg complex (no lag phase in Pn generation was observed upon addition of Sk<sup>E</sup> to human Pg, authors' unpublished observation) as compared to the Sk<sup>U</sup>-Pg complex.

#### ACKNOWLEDGMENT

We thank Margit S. Rasmussen for excellent technical assistance.

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BI9922502