Functional Roles of Streptokinase C-Terminal Flexible Peptide in Active Site Formation and Substrate Recognition in Plasminogen Activation†

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Received August 27, 2002; Revised Manuscript Received October 14, 2002

ABSTRACT: The bacterial protein streptokinase (SK) activates human plasminogen (Pg) into the fibrinolytic protease plasmin (Pm). Roughly 40 residues from the SK C-terminal domain are mobile in the crystal structure of SK complexed with the catalytic domain of Pm, and the functions of this C-tail remain elusive. To better define its roles in Pg activation, we constructed and characterized three C-terminal truncation mutants containing SK residues 1−378, 1−386, and 1−401, respectively. They exhibit gradually reduced amidolytic activity and Pg-activator activity, as well as marginally decreased binding affinity toward Pg, as more of the C-terminus is deleted. As compared with full-length SK, the shortest construct, SK^{1-378}, exhibits an 80% decrease in amidolytic activity (k_{cat}/K_M), an 80% decrease in Pg-activator activity, and a 30% increase in the dissociation constant toward the Pg catalytic domain. The C-terminal truncation mutations did not attenuate the resistance of the SK−Pm complex to α_2-antiplasmin. Attempts at using a purified C-tail peptide to rescue the activity loss of the truncation mutants failed, suggesting that the integrity of the SK C-terminal peptide is important for the full function of SK.

Streptokinase (SK)[1], a 414-residue multidomain protein secreted by many hemolytic strains of streptococci, is a thrombolytic agent widely used for the clinical treatment of blood clotting disorders, especially acute myocardial infarction (*1*, *2*). The treatment is based on the ability of this bacterial protein to bind plasminogen (Pg), forming an activator complex that proteolytically activates other Pg molecules into the fibrinolytic protease plasmin (Pm) (*3*, *4*). Physiological Pg activators, such as tissue type Pg activator and urokinase, activate Pg by hydrolyzing its $Arg^{561}-Val^{562}$ peptide bond. By contrast, SK does not possess proteolytic activity. Such activity is attributed to the Pg moiety in the activator complex. The ability of SK to generate an active site in Pg through nonproteolytic mechanisms is of general interest for studying protease activation. Two activation mechanisms have been proposed, namely N-terminus insertion (*5*, *6*) and contact activation (*7*, *8*). Under certain experimental conditions, it has been shown that these mechanisms can function independently, although they are likely to work cooperatively in vivo (*9*, *10*). SK also forms an activator complex with Pm with much higher affinity than with Pg (*11*). In such an activator complex, the proteolytic activity comes from Pm, and SK serves as a cofactor providing substrate specificity toward Pg molecules. Therefore, functions of SK include forming a complex with Pg, generating an active site in Pg through nonproteolytic mechanisms, and providing the complex with the specificity to bind substrate Pg molecules. High-affinity SK-binding sites on human Pg (hPg) are present only within its serine protease domain (*10*, *12*), and this domain (μPg) is capable of being activated by Pg activators, including SK (*13*), and thus is used as a model system for studying Pg activation.

SK contains three major domains (*7*, *14*), referred to as SKα, SKβ, and SKγ. The C-terminal domain, SKγ, consists of amino acid residues 290−414, including a well-defined β-grasp folding domain (residues 290−372). A crystallography study on the complex of SK and the Pm serine protease domain (μPm) illustrates that SKγ is located in the vicinity of the so-called activation subdomain of μPm, which includes the activation loop and activation pocket (*7*). It was therefore proposed that SKγ plays a major role in the contact activation mechanism of active site formation. This hypothesis is supported by subsequent biochemical and mutagenesis studies (*10*, *15*). In addition, SKγ is proposed to contribute, together with the SKβ domain, to the species specificity of SK when forming a Pg-activator complex (*7*, *16*, *17*), which explains why SK from human-hosted streptococcus have lower or no activation capability on Pg from some other mammalian species (*18*). NMR studies indicate the presence of a less-structured C-terminal tail in SK (*14*). This tail, residues 375−414, appears mobile in the crystal structure of the SK−μPm complex (*7*) and is susceptible to proteolysis (*14*, *19*). These observations raise questions on the functional roles of this apparently unstructured C-tail during Pg activation. In addition, several previous studies suggest that SK with a truncated C-terminal peptide could activate hPg as efficiently as full-length SK (*20*, *21*). In the present study, we investigated the effects of truncation of the SK C-tail on

† This work was supported by NIH Grant HL60626.

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[1] Abbreviations: SK, streptokinase; Met^0-SK, recombinant SK with an extra N-terminal methionine residue; Pm(Pg), plasmin(ogen); μPm(μPg), microplasmin(ogen) (i.e., the catalytic domain of plasmin(ogen)); hPm(hPg), human-plasmin(ogen); α_2AP, α_2-antiplasmin; *N*-*p*-tosyl-GPK-*p*NA, *N*-*p*-tosyl-glycine-proline-lysine-*p*-nitroanilide; SPR, surface plasmon resonance; CD, circular dichroism.

10.1021/bi026746m CCC: $25.00
Published on Web 12/10/2002

Pg activation. The results indicate that the C-terminal peptide plays a significant role in generating an active site in the Pg moiety of the activator complex and in the recognition of substrate Pg molecules by the activator complex.

MATERIALS AND METHODS

Mutation Construction. Three C-terminal truncation variants of SK (SK^{1-378}, SK^{1-386}, and SK^{1-401}) were generated by introducing a stop codon at a selected position in the previously described full-length SK gene (*10*) using the polymerase chain reaction (PCR) method. Expressed in *Escherichia coli*, all SK constructs contained an extra methionine residue (Met^0) at their N-terminus derived from the starting codon. In addition, they all contained a Trp to Ala substitution at position 6, which is located in their flexible N-terminal peptide and was inherited from the parental SK gene (*10*). All constructs were verified by DNA sequencing. Two previously described variants of human μPg (Pg residues 542−791) were used in this study (*22*). The first variant, μPg^{R561A}, contained an Arg^{561} to Ala substitution at the activation bond, and thus, is resistant to proteolytic activation. In addition to this point mutation, the second μPg variant, $\mu Pg^{R561A,S741A}$, contained an active site Ser^{741} to Ala substitution, thus, abolishing proteolytic activity. Each of the recombinant μPg proteins contained a N-terminal peptide of the sequence MASMT GGQQM GRGSG S, adopted from the expression vector.

Protein Purification and Characterization. All SK and μPg variants were overexpressed as inclusion bodies from pET11a vector (Novagen, Madison, WI) in *E. coli* strain BL21 (DE3). The rapid dilution refolding procedure used was essentially the same as described previously (*10*). All SK truncation mutants were purified over Sephacryl-200-HR (Amersham Biosciences, Piscataway, NJ) in a buffer containing 20 mM Tris-HCl (pH 8.0) and 0.4 M urea. Each was further purified using Resource-Q anion-exchange chromatography (Amersham Biosciences, Piscataway, NJ) in 20 mM HEPES (pH 7.5) and eluted with a 0−1.0 M NaCl gradient in the same buffer. The μPg variants were purified over a Sephacryl-200-HR column in a buffer containing 20 mM HEPES (pH 7.0) and 0.4 M urea. Protein concentrations were determined using optical absorbance at a wavelength of 280 nm with the following extinction coefficients: 17 832 (AU/M) for SK^{1-378}, 21 815 for SK^{1-386}, 32 556 for SK^{1-401}, 32 588 for SK, and 50 551 for μPg and its point mutation variants (determined by mass-spectroscopic amino acid analysis) (*10*). All protein samples were dialyzed against 20 mM HEPES (pH 7.5), concentrated to ∼3 and ∼30 mg/mL for μPg and SK variants, respectively, and stored on ice. In addition, a SK C-terminal peptide of residues 376−414 ($SK^{376-414}$) was expressed as a soluble GST-fusion protein and purified by Glutathione Sepharose 4B affinity chromatography (Amersham Biosciences, Piscataway, NJ). The peptide was cleaved by thrombin and separated from immobilized GST by centrifugation. The supernatant was ultrafiltrated through a 30 kDa cutoff membrane (Millipore Co., MA) to remove the thrombin, dialyzed against 20 mM HEPES (pH 7.5), and concentrated to ∼2.5 mg/mL. Native hPg was purified from plasma following previously published procedures (*23*). To remove trace plasmin, the Pg sample was further purified using an aprotinin agarose column (Sigma-Aldrich, St. Louis, MO). SDS−PAGE analysis verified that the final purified sample consisted of Glu^1-Pg. Given its extinction coefficient (ϵ_{280}) of 187 156 AU/M as determined by mass-spectroscopic amino acid analysis, it was estimated that near 100% of catalytic sites of the hPg sample could be titrated (*24*, *25*).

Analysis of SK Variants by Human Plasmin-Affinity Chromatography. A human-plasmin (hPm) affinity column was prepared both to verify the correct conformation of refolded SK variants and to be used as a tool for SK purification. Approximately 16 mg of hPg was activated to hPm by adding a catalytic amount of human tissue-type Pg activator (500:1 molar ratio; Calbiochem, La Jolla, CA). The conversion was verified by SDS−PAGE under reducing conditions. The hPm in solution was then irreversibly inhibited by adding aliquots of a stock solution (100 mM) of 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich, St. Louis, MO) at several time points up to 20 h and until amidolytic activity was no longer discernible. The inhibited hPm was coupled to CNBr-activated sepharose 4B following the recommended coupling procedure of the manufacturer (Amersham Biosciences, Piscataway, NJ). The coupling efficiency was estimated to be about 95% based on the collected unbound hPm. The bound resin was packed as a 6 mL column. To ensure that all hPg had been activated to hPm and irreversibly inhibited, 75 μM recombinant SK and 0.6 mM AEBSF were applied to the column and incubated overnight. Subsequent SK affinity analysis was performed by FPLC. Each SK variant was dialyzed against 20 mM HEPES (pH 7.4) and filtered through a 0.2-μm pore size filter before application to the affinity column. Approximately 1.5 mg of each variant was loaded onto the column and eluted with a 0−2.0 M NaCl gradient in 20 mM HEPES (pH 7.4); fractions were analyzed by SDS−PAGE.

Removal of the N-Terminal Methionine from Recombinant SK Variants. The N-terminal Met^0 of the recombinant SK variants was removed using the *E. coli* enzyme methionine aminopeptidase (Met-AP) (*26*). The method, adopted from the published procedure, consisted of a reaction mixture of SK (40 μM) and Met-AP (2.0 μM) in a reaction buffer of 20 mM bis-tris-propane (pH 7.1), 50 mM KCl, and 2 mM $CoCl_2$. The reactions were carried out at 30 °C for 4 h and stopped by addition of 2 mM EDTA (pH 8.2) for 15 min at 22 °C. The samples were dialyzed overnight against water at 4 °C. The completeness of each reaction was determined using N-terminal sequence analysis. It is estimated that near 100% Met^0 was removed from SK, 86% from SK^{1-401}, 89% from SK^{1-386}, and 60% from SK^{1-378}.

Binding Affinity Study Using Surface Plasmon Resonance. Association and dissociation between μPg variants and immobilized SK variants were measured using surface plasmon resonance (SPR) with a BIAcore 1000 biosensor (BIAcore Inc., Sweden) as described previously (*10*). In these experiments, each SK variant was separately immobilized onto the surface of a carboxylated dextran matrix sensor chip. Various concentrations of $\mu Pg^{R561A,S741A}$ (0.1−2.0 μM) in a running buffer of 10 mM HEPES (pH 7.2), 150 mM NaCl, and 0.005% (v/v) surfactant P-20 were injected over the sensor surface at a flow rate of 30 μL/min, and the binding interactions were monitored in the form of sensorgrams. Association was measured during sample injection (80 s); dissociation was measured during injection of the running buffer alone (100 s). After each cycle, the sensor surface

was regenerated by injecting 3.5 M urea in 0.1 M Tris-HCl (pH 7.2) followed by equilibration in running buffer. After subtraction of the nonspecific refractive index component, the association rate constants (k_{on}) and dissociation rate constants (k_{off}) were calculated from the sensorgrams by nonlinear fitting of the association and dissociation data to a 1:1 binding model. Equilibrium dissociation constants (K_D) were then calculated using the equation $K_D = k_{off}/k_{on}$.

Amidolytic Activity Assay. Equimolar concentrations of μPg^{R561A} and SK variants, with or without Met^0 (final concentration 1 μM), were preincubated in a buffer containing 0.1 M HEPES (pH 7.4) and 0.1% (w/v) poly(ethylene glycol) (PEG) 2K in a quartz microcuvette for 3 min at 22 °C. Chromogenic substrate *N-p*-tosyl-glycine-proline-lysine-*p*-nitroanilide (*N-p*-tosyl-GPK-*p*NA, Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 0.5 mM. The reaction was immediately monitored at 406 nm for substrate hydrolysis using a Beckman DU-640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). The hydrolysis rate was calculated from the initial linear phase of the reaction. Further experiments were performed with the Met^0-SK variants to determine their kinetic parameters. With chromogenic substrate concentrations varying from 0.25 to 10 mM, the hydrolysis rate of the linear phase of each assay was plotted as a function of substrate concentration. The measurements were made at 430 nm for reduced background readings (*22*). At concentrations higher than 10 mM, *N-p*-tosyl-GPK-*p*NA appeared to exhibit a slight inhibitory effect on the reaction. The K_M and V_{max} (i.e., $k_{cat}[E]$) of the Michaelis−Menten equation were calculated using nonliner regression analysis with Prism software (GraphPad, San Diego, CA). The active site concentration of μPg^{R561A} ([*E*]) was 0.7 μM, 70% of the protein concentration, as determined by the active site titration.

One-Stage Plasminogen Activation Assays. Full-length SK or its C-terminal truncation variants (final concentration 1 nM) were mixed with hPg (0.25 μM) and chromogenic substrate *N-p*-tosyl-GPK-*p*NA (0.5 mM) in a buffer consisting of 0.1 M HEPES (pH 7.2) and 0.1% (w/v) PEG 2K. The generation of amidolytic activity was measured at 22 °C by recording the absorption at 406 nm as a function of time. The absorption data from the rapid-rising phase (0.05−1.0 AU) of the assay was fitted to the equation $A = 0.5 \cdot a \cdot (t - t_0)^2$, where *A* is the optical absorption, *a* is the acceleration rate, and t_0 is the so-called lag time for the complex to obtain maximum Pg-activator activity. Given the extinction coefficient of *p*NA at 406 nm of 10^4 AU/M (*27*), the maximum consumption of the chromogenic substrate during the entire assay was below 20%. By comparing the chromogenic substrate hydrolysis rate of fully activated hPm under a similar condition, the maximum consumption of hPg by the most active SK variant was determined to be below 20%. Since only data from the rapid-rising phase was used, the result approximately corresponds to the maximal stable activity of the complex. Under such an assumption, the acceleration rate, *a*, was proportional to the velocity of Pm generation by the Pg activator (i.e., $k_{cat}/([hPg] + K_M) \cdot [hPg] \cdot [SK]$). In this assay, for Met^0-SK samples, the lag time (t_0) was inversely related to the concentration of SK.

α_2-Antiplasmin Inhibition Assay for Complexes of μPg and SK Variants. Met^0-SK variants were mixed with μPg^{R561A} at equimolar concentrations (final concentration 1 μM) in a buffer consisting of 0.1 M HEPES (pH 7.2) and 0.1% (w/v) PEG 2K and then preincubated at 22 °C for 3 min. For assays without α_2-antiplasmin (α_2AP), the chromogenic substrate *N-p*-tosyl-GPK-*p*NA was then added to a final concentration of 0.5 mM. The initial rate of absorption increase was recorded at 406 nm. For assays with α_2AP, after the preincubation, α_2AP (Sigma-Aldrich, St. Louis, MO) (final concentration 1 μM) was added to the mixture, which was further incubated at 22 °C for 1 min. The chromogenic substrate was then added to a final concentration of 0.5 mM followed by recording of the initial reaction rate. As a positive control, full-length recombinant SK (25 nM) and hPg (0.25 μM) were mixed and preincubated at 37 °C for 10 min and assayed both in the presence and absence of α_2-AP (0.25 μM). Inhibition of Pm amidolytic activity by α_2-AP was 65% in this control assay.

Rescuing Truncation Mutant SK^{1-378} with C-Terminal Peptide $SK^{376-414}$. Equimolar (final concentration 1 μM) μPg^{R516A} and Met^0-SK^{1-378} were preincubated in a buffer containing 0.1 M HEPES (pH 7.4) and 0.1% (w/v) PEG 2K for 3 min at 22 °C in either the presence or absence of C-terminal peptide $SK^{376-414}$ (final concentration 50 μM). Chromogenic substrate *N-p*-tosyl-GPK-*p*NA was then added to a final concentration of 0.5 mM. The reaction was recorded immediately at 406 nm, and the rate of substrate hydrolysis was calculated from the initial linear phase of the reaction. A rate increase, if any, in the presence of $SK^{376-414}$ peptide would indicate a rescuing effect of the peptide on the loss of activity of the truncated mutant. In addition, the C-terminal peptide sample was analyzed with circular dichroism (CD) using a J-715 spectropolarimeter (JASCO Inc., Easton, MD).

RESULTS

Characterization of SK Variants. Three SK variants, SK^{1-378}, SK^{1-386}, and SK^{1-401}, consisting of residues 1−378, 1−386, and 1−401, respectively, were constructed to study the functional roles of the SK C-terminal peptide. The C-terminal positions of these mutants were beyond the visible C-terminus of SK in the crystal structure of the SK−μPm complex (*7*) and were chosen based on previously reported proteolytic products that maintain substantial activity (*19*, *20*, *28*). In the final step of purification, each SK variant, as well as full-length SK, eluted as a single peak off of an hPm-affinity column at relatively high salt concentrations, suggesting that the recombinant SK variants were refolded correctly. For a given SK variant, the position of the elution peak was independent of the amount of sample loaded. The order of elution peaks observed in this analysis from the lowest to highest salt concentration was SK^{1-378} (720 mM), full-length SK (800 mM), SK^{1-386} (840 mM), and SK^{1-401} (900 mM). It is interesting to note that full-length SK eluted at a midrange point instead of at the highest salt concentration.

SK C-Terminal Peptide Plays No Important Roles in SK−μPg Complex Formation. To address the question whether the C-terminal peptide of SK is involved in the formation of an activator complex with Pg, we studied the binding interactions between $\mu Pg^{R561A,S741A}$ and three SK truncation variants using SPR. Here, $\mu Pg^{R561A,S741A}$ was employed to eliminate potential proteolytic degradation of SK by mutating both the activation bond and the active site serine residue in

Table 1: Kinetic and Affinity Data of SK C-terminal Truncation Variants Binding to μPg[a]

	k_{on} ($\times 10^5$) ($M^{-1}\,s^{-1}$)	k_{off} ($\times 10^{-3}$) (s^{-1})	K_D (nM)
Met^0-SK	5.6 ± 0.3	12.5 ± 0.1	22.3 ± 1.3
Met^0-SK$^{1-401}$	6.6 ± 0.2	12.4 ± 0.1	18.8 ± 0.6
Met^0-SK$^{1-386}$	5.4 ± 0.2	13.2 ± 0.1	24.4 ± 1.0
Met^0-SK$^{1-378}$	5.7 ± 0.4	16.2 ± 0.1	28.4 ± 2.1

[a] Data were derived from measurements using a BIAcore1000 biosensor. SK variants were immobilized to the sensor chip, and μPg^{R516A,S741A} samples (0.1−2.0 μM) were injected in the flow cell. Standard error values are based on the global fitting of association and dissociation data.

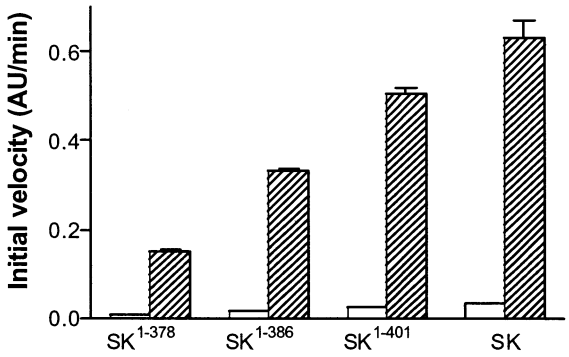

FIGURE 1: Amidolytic activity of μPg^{R561A} complexed with SK variants. The open bars represent the Met^0-SK variants, and the shaded bars represent SKs with the native N-terminus. Equimolar μPg and SK variants (1.0 μM) were preincubated for 3 min followed by addition of 0.5 mM chromogenic substrate. Amidolytic activity was determined by measuring the rate of increase of absorption at 406 nm. The results were based on duplicate measurements, and error bars represent standard error of the mean.

μPg (residues 542−791 of human Pg). Results of the SPR study are summarized in Table 1. The equilibrium dissociation constants of the three truncation variants ranged between 18.8 and 28.4 nM, which are comparable with that of full-length recombinant SK. Among the three variants, higher equilibrium dissociation constants were associated with a longer C-terminal truncation, although the effect is marginal. These results are in line with the above observations from the hPm-affinity chromatography.

SK C-Terminal Tail Is Required for High Amidolytic Activity of the SK−μPg Complex. To study the functional roles of the SK C-terminal peptide during the two hypothesized mechanisms of active site generation in a SK−Pg complex, we measured the amidolytic activity of μPg^{R561A} complexed with SK C-terminal truncation variants using the chromogenic substrate *N*-*p*-tosyl-GPK-*p*NA (Figure 1). The activation bond mutant, μPg^{R561A}, was used to eliminate proteolytic activation of μPg, such that any observed amidolytic activity came directly from mechanisms associated with SK binding. SK variants both with and without the N-terminal Met^0 were assayed. In both cases, the results showed the same trend: the longer the C-tail truncation, the lower the amidolytic activity of the complex. Particularly, in each group the activity of full-length SK is about 4-fold higher than that of the shortest variant, SK$^{1-378}$. Comparing the two groups, SK variants with a wild-type N-terminus consistently exhibited ~20-fold higher amidolytic activity when complexed with μPg^{R561A} than the corresponding variants with a Met^0-blocked N-terminus. Further kinetic studies on the enzymatic activity of the complexes suggest that for the truncation of SK residues 402−414, the loss of amidolytic activity is attributed mainly to an increase in K_M, while for further truncations it is additionally attributed to decreases in k_{cat} (Table 2).

Table 2: Kinetic Data of the Amidolytic Activity of μPg^{R561A} Complexed with SK Variants[a]

	K_M ($\times 10^{-3}$ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\,s^{-1}$)
Met^0-SK	4.8 ± 0.7	0.83 ± 0.05	173 ± 36
Met^0-SK$^{1-401}$	8.4 ± 1.0	0.74 ± 0.05	89 ± 16
Met^0-SK$^{1-386}$	8.4 ± 1.4	0.51 ± 0.05	61 ± 16
Met^0-SK$^{1-378}$	10.9 ± 2.0	0.30 ± 0.03	27 ± 8

[a] The hydrolysis rates of a chromogenic substrate were recorded at a wavelength of 430 nm. The results were based on triplicate measurements.

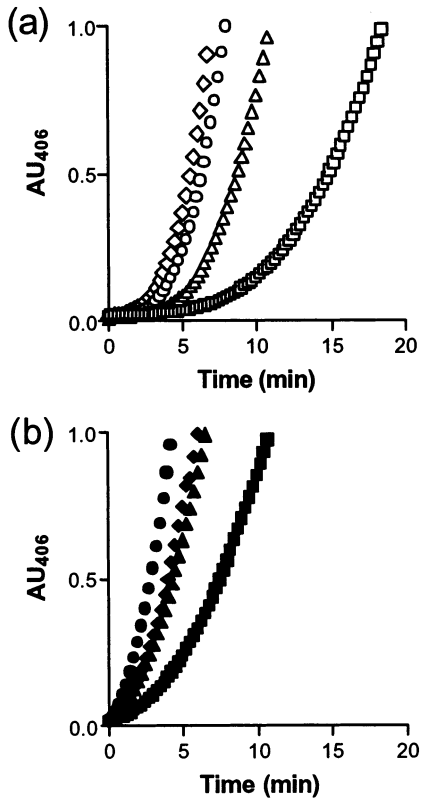

FIGURE 2: Activation of hPg by SK variants. Human Pg (0.25 μM) was activated by SK variants (1.0 nM) in the presence of 0.5 mM chromogenic substrate. Hydrolysis of the chromogenic substrate by activated hPm was recorded at 406 nm as a function of time. (a) Reaction with Met^0-SK$^{1-378}$ is represented by (□), Met^0- SK$^{1-386}$ by (△), Met^0-SK$^{1-401}$ by (○), and Met^0-SK by (◇). (b) SK$^{1-378}$ by (■), SK$^{1-386}$ by (▲), SK$^{1-401}$ by (●), and SK by (◆). Each pair of SK variants of the same C-terminal truncation, with or without N-terminal Met^0, appears to have similar final acceleration rates.

SK C-Tail Is Involved in Recognition of Substrate Pg. One of the functions of SK is to change the substrate specificity of hPm such that Pg molecules become a preferred substrate (*3*). Part of this function has been attributed to the N-terminal domain of SK (*7*). To evaluate the role of the SK C-tail in recognition of substrate Pg, we measured the efficiency of Pg activation by SK variants of varied C-tail lengths. The results are illustrated in Figure 2 and summarized in Table 3. During the maximum stable activity stage, the difference of acceleration rates between Met^0-attached and Met^0-removed variants of a given C-terminal truncation is much

Table 3: Plasminogen Activation Efficiency of SK Variants[a]

	a ($\times 10^{-3}$ AU/min^2)	t_0 (min)
Met^0-SK	75.2 ± 10.8	1.9 ± 0.2
Met^0-SK^{1-401}	48.8 ± 3.1	2.3 ± 0.4
SK^{1-401}	79.0 ± 2.8	0
Met^0-SK^{1-386}	36.0 ± 6.0	3.4 ± 0.2
SK^{1-386}	37.0 ± 5.4	0
Met^0-SK^{1-378}	12.0 ± 1.2	5.6 ± 0.1
SK^{1-378}	14.4 ± 2.4	0

[a] Activation of 0.25 μM hPg by 1.0 nM SK variants were separately measured using one-stage activation assays at 22 °C. The generation of Pm activity was monitored using substrate *N*-*p*-tosyl-GPK-*p*NA at a wavelength of 406 nm. The results were based on duplicate measurements.

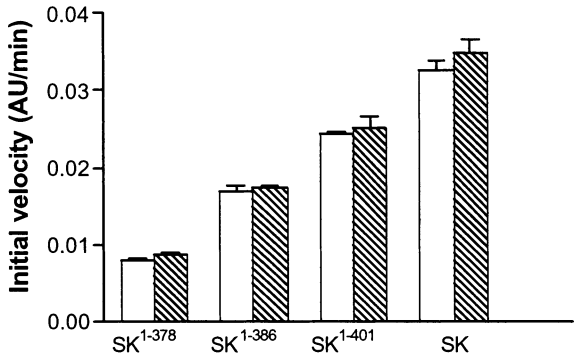

FIGURE 3: Resistance to α_2AP inhibition by μPg−SK variant complexes. Equimolar μPg^{R561A} and Met^0-SK variants (1.0 μM) were preincubated, followed by addition of chromogenic substrate (0.5 mM) in the presence (shaded bar) and absence (open bar) of α_2AP. The initial rates of hydrolysis are shown in the vertical axis with error range derived from duplicate measurements.

smaller than that in their amidolytic activities, suggesting that Pm contributes the active site in both cases. Furthermore, the Pg-activator activity decreases as more of the SK C-terminal peptide is deleted, indicating that the C-terminal peptide may be involved in binding substrate Pg or affect other structural moieties necessary for substrate recognition.

Resistance of the SK−Pm Complex to α_2-Antiplasmin Does Not Require the SK C-Tail. One of the distinct characteristics of the SK−Pm complex is the resistance of its proteolytic activity to α_2AP inhibition. This resistance is probably due to an overlap of binding sites of α_2AP and SK on Pm. To test whether the C-terminal peptide of SK contributes to this resistance, complexes of μPg with the SK variants were evaluated for α_2AP inhibition. In this experiment, μPg^{R561A} was used to ensure that a Pg enzymatic active site, which presumably reacts with and is inhibited by α_2AP, exists only in a complex with SK. The results are shown in Figure 3. As with full-length SK, no inhibition was observed for complexes containing any of the three SK C-tail truncation variants, indicating that the C-terminal peptide (residues 378−414) does not contribute to the resistance of the SK−Pm complex to α_2AP.

Isolated SK C-Tail Peptide Cannot Compensate for the Amidolytic Activity Loss of an Activator Complex Containing Truncated SK. To test whether the amidolytic activity loss of an activator complex containing C-terminal truncated SK^{1-378} can be rescued by an isolated SK C-terminal peptide, we made the recombinant peptide, $SK^{376-414}$, from a GST-fusion construct. This peptide did not show any transition during a 220 nm CD temperature scan between 20 and 90 °C (2 °C/min). In addition, its CD wavelength scan did not produce a spectra characteristic of a structured polypeptide (data no shown). Amidolytic activity assays in the presence of excessive C-tail peptide, relative to that of the μPg^{R561A}—SK^{1-378} complex, essentially showed no difference in the rate of substrate hydrolysis as compared to those in the absence of the C-tail peptide. Similarly, the peptide showed no effect on isolated μPg^{R561A}.

DISCUSSION

SK from *Streptococcus equisimilis* contains three well-ordered β-grasp folding domains and a flexible C-terminal tail of ~40 residues (*7*, *14*). This C-tail peptide is among the early proteolysis targets in SK; for example, Pm cleaves the C-terminal peptide at bonds Lys^{386}−Asp^{387}(*19*) and Arg^{401}−Tyr^{402} (*28*). An isolated C-terminal peptide, $SK^{376-414}$, does not show any stable folding or recognizable secondary structure in our CD spectroscopic analysis, indicating that this peptide is unlikely to form the fourth independently folded domain in SK as suggested by some spectroscopic studies (*29*, *30*). Although it is generally considered that this unstructured C-tail is dispensable for SK function (*14*, *15*, *31*), its close proximity to the activation loop and activation pocket of Pg argues that it could play important roles in Pg activation. Particularly, a functional conformation of this C-tail peptide may be induced upon binding to either the proenzyme or substrate Pg. If it is indeed functional, the C-tail could affect a SK−Pg complex in one or more ways, including complex formation, contact activation, N-terminus-insertion activation, and substrate recognition.

Using both affinity chromatography and SPR, we demonstrate that deletion of the C-terminal peptide has little effect on the affinity between SK and Pg (and μPg). In both cases, a marginally higher affinity is associated with a longer C-terminal peptide, except that in both experiments, SK^{1-401} has higher affinity than full-length SK toward Pg. This could suggest that the tip of the SK C-tail (~residues 402−414) interferes with the formation of a complex with Pg. The overall small effect of the C-terminal peptide on complex formation is consistent with the structural observation that the region of SK residues 373−414 is mobile in the crystal structure of the SK−μPm complex (*7*). Since the affinity between SK and Pm (Pg) within a Pg-activator complex is a few logs higher than that between the complex and its substrate Pg (*11*, *28*, *32*), results of the binding experiments are interpreted based on the 1:1 complex model.

SK-induced active site formation within an activator complex involves two mechanisms, namely N-terminus insertion (*5*, *6*) and contact activation (*10*, *11*, *15*). The extra N-terminal methionine (i.e., Met^0) in our recombinant SK proteins, which can be enzymatically removed at will, provides an ideal tool to dissect the functional roles of the C-terminal peptide in each mechanism. In the presence of Met^0, the insertion mechanism is impaired because of lack of native N-terminus, leaving the contact activation as the only viable mechanism (*10*). The fact that Met^0-SK truncation variants exhibit a decreased ability in forming an active site in μPg (see Table 2) suggests that the C-terminal peptide is involved in the contact activation mechanism in which the active site is induced and stabilized by complex formation between the proenzyme and the cofactor. In the presence of

a native SK N-terminus, both N-terminus-insertion and contact-activation mechanisms should work. In this case, larger activity decreases were observed in association with C-terminal truncations, and the changes are roughly proportional to those observed with the Met^0-blocked N-terminus (see Figure 1). Clearly, the N-terminus-insertion mechanism is attenuated but not abolished by the truncation of the C-terminal peptide. This fact is consistent with the observation that SKγ reduces the lag phase in both active site generation and Pg activation by SK-uberis from *Streptococcus uberis* (*9*), a two domain SK variant containing equivalent α and β domains but lacking a homologous γ domain.

In the hPg activation assay, we demonstrate that the SK C-terminal peptide is important for the Pg-activator activity of SK. The increase in lag time observed for the SK C-terminal truncation mutants during Pg activation (Figure 2) is a result of the decrease in ability of these mutants to generate an active site within the activator complex. During the maximum stable activity stage of Pg activation, however, the observed acceleration rates (Table 3) for a pair of Met^0-attached and Met^0-removed variants of the same C-terminal truncation are much closer to each other than their amidolytic activities. Considering the significantly higher Pm-SK affinity than that of Pg-SK (*11*), this observation suggests that Pm contributes the active site in both cases. Under this assumption, at this stage, the active site is preformed in the activator complex, and SK primarily contributes to the specificity in recognizing substrate Pg. The decrease in Pg-activator activity because of C-terminal truncation (see Table 3) may come from two effects: (i) direct loss of substrate Pg binding exosite(s) in the deleted peptide and (ii) destabilizing other substrate Pg binding sites because of deletion of the peptide. At this point, we are unable to distinguish between these two possibilities.

α_2AP is a physiologic Pm inhibitor that keeps incidental Pm activity in check by setting a threshold in plasma. SK, as a Pg-activation cofactor, circumvents this regulation by forming a Pg-activator complex resistant to α_2AP. Such a mechanism probably gives the hemolytic strains of streptococcus an advantage during pathogenic invasion. Here, we demonstrate that the SK C-terminal peptide (SK residues 370−414) does not contribute to the resistance of the SK−Pm complex to α_2AP inhibition. This observation is consistent with previous reports that α_2AP resistance is found in SK−uberis (the $\alpha\beta$ two-domain version of SK) (*9*) but not in staphylokinase from *Staphylococcus aureus* (a SKα homolog) (*33*), which suggests that the binding sites of the SKβ domain and α_2AP on Pm may overlap with each other.

SK remains the most potent Pg activator known in vitro. Initially, it promotes a conformational change of Pg from a closed form resistant to activation to an open form ready to be activated (*10*, *34*). Through multiple activation mechanisms it rearranges the latent active site of Pg to make it catalytically active (*6*, *11*, *35*). In this process, it provides Pm(Pg) with specificity toward substrate Pg (*18*) and makes them resistant to physiological Pm inhibition (*17*, *36*). The more sophisticated structure of SK has been providing explanations for its superb Pg-activation ability (*7*, *8*, *10*). The apparently flexible C-terminal tail of SK is demonstrated to contribute significantly to active site formation and substrate recognition, thus enhancing the overall performance of SK in Pg activation.

ACKNOWLEDGMENT

We thank Dr. K. Rodger for helping in the CD experiments. PCR primer synthesis and N-terminal sequencing analysis were performed by the Molecular Biology Resource Facility, William K. Warren Medical Research Institute, University of Oklahoma Medical Center.

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BI026746M