Mutation of Lysines in a Plasminogen Binding Region of Streptokinase Identifies Residues Important for Generating a Functional Activator Complex†

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ABSTRACT: Through a unique but poorly understood mechanism, streptokinase (SK) interacts with human plasminogen to generate an "activator complex" that efficiently cleaves substrate plasminogen molecules. Previous studies have suggested that lysine residues in SK may play a role in the binding and function of the activator complex. To investigate this hypothesis, 10 different lysine residues in the plasminogen binding region of SK were altered to construct 8 recombinant (r) SK mutants. Only one double mutant, $rSK_{K256,257A}$ (replacing Lys with Ala at residues 256 and 257), showed a statistically significant reduction (63%) in binding affinity for Glu-plasminogen. This mutant also displayed a lagtime in the appearance of maximal activity, and modest impairments (2−5-fold) in kinetic parameters for amidolytic and plasminogen activator activity compared to rSK. In contrast, another mutant, $rSK_{K332,334A}$, formed an activator complex with profound and nearly selective defects in the catalytic processing of substrate plasminogen molecules. When compared to rSK in kinetic assays of plasminogen activation, the $rSK_{K332,334A}$ mutant formed an activator complex that bound substrate plasminogens normally (normal K_m), but its ability to activate or cleave these molecules (k_{cat}) was reduced by 34-fold. In contrast, in amidolytic assays, the kinetic parameters of $rSK_{K332,334A}$ showed only minor differences (<2-fold) from rSK. Similarly, the binding affinity of this mutant to human Glu-plasminogen was indistinguishable from rSK [$(2.6 \pm 0.8) \times 10^9$ vs $(2.4 \pm 0.2) \times 10^9$ M^{-1}, respectively]. In summary, these experiments have identified lysine residues in a plasminogen binding region of SK which appear to be necessary for normal high-affinity binding to plasminogen, and for the efficient catalytic processing of substrate plasminogen molecules by the activator complex.

Streptokinase (SK),[1] a 47 kilodalton single-chain, bacterial protein, is widely used as a plasminogen activator for the treatment of myocardial infarction. However, unlike other routinely used plasminogen activators (tissue plasminogen activator and urokinase), SK itself is not catalytically active. Rather, through a unique mechanism, SK binds and interacts with plasminogen to generate an active enzyme complex (Blatt et al., 1964; De Renzo et al., 1967; Reddy & Markus, 1972; Schick & Castellino, 1973). This enzyme, or "activator complex", then catalyzes the cleavage of the single-chain zymogen plasminogen to the active two-chain enzyme plasmin.

The molecular role of SK in generating the activator complex is only partially understood. Following the tight binding of SK to plasminogen, the SK−plasminogen activator complex is generated when the latent active site within the plasminogen moiety is exposed and becomes functional (Summaria et al., 1968; Reddy & Markus, 1972; Schick & Castellino, 1974). Through undefined mechanisms, SK is able to generate or unmask this active site without cleavage (McClintock & Bell, 1971). Previous studies are consistent with the hypothesis that streptokinase has at least three functions in the generation of a functional plasminogen activator complex: (1) high-affinity binding to plasminogen to form a complex; (2) interacting with plasminogen to generate the active site; (3) binding and processing of substrate plasminogen molecules (Buck & Boggiano, 1971; Reed et al., 1995). Unfortunately, the molecular mechanisms underlying these functions remain unknown.

Recent studies have identified a region of SK, between lysine residues 244 and 352, which mediates the tight binding of SK to plasminogen (Reed et al., 1995). Recombinant SK fragments, containing the 244−352 region, inhibit SK from forming a functional SK−plasminogen activator complex, underlining the critical relationship between binding and function. Further studies have suggested that lysine residues may play an important functional role in the binding of SK to plasminogen and in the generation of the activator complex. For example, Alkjaersig et al. (1959) showed that a lysine analog, ϵ-aminocaproic acid (EACA), markedly inhibited the ability of SK to activate plasminogen. We have

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[1] Abbreviations: SK, streptokinase isolated from group C *Streptococcus*; rSK, recombinant SK fusion protein containing the sequences of native SK; $rSK_{K256,257A}$, rSK mutant with K256 and K257 replaced with A; $rSK_{K256,257A}$, rSK mutant with K332 and K334 replaced by A; SAC, *Staphylococcus aureus* cells; MBP, maltose binding protein; EACA, ϵ-aminocaproic acid; assay buffer, 50 mM Tris-HCl, 100 mM NaCl, pH 7.4.

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subsequently found that EACA interferes with the generation of a functional activator complex by inhibiting the primary binding interactions between SK and plasminogen (Lin et al., 1995). These two lines of evidence led to the hypothesis that lysine residues, in the plasminogen binding domain of SK, may be important for binding and complex formation, and for the functional processing of plasminogen substrate molecules by the SK−plasminogen activator complex. In the present study, we have investigated this hypothesis by serially mutating 10 lysines in this region of SK. The purified recombinant (r)SK mutants were analyzed by saturation binding assays and kinetic studies to ascertain the roles of the mutated lysine residues in plasminogen binding, active site formation, and plasminogen activation.

EXPERIMENTAL PROCEDURES

Mutagenesis. The gene coding for SK was ligated into the pMAL-C vector (New England Biolabs, Inc., Beverly, MA) as described (Reed et al., 1993) to create a rSK. Mutagenesis was performed using the unique site elimination technique (Deng & Nickoloff, 1992; Pharmacia Biotech Inc., Piscataway, NJ). Briefly, the pMAL-C vector carrying the rSK gene was annealed with two primers: the target mutagenic primer (Genosys, Woodlands, TX) which introduces the desired mutations into the rSK DNA and the selection primer that eliminates the unique *Sca*I restriction site in the amp^r region of the plasmid. The elimination of the *Sca*I site serves as the basis for selection of mutated plasmids. The sequences of the target mutagenic primers are shown in Table 1. Following the synthesis of both strands of mutated DNA, the mutations were selected by unique restriction endonuclease digestions and confirmed by double-stranded DNA sequencing (Sanger et. al., 1977; U.S. Biochemicals, Cleveland, OH).

Expression and Purification of MBP−SK Fusion Proteins. rSK wild-type and site-directed rSK mutants were expressed in *Escherichia coli* as inducible fusion proteins via the pMALc expression vector (New England Biolabs) as described (Maina et al., 1988; Reed et al., 1993). In this system, typical expression levels for the rSKs were approximately 70−80 mg/L of bacterial culture. The rSKs were purified by affinity chromatography on amylose resin as described (Reed et al., 1995). Protein fractions were pooled and concentrated using ultrafiltration membranes (YM30, Diaflo; Amicon Division, Beverly, MA) or Amicon Centricon-30 concentrators (Amicon, Inc., Beverly, MA) and dialyzed into assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4). Samples were aliquoted and stored at −80 °C to avoid repeated freezing and thawing. The antigen concentration of purified rSK proteins was determined by radioimmunoassay as described (Reed et al., 1995), and active site concentration was determined by active site titration (see below).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblotting. After rSK fusion proteins were expressed and purified, they were analyzed by SDS−PAGE on 8% gels as described by Laemmli (1970). Immunoblotting experiments were used to quantify the amounts of Glu-plasminogen and plasmin in the activator complex formed by different rSKs under the same reaction conditions used for the kinetic studies reported below. Prior to the experiments, the molar quantities of Glu-plasminogen and rSK were determined by active site titration. Then stoichiometric rSK−plasmin(ogen) complexes were incubated for the times specified below (plasminogen activation assays). The activator complex was then immediately boiled for 5 min in sample buffer with 5% β-mercaptoethanol (Laemmli, 1970). The activator complexes were electrophoresed on 10% SDS−polyacrylamide gels and electroblotted onto poly(vinylidene difluoride) membranes. After blocking nonspecific protein binding sites with 5% nonfat milk, the blots were probed with rabbit anti-plasminogen antibody (10 μg/mL; Boehringer Mannheim, Indianapolis, IN) for 1 h at 21 °C. After washing, the bound anti-plasminogen antibody was detected by incubation with ^{125}I-protein A (2 000 000 cpm) for 1 h. Washed membranes were exposed in a phosphorimager (Molecular Dynamics, Sunnyvale, CA), and the amounts of plasminogen and plasmin were quantitated by radioimmunoassay by comparison to known standard amounts of plasmin(ogen). The percentage of the activator complex composed of plasminogen or plasmin is reported in the legend to Table 3.

Immunoblotting experiments were also used to determine the amount of Glu- and Lys-plasminogen present in the plasminogen preparations. In these experiments, different amounts of Glu-plasminogen (American Diagnostica, Greenwich, CT; or Pharmacia Hepar, Franklin, OH) or Lys-plasminogen (American Diagnostica) were electrophoresed under reducing conditions on 6% gels. The proteins were transferred to poly(vinylidene difluoride) membranes and immunoblotted as just described. Two bands were detected at ∼90 kDa and at ∼83 kDa corresponding to Glu- and Lys-plasminogen. The amount of Glu- and Lys-plasminogen in each preparation was determined by radioimmunoassay as above.

Protein Labeling. Purified proteins were radioiodinated by the Iodogen method (Fraker & Speck, 1978), and the specific radioactivity was determined as described (Lukacova et al., 1991).

Saturation Binding Studies. The binding affinities of wild-type rSK and site-directed rSK fusion proteins to ^{125}I-Glu-plasminogen were measured in solution using a method previously described in detail (Reed et al., 1995). The Glu-plasminogen was reported as 98% Glu-type by the manufacturer (American Diagnostica) and found to be 97% Glu-type and 3% Lys-plasminogen by our immunoblotting assays. In a typical assay, rSK or rSK mutant (100 μL; 2×10^{-10} M) was mixed with ^{125}I-Glu-plasminogen (100 μL) at final concentrations ranging from 2×10^{-9} to 4×10^{-11} M in binding buffer (10 mM KH_2PO_4, 150 mM NaCl, with 0.1% BSA and aprotinin, 100 kallikrein inhibitor units/mL) for 15 min at room temperature (21 °C). Then the samples were placed on ice, and 2 μL of rabbit, affinity-purified, anti-MBP antibodies was added for 1 h. Subsequently, 10 μL of protein A containing *Staphylococcus aureus* cells (SAC; heat-killed and fixed; Boehringer Mannheim Co.) was added, and the mixture was allowed to incubate for another 1 h on ice. Then 2 mL of ice-cold binding buffer was added as a wash to the tubes, and the samples were centrifuged at 3500 rpm at 4 °C for 30 min. The supernatant was removed, and the precipitated ^{125}I-Glu-plasminogen was measured in a gamma counter. The amount of nonspecific binding was estimated by addition of 1000-fold molar excess of wild-type SK or 100 mM concentration of EACA. The data were analyzed using the Ligand program (Munson & Rodbard, 1980). It

is important to note that the binding assay conditions described above were derived through experiments designed to optimize immunoprecipitation and to establish the incubation time necessary for maximal rSK−Glu-plasminogen binding. These experiments established that under these conditions, ^{125}I-rSK could be quantitatively immunoprecipitated at 10^{-10} M concentration using affinity-purified, polyclonal rabbit anti-MBP antibody. Studies also established that SK−Glu-plasminogen binding was rapid, achieving a maximum steady level by 2 min, which was stable for at least 30 min at 21 °C. To examine the impaired binding of the $rSK_{K256,257A}$ mutant noted in these assays, the primary binding reaction between the mutant and ^{125}I-Glu-plasminogen was studied under conditions in which it formed a complex with Glu-plasminogen that had maximal, stable catalytic activity (37 °C for 20 min). The binding data were analyzed with the aid of the Ligand Program (Munson & Rodbard, 1980) to obtain estimates of the binding affinity (K_A) and standard error. The Student's *t*-test (Wilkinson, 1989) was used to determine whether a mutant's measured binding affinities were statistically different (i.e., $p < 0.05$) from those of rSK.

Binding Studies of Conformationally Sensitive MAbs to Mutant SK. The binding of conformationally sensitive and nonconformationally dependent anti-SK monoclonal antibodies (MAbs) to rSK and to the $rSK_{K256,257A}$ mutant was measured. These antibodies were derived from a well-characterized panel of MAbs which has previously been described (Reed et al., 1993). Wells of a microtiter plate were coated with 10 μg/mL wild-type rSK or the $rSK_{K256,257A}$ mutant for 1 h at 21 °C. After rinsing, nonspecific protein binding sites were blocked with 1% bovine serum albumin (Sigma, St. Louis, MO) in PBS for 1 h. The wells were then incubated with 25 μL of hybridoma supernatants from different anti-SK MAbs, or a negative control (anti-digoxin) MAb. Following 1 h incubation, the wells were washed. Then ^{125}I-labeled goat anti-mouse antibody (25 μL; 50 000 cpm) was added for 1 h to detect the bound anti-SK MAb. After washing, the amount of bound antibody was measured by gamma scintillation counting.

Active Site Titration. The molar quantity of active sites generated by the various rSK−plasminogen activator complexes was determined at 25 °C in a Hitachi 2000 fluorescence spectrophotometer by active site titration (Chase & Shaw, 1969; McClintock & Bell, 1971) using the fluorogenic substrate 4-methylumbelliferyl *p*-guanidinobenzoate (Sigma) as we have previously described (Reed et al., 1995).

Amidolytic and Plasminogen Activation Assays. The rate at which the different mutants formed a rSK−plasminogen activator complex with maximal stable activator function was determined. Human Glu-plasminogen was premixed with the various rSKs in equimolar ratios for 0, 5, 15, 20, and 60 min at 37 °C, and 5 nM (final) activator complex was added to the wells of a microtiter plate containing assay buffer, human Glu-plasminogen (100 nM), and S-2251 (0.5 mM) in a final volume of 150 μL at 37 °C. The change in absorption at 405 nm was continuously recorded for 20 min in a Thermomax microtiter plate reader (Molecular Devices, Palo Alto, CA). The time of preincubation which yielded maximal stable plasminogen activator activity was used in subsequent experiments and varied between 5 and 20 min.

(A) Amidolytic Assays of SK−Glu-Plasminogen Complexes. The amidolytic activity of SK or rSK was studied in a manner similar to that described by Wohl et al. (1980). The reaction temperature (37 °C) was thermostatically controlled in a Hewlett Packard 8451 A diode array spectrophotometer using a thermocycler (VWR Scientific, Model 1136). Purified wild type rSK or site-directed mutants (40 nM) were incubated with 20 nM of Glu-plasminogen at 37 °C for 5−20 min to form a maximally active plasminogen activator complex. [This plasminogen was reported as ≥95% Glu-type by the manufacturer (Pharmacia Hepar) and found to be 99% Glu-type and 1% Lys-type by immunoblotting as described above.] A molar excess of rSK was used to eliminate any chance of free plasmin occurring in the reaction (Wohl et al., 1980) as described. The mixture was then transferred to a quartz cuvette containing assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) and various concentrations (100−800 μM) of H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251; KabiVitrum, Stockholm, Sweden) in a total volume of 600 μL. The change in absorbance was continuously monitored at 405 nm for 5 min. Lineweaver−Burk plots (Lineweaver & Burk, 1934) were constructed as described (Wohl et al., 1980; Fears et al., 1985) and used to estimate kinetic parameters.

(B) Plasminogen Activation Assays. The kinetics of plasminogen activation by rSK and rSK mutants were also studied as described by Wohl et al. (1980). Glu-plasminogen (Pharmacia Hepar) and rSK (2−20 nM, depending on the mutant) were mixed in stoichiometric ratios and preincubated at 37 °C to form an activator complex as described above. They were then added to a quartz cuvette containing assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4), 500 μM H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251; KabiVitrum), and various concentrations of Glu-plasminogen [$(0.3-5) \times K_m$ for SK; Wohl et al., 1980]. The change in absorbance at 405 nM was monitored at 37 °C in a Hewlett Packard 8451 A diode array spectrophotometer using a thermocycler (VWR Scientific, Model 1136). Initial reaction rates were obtained from the first 300 s by plotting A_{405} vs time^2 and the apparent Michaelis constants and catalytic rate constants were calculated by construction of Lineweaver−Burk plots as outlined (Wohl et al., 1980; Fears et al., 1985) or by hyperbolic fits of the data as described (Cleland, 1979; Robertson, 1989).

RESULTS

To investigate the potential functional roles played by lysine residues in a plasminogen binding region of SK (spanning amino acids 244−352), 10 lysine residues were mutated using the primers shown in Table 1. Four mutants with single mutations and four with double mutations were obtained by site-directed mutagenesis. DNA sequencing confirmed the expected nucleotide sequence changes. The size and relative purity of the rSK proteins were analyzed by SDS−PAGE on 8% gels (Figure 1). All rSKs were the expected molecular mass of 89 kDa.

Liquid phase saturation binding assays were performed to determine the effects of these lysine mutations on the binding affinity of SK for plasminogen. The binding experiments were performed at 21 °C with aprotinin to inhibit proteolysis. These conditions were previously shown to yield stable estimates of the binding affinities of these two molecules (Reed et al., 1995). The binding of all of the rSK mutants to plasminogen was saturable and could be specif-

Table 1: Mutagenic Primers Used To Construct Site-Directed SK Mutants[a]

mutant	mutagenic primer	restriction site
rSK_{K273R}	ATC TCT GAG ArA TAT TAC GTC CTT ArA ArA GGG GAA ArG CCG TAT GAT	−
rSK_{K278R}	ATC TCT GAG ArA TAT TAC GTC CTT ArA ArA GGG GAA ArG CCG TAT GAT	−
rSK_{K279R}	ATC TCT GAG ArA TAT TAC GTC CTT ArA ArA GGG GAA ArG CCG TAT GAT	−
rSK_{K282R}	ATC TCT GAG ArA TAT TAC GTC CTT ArA ArA GGG GAA ArG CCG TAT GAT	−
$rSK_{K279,282R}$	ATC TCT GAG ArA TAT TAC GTC CTT ArA ArA GGG GAA ArG CCG TAT GAT	−
$rSK_{K256,257A}$	GCT TAT AGG ATC AAT gcg gcc TCT GGT CTG AAT G	*Hae*III
$rSK_{K293,298A}$	GCA GTC ACT TGg ccC TGT TCA CCA TCg cAT ACG TTG ATG TCG	*Hae*III
$rSK_{K332,334A}$	CGA TCC TCG TGA Tgc cGC ggc ACT ACT CTA CAA C	*Sac*II

[a] Mutated nucleotides are shown as lower case letters. Mutagenic primers were degenerate in sequence at different nucleotide positions to yield more that one mutant per primer. Mutations yielding new restriction sites are underlined.

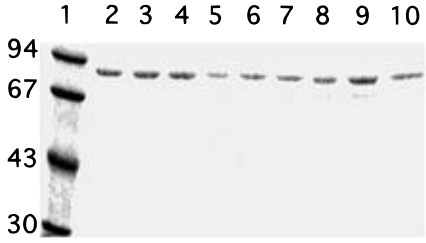

FIGURE 1: SDS−PAGE analysis of purified rSKs used in this study. Proteins were electrophoresed on 8% acrylamide gels and stained with Coomassie blue dye. Lane 1, molecular weight marker; lane 2, rSK_{K273R}; lane 3, rSK_{K278R}; lane 4, rSK_{K279R}; lane 5, rSK_{K282R}; lane 6, $rSK_{K279,282R}$; lane 7, $rSK_{K256,257A}$; lane 8, $rSK_{K293,298A}$; lane 9, $rSK_{K332,334A}$; lane 10, rSK.

Table 2: Binding Affinity Constants (K_A) for the Binding of rSKs to Glu-Plasminogen[a]

rSK or mutant	plasminogen binding affinity (K_A) ($\times 10^9$ M^{-1}, ±SD)	no. of expt
rSK	2.4 ± 0.24	5
$rSKK_{273R}$	2.0 ± 0.54	1
$rSKK_{278R}$	2.5 ± 0.46	2
$rSKK_{279R}$	2.5 ± 0.42	2
$rSKK_{282R}$	2.7 ± 0.27	2
$rSKK_{279,282R}$	1.8 ± 0.15	2
$rSKK_{256,257R}$	2.9 ± 0.83	6
$rSKK_{293,298A}$	3.7 ± 0.80	2
$rSKK_{332,334A}$	2.6 ± 0.79	3
$rSKK_{256,257A}$[b]	0.9 ± 0.19	8

[a] Saturation binding studies were performed at 21 °C to measure the binding affinity of rSK to ^{125}I-Glu-plasminogen in solution as described in detail under Experimental Procedures. The binding data were analyzed with the aid of the Ligand program (Munson & Rodbard, 1980). [b] The measured binding affinity for this mutant was different from rSK ($p < 0.001$).

ically inhibited by unlabeled SK (data not shown). Table 2 summarizes the binding affinities obtained for rSK and the mutants. A *t*-test was used to objectively assess whether the binding affinity of a mutant differed significantly ($p < 0.05$) from the binding affinity of rSK. By this criterion, only the binding affinity of $rSK_{K256,257A}$ was significantly different, in a statistical sense, from that of rSK ($p < 0.001$). Figure 2 shows a Scatchard transformation of a typical saturation binding experiment for the $rSK_{K256,257A}$ mutant, and rSK as a reference. For both rSKs, the Scatchard transformation was linear, suggesting a single class of binding sites for both proteins. Since previous studies (Reed et al., 1995) have shown that complex formation between rSK with plasminogen was rapid, with stable, maximal binding achieved within 2 min at 21 °C, we explored whether the reduced apparent binding affinity of the $rSK_{K256,257A}$ may be due to the fact that it achieved maximal stable binding to Glu-plasminogen at a slower rate than rSK. However with

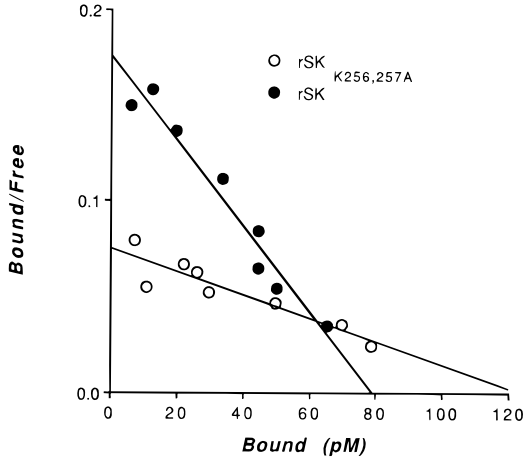

FIGURE 2: Representative Scatchard transformations of the saturation binding isotherms of rSK and $rSK_{K256,257A}$ to Glu-plasminogen. The studies were performed as described under Experimental Procedures. The results were analyzed with the aid of the Ligand program (Munson & Rodbard, 1980).

longer incubation times at 21 °C, the binding affinity still did not improve, and when binding experiments were performed under experimental conditions that were found to yield maximal amidolytic activity of the $rSK_{K256,257A}$−Glu-plasminogen complex (20 min incubation at 37 °C, see below), the binding affinity of the mutant was still reduced [$K_A = (0.1 \pm 0.01 \times 10^9$ M^{-1}; $n = 3$].

Given the reduced binding affinity of $rSK_{K256,257A}$ for Glu-plasminogen, we attempted to detect changes in the folding of the mutant, as compared to rSK, by probing with conformation-sensitive monoclonal anti-SK MAbs. These monoclonal antibodies recognized discrete epitopes throughout the SK molecule (Reed et al., 1993). Figure 3 shows that, in a pairwise comparison, the conformation-sensitive and, for contrast, the non-conformation-dependent MAbs bound equally well to the mutant $rSK_{K256,257A}$ and to the wild-type rSK, indicating that no conformational difference between the mutant and rSK was detected.

Screening assays were performed to determine which lysine mutations may affect the ability of SK to form a functional plasminogen activator complex with Glu-plasminogen. Only the $rSK_{K256,257A}$ and $rSK_{K332,334A}$ mutants showed an impaired capacity to form an SK−PAC which could activate Glu-plasminogen (see Figure 4). Studies were performed to determine the amount of time necessary for the rSK mutants to generate a plasminogen activator complex with maximal, stable catalytic activity. These studies showed that the activator complexes formed by rSK and $rSK_{K332,334A}$ were maximally active by 5 min of incubation, while the activator complex formed by $rSK_{K256,257A}$ developed maximal

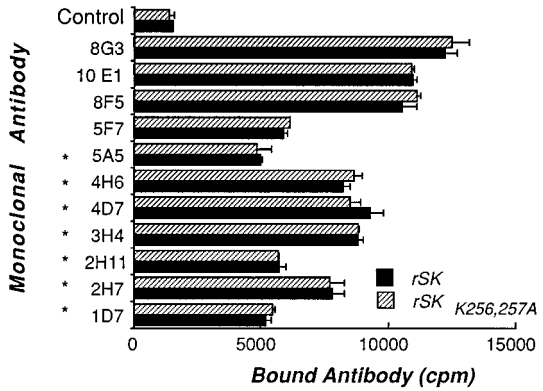

FIGURE 3: Comparative binding of anti-SK monoclonal antibodies to rSK and $rSK_{K256,257A}$. Wells of a microtiter plate were coated with rSK, or $rSK_{K256,257A}$ (10 μg/mL), and then nonspecific protein binding sites were blocked. Conformation-sensitive (*) anti-SK MAbs, non-conformation-dependent anti-SK MAbs, or control (anti-digoxin) MAbs were added to the wells. After washing, the bound antibody was detected by ^{125}I-labeled goat anti-mouse antibody, followed by gamma scintillation counting. The data represent the mean ± SD.

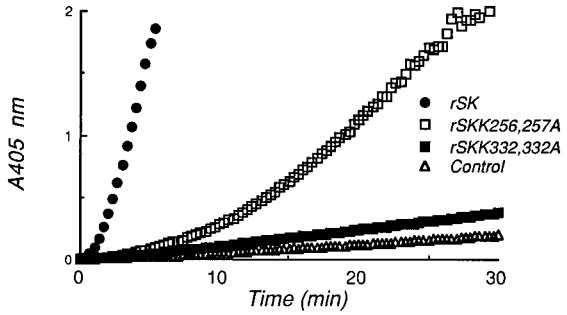

FIGURE 4: Plasminogen activation by rSK and rSK mutants. Different rSKs (10 nM) or no rSK (control) was added to a quartz cuvette containing assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4), 500 μM S-2251, and 0.3 μM human Glu-plasminogen in a total volume of 300 μL. Plasminogen activation was detected by continuously monitoring the change in absorbance at 405 nm in a spectrophotometer.

Table 3: Enzyme Kinetic Parameters for Plasminogen Activation by Recombinant SK (rSK), and rSK Site-Directed Mutants[a]

	activation parameters		
SK type	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
rSK	0.24 ± 0.06	2.37 ± 0.27	9.9
$rSKK_{256,257A}$	0.34 ± 0.08	0.50 ± 0.07	1.5
$rSKK_{332,334A}$	0.24 ± 0.08	0.07 ± 0.02	0.3

[a] Activation experiments were carried out at 37 °C in 0.3 mL volumes with activator complex concentrations of 6.7−20.0 nM. Activator complexes were formed by incubation of stoichiometric amounts of the different rSKs and Glu-plasminogen for different times as necessary to achieve maximum stable activity: rSK (6.7 nM, 5 min), $rSK_{K256,257A}$ (10 nM, 20 min), and $rSK_{K332,334A}$ (20 nM, 5 min). Under these conditions, the activator complex formed by rSK was 99% plasminogen (1% plasmin), $rSK_{K256,257A}$ 61% plasminogen (39% plasmin), and $rSK_{K332,334A}$ 86% plasminogen (14% plasmin). The values represent the mean ± SE.

stable activity after 20 min of incubation at 37 °C. Using these data, kinetic assays were performed to study the ability of the mutants to activate Glu-plasminogen. These kinetic assays (Table 3) revealed that the K_m and k_{cat} for the activator complex formed by rSK were consistent with reported estimates for the activator complex formed with nonrecombinant SK in terms of K_m and k_{cat} (Wohl et al., 1980; Fears et al., 1985; Collen et al., 1993; Shi et al., 1994). In contrast,

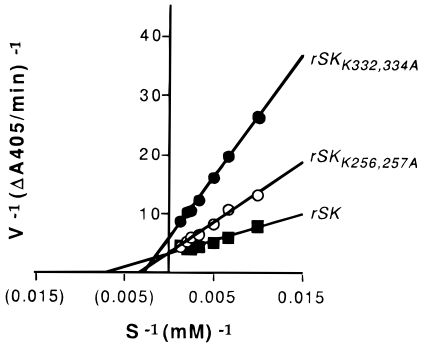

FIGURE 5: Lineweaver−Burk plots of amidolysis kinetics by rSK, and rSK mutants. Various concentrations (100−800 μM) of S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride) were hydrolyzed at 37 °C by a final concentration of 20 nM activator complex. The absorption at 405 nm was continuously recorded for 7 min to obtain reaction velocities. Lineweaver−Burk plots of V^{-1} $[(A_{405}/min)^{-1}]$ vs S^{-1} (μM^{-1}) from typical experiments are shown.

Table 4: Enzyme Kinetic Parameters for the Amidolytic Activity of Recombinant SK (rSK), and rSK Site-Directed Mutants[a]

	amidolytic parameters		
SK type	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
rSK	188 ± 12	42.2 ± 1.0	0.22
$rSKK_{256,257}A$	310 ± 21	33.6 ± 1.0	0.11
$rSKK_{332,334}A$	309 ± 31	17.9 ± 0.8	0.06

[a] Amidolytic assays were carried out at 37 °C in a total volume of 0.6 mL. Activator complexes (20 nM) were formed by mixing rSK or rSK mutant together with Glu-plasminogen (see Experimental Procedures). The activator complex was added to various concentrations (100−800 μM) of H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride, and the rate of product formation was determined by the change in absorbance at 405 nm. Kinetic parameters were determined as described under Experimental Procedures. The values represent the mean ± SE.

for $rSK_{K256,257A}$ the k_{cat} of the activator complex was reduced 4.7-fold compared to rSK while the K_m was minimally if at all changed. Similarly, when compared to rSK, the K_m of the activator complex formed by $rSK_{K332,334A}$ was not grossly altered though the k_{cat} was markedly reduced (34-fold).

To determine the mechanism(s) responsible for the reduced plasminogen activator function of these two mutants, we examined their capacity to form a SK−plasminogen complex which could cleave the peptide substrate H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251). Figure 5 shows representative Lineweaver−Burk plots obtained for the activator complexes formed by rSK, $rSK_{K256,257A}$, and $rSK_{K332,334A}$ in enzyme kinetic studies. Table 4 shows the average kinetic parameters obtained for these rSKs in these studies. The kinetic parameters for rSK were consistent with reported values for nonrecombinant SK under similar conditions (Wohl et al., 1980). When compared to rSK, the K_m for $rSK_{K256,257A}$ was minimally higher (1.6-fold), and the k_{cat} was nearly the same and the k_{cat}/K_m was slightly lower (2.0-fold). Similarly, when compared to rSK, the K_m of $rSK_{K332,334A}$ was ∼1.6-fold higher and the k_{cat} was 2.4-fold lower, making a k_{cat}/K_m 3.7-fold lower. Thus, overall the amidolytic kinetic parameters of the activator complexes formed by the $rSK_{K332,334A}$ and $rSK_{K256,257A}$ mutants were only modestly different from that formed by rSK.

DISCUSSION

Previous experiments have suggested that lysine residues in a plasminogen binding domain of SK may have important

functional roles in the generation of the SK−PAC. To examine this hypothesis, we mutated 10 lysine residues in this region and tested their effect on 3 putative functions of SK in the activator complex: (1) initial high-affinity binding to plasminogen to create the SK−plasminogen complex; (2) interaction with plasminogen in the complex to generate an active site; and (3) participation in the binding and processing of substrate plasminogen molecules.

Of the original mutants studied, only $rSK_{K256,257A}$ showed a statistically significant reduction in binding affinity for Glu-plasminogen at 21 °C. This binding affinity did not improve even with prolonged incubation at 37 °C, although these conditions were sufficient to induce maximal catalytic function in the activator complex formed by this mutant. Nevertheless, $rSK_{K256,257A}$ still bound to Glu-plasminogen tightly, indicating that the high-affinity binding between these two proteins is due to the net effect of many interactions between several residues in SK and plasminogen. This result was anticipated because it has been difficult to find fragments of SK which have the same high binding affinities to plasminogen as does full-length SK (Reed et al., 1995) and crystallographic studies (in other systems) have shown that high-affinity binding typically involves interactions between several different residues in both molecules (Clothia et al., 1986). Mutations of Lys to Ala at residues 293 and 298 and 332 and 334 did not produce significant decreases in the K_A, indicating that these residues may not play a major role in binding interactions between these two molecules. Mutations of Lys residues 273, 278, 279, and 282 to Arg also did not significantly reduce the binding affinity for Glu-plasminogen. However, by analogy to the normal binding affinity of the $rSK_{K256,257R}$ mutant, these Lys to Arg mutations may have been too conservative to elicit the possible contribution of these particular residues to binding or function.

In addition to its decreased binding affinity, the $rSK_{K256,257A}$ mutant displayed a lag phase in the development of maximal, stable catalytic function. However, once stable catalytic function was achieved, the SK−PAC formed by $rSK_{K256,257A}$ showed mild impairments in K_m and k_{cat} in amidolytic function (<2-fold) and moderate reductions in k_{cat} (4.7-fold) for plasminogen activation. Since the high-affinity binding of SK to plasminogen is the first step toward the generation of a functional activator complex, these modest reductions in the catalytic activity of the activator complex formed by the $rSK_{K256,257A}$ mutant may be the direct consequence of its reduced binding affinity for plasminogen.

In contrast, another mutant, $rSK_{K332,334A}$, had normal binding affinity for plasminogen but produced an activator complex with a selective defect in the cleavage of plasminogen. Like $rSK_{K256,257A}$, $rSK_{K332,334A}$ formed a activator complex which had mildly impaired (<2.5-fold) K_m and k_{cat} in amidolytic assays. However, despite a normal K_m, the catalytic efficiency of plasminogen activation by the activator complex formed by $rSK_{K332,334A}$ was markedly reduced (34-fold). These findings suggest that K_{332} and/or K_{334} are necessary for efficient catalytic processing of substrate plasminogen substrates by the activator complex; however, these residues probably do not play an important role in (1) the high-affinity binding to plasminogen, (2) the amidolytic function of the activator complex, or (3) the binding of substrate plasminogen molecules to the activator complex.

In the last few years, there have been several studies with proteolytic or recombinant fragments of SK which have yielded important insights into the structural features of the molecule which are important for the generation of a fully functional activator complex. On the basis of NMR and proteolytic studies, it appears that SK may consist of three domains which are structurally autonomous and span residues 1−146, 147−287, and 293−380 (Teuten et al., 1993; Parrado et al., 1996). A high-affinity plasminogen binding region appears to be present in the middle and perhaps carboxyl domains of SK (Rodriguez et al., 1995; Reed et al., 1995). The minimal sequences necessary for detectable plasminogen activation have been reported to involve both the middle and carboxyl domains (Parrado et al., 1996; Rodriguez et al., 1995), though measurable significant plasminogen activation (i.e., >1% activity) would appear to require all three domains (Brockway & Castellino, 1974; Parrado et al., 1996; Shi et al., 1994). This would suggest the hypothesis that the different domains of SK interact with each other, as well as with plasminogen, to modulate the activity and stability of the activator complex. Further insights into SK action will require precise functional studies that dissect the effect of different mutated residues of SK on binding, amidolysis, and plasminogen activation by the activator complex. Needless to say, these structure−function studies would be greatly aided by three-dimensional structural information on SK and the activator complex. A better understanding of the structural elements responsible for SK function might also lead to a way to modify the activity, degradation, antigenicity, etc. of the activator complex to enhance its efficacy in thrombotic disease.

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