Identification of a Plasminogen Binding Region in Streptokinase That Is Necessary for the Creation of a Functional Streptokinase−Plasminogen Activator Complex†

Guy L. Reed,*,‡,§,∥ Lee-Fong Lin,‡ Behnaz Parhami-Seren,§,∥ and Paul Kussie§,∥,⊥

Cardiovascular Biology Laboratory, Harvard School of Public Health, Boston, Massachusetts 02115, Massachusetts General Hospital, Boston, Massachusetts 02114, and Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Streptokinase is a plasminogen activator widely used to treat patients with myocardial infarction. However, streptokinase is not a protease, and must first bind and interact with plasminogen to form an enzymatic complex. By measuring the binding of recombinant streptokinase fragments to plasminogen, we have sought, first, to identify a plasminogen binding region in streptokinase and, second, to explore the relation between binding (via this region) and the generation of a functional streptokinase−plasminogen activator complex. Recombinant streptokinase bound in a saturable and specific manner to human Glu-plasminogen with a dissociation constant of 4.2×10^{-10} M. Recombinant streptokinase fragments spanning amino acids 1−127 and 1−253 could not be shown to bind to Glu-plasminogen, whereas fragments spanning amino acids 1−352, 120−352, and 244−414 bound tightly to plasminogen and each fragment completely inhibited the binding of full-length streptokinase to plasminogen. Although these latter streptokinase fragments formed a complex with plasminogen, enzymatic assays indicated that none of them was capable of generating an active site. When the streptokinase region shared by these three fragments, spanning residues 244−352, was expressed, it also bound plasminogen and competitively inhibited the formation of a functional plasminogen activator complex by full-length streptokinase. Taken together, these data indicate that streptokinase binds to plasminogen with high affinity, that a primary binding region for plasminogen is located within amino acids 244−352, and that binding via this region is necessary for the generation of a functional plasminogen activator complex.

The plasminogen activator streptokinase (SK)[1] is widely used in humans to dissolve the thrombi that cause myocardial infarctions. SK is a single-chain polypeptide of 414 amino acids derived from different strains of streptococcus (Malke et al., 1985). SK differs significantly from the human plasminogen activators urokinase and tissue plasminogen activator. These human plasminogen activators are serine proteases that cleave a peptide bond in plasminogen to convert it to the active enzyme plasmin. In contrast, SK has no intrinsic enzymatic activity. Several theories have been proposed to explain its mechanism of action (e.g., Taylor & Beisswenger, 1973; Kosow, 1975; Jackson & Tang, 1982; Nikandrov, 1992), but the preponderance of data favors an "activator complex" model of SK function [reviewed by Castellino (1981) and Reddy (1988)]. In this model, SK forms a 1:1 molar complex with plasminogen, altering its conformation to produce, without proteolytic cleavage, an active site in the zymogen (McClintock & Bell, 1971; Reddy & Markus, 1972; Schick & Castellino, 1973; Summaria et al., 1982). Studies with diisopropyl[^{32}P]phosphorofluoridate suggest that the active site Ser generated by SK in plasminogen after complex formation is the same as that identified in the active enzyme plasmin (Buck et al., 1968; Groskopf et al., 1969). Yet despite similar active sites, the SK−plasminogen activator complex (SK−PAC) and plasmin show markedly different abilities to process substrates (Reddy & Markus, 1972; Schick & Castellino, 1973). In particular, the SK−plasminogen activator complex efficiently "activates" plasminogen by cleavage of its Arg 561−Val bond, whereas plasmin cannot.

Although the kinetic properties of the SK−PAC and the role of plasminogen in the complex have been extensively studied, the role of SK in the creation and catalytic function of the SK−PAC is poorly understood. It has been shown that after binding and interacting with SK, a "virgin" or uncleaved plasminogen can be isolated from the SK−PAC which has amidolytic activity that is similar to plasmin though it cannot activate other plasminogen molecules (Summaria et al., 1982; Shi et al., 1993). Taken together, these results suggest that SK may have three roles in the SK−PAC: (1) binding to plasminogen to form the complex; (2) generation of the active site in plasminogen; and (3) enabling the binding and processing of substrate plasminogen molecules by the SK−PAC (Buck & Boggiano, 1971). These potential functions of SK in the SK−PAC might be

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* To whom correspondence should be addressed at the Harvard School of Public Health, 677 Huntington Ave., Boston, MA 02115. Telephone: (617) 432-4992. FAX: (617) 432-0033.

‡ Cardiovascular Biology Laboratory, Harvard School of Public Health.

§ Massachusetts General Hospital.

∥ Harvard Medical School.

⊥ Current address: Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

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[1] Abbreviations: SK, streptokinase; t-PA, tissue plasminogen activator; PCR, polymerase chain reaction; SAC, *Staphylococcus aureus* cells; MBP, maltose binding protein; BSA, bovine serum albumin. "Natural SK" refers to naturally occurring SK, and "full length" SK refers to recombinantly expressed SK that contains the complete amino acid sequence of natural SK.

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elucidated by structure−function studies with SK fragments. Studies of purified proteolytic fragments of SK have shown that a 36 kDa fragment, spanning amino acids 60−387, binds plasminogen, generates an active site, and forms a functional activator complex (Brockway & Castellino, 1974; Shi et al., 1994). Although some structure−function studies have been performed recently with other proteolytic fragments of SK (Shi et al., 1994), it has not been possible to clearly localize binding, active site generation, or activator complex function to significantly smaller regions of SK. To begin to examine the potential functions of different regions of SK, recombinant fragments of the SK have been constructed and expressed. Experiments have been performed to identify SK fragments that bind to plasminogen and to measure their binding affinity. SK fragments that complex with plasminogen have been tested for their ability to generate an amidolytic active site. A binding region for plasminogen in SK has been identified, and the corresponding recombinant SK fragment has been expressed. Inhibition experiments with this fragment suggest that binding interactions via this region are important for the generation of an active site in the SK−plasminogen activator complex.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Streptokinase. The SK gene was cloned from *Streptococcus equisimilis* by the polymerase chain reaction (PCR) (Saiki et al., 1988) as we have described (Reed et al., 1993). The cloned SK gene was sequenced (U.S. Biochemicals, Cleveland, OH; Sanger et al., 1977) and found to be identical to the published sequence (Malke et al., 1985). Truncated portions of the SK gene were generated in a similar manner using PCR and specific internal primers. The following primers (Genosys, The Woodlands, TX) were used for amplification and cloning of SK fragments [numbers refer to the amino acid residues; primer restriction endonuclease sites (*Eco*RV or *Pst*I) are underlined]: SK amino terminus, 5′-d(GC GAT ATC GCT GGA CCT GAG TGG); SK carboxyl terminus, 5′-d(GC CTG CAG TCA TTA TTT GTC GTT AGG); SK 244−248, 5′-d(GC GAT ATC CGT GTT AAA AAT CGG G); SK 127−122, 5′-d(GC CTG CAG TCA TTA CAA GGT TAC CGA ACC ATC); SK 253−248, 5′-d(GC CTG CAG TCA TTA CCT ATA AGC TTG TTC CCG); SK 352−347, 5′-d(GC CTG CAG TCA TTA TCC AGT TAA GGT ATA GTC); SK 120−124, 5′-d(GC GAT ATC GAC AAA GAT GGT TCG).

After the DNA sequence corresponding to full-length SK, or to the desired SK fragment, had been cloned, it was ligated into the pMAL expression vector (New England Biolabs, Beverly, MA) and expressed as a fusion protein in *Escherichia coli* [with maltose binding protein (MBP; Maina et al., 1988)]. SK fusion proteins were purified by affinity chromatography on an amylose resin (New England Biolabs) as described by the supplier, by affinity chromatography on a plasminogen−Sepharose column, or by preparative gel electrophoresis on a BioRad Model 491 Prep Cell (BioRad, Richmond, CA). The purity of the recombinant SK fusion proteins was assessed by SDS−PAGE (Laemmli, 1970). For some experiments, the SK fusion proteins were cut with factor Xa as described (Maina et al., 1988), and the MBP was removed by affinity chromatography on an amylose resin. After purification, the amount of recombinant SK 1−414 was determined by using an absorption coefficient of 7.5 for a 1% solution at 280 nm (Reddy, 1988). The relative concentrations of the cleaved, purified SK fragments were determined by comparative radioimmunoassay with fragment-specific monoclonal antibodies (Reed et al., 1993). Briefly, wells of a microtiter plate were coated with various concentrations of natural SK (0, 2.5, 5, 10, 20, and 40 μg/mL). After nonspecific binding sites had been blocked with 1% bovine serum albumin, fragment-specific monoclonal antibodies were added to each well in duplicate. After a 1-h incubation, the wells were washed and probed with ^{125}I-labeled goat anti-mouse antibody (Cappel Organon Teknika, Durham, NC) for 1 h. After another wash, the amount of bound antibody was determined by γ counting. A standard curve relating antibody binding to natural SK concentration was derived for each fragment-specific monoclonal antibody. The assay was then repeated with unknown concentrations of purified recombinant SK fragments and appropriate fragment-specific monoclonal antibodies. The concentration of a recombinant SK fragment was determined by reference to the standard curve.

Protein Labeling. Human Glu-plasminogen (~98% Glu-type, American Diagnostica, Greenwich, CT, product 410A) was labeled by the Iodogen method (Pierce, Rockford, IL; Fraker & Speck, 1978) to a specific activity ranging from 0.80 to 1.35 million cpm/pmol. Goat anti-mouse antibody was similarly labeled, with specific activities ranging from about 7000 to 10 000 cpm/ng.

Ligand Blotting. Purified SK fusion proteins were subjected to electrophoresis on 10% polyacrylamide gels under reducing conditions (Laemmli, 1970). Proteins were stained with Coomassie blue dye or transferred to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA) by semi-dry electroblotting as described (Kyhse, 1984). The membranes were blocked in 5% nonfat milk and probed with ^{125}I-plasminogen (2 000 000 cpm) for 1 h. After the blots had been washed, they were subjected to autoradiography.

Solution Binding Assays. Saturation binding assays were performed to study the binding of full-length MBP−SK (1−414) to ^{125}I-Glu-plasminogen in solution using immunoprecipitation to separate the bound MBP−SK−^{125}I-Glu-plasminogen from the unbound ^{125}I-Glu-plasminogen. In a typical assay, full-length MBP−SK (100 μL; 2×10^{-10} M) was mixed with ^{125}I-Glu-plasminogen (100 μL) at final concentrations ranging from 2×10^{-9} M 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., Indianapolis, IN) 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 γ 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). Because of their apparent lower affinity for plasminogen in preliminary binding experiments, the binding of MBP−SK fragments (1−352, 120−352, 244−414) to plas-

minogen was measured in a cold inhibition assay format (Munson & Rodbard, 1980). MBP−SK fragments (25−50 nM final concentration) were incubated with unlabeled Glu-plasminogen with concentrations ranging from 0.038 to 8.33 pmol and ^{125}I-Glu-plasminogen (200 000 cpm). The remainder of the binding experiment was conducted as previously described, and the binding affinity of these MBP−SK fragments was determined using the Ligand program.

It is important to note that the binding assay conditions described above were derived by experiments designed to optimize immunoprecipitation and to establish the incubation time necessary for maximal MBP−SK−Glu-plasminogen binding. These experiments established that under these conditions, ^{125}I-MBP−SK 1−414 could be quantitatively immunoprecipitated at 10^{-10} M concentration using affinity-purified, polyclonal rabbit anti-MBP antibody. Preliminary studies also established that SK−Glu-plasminogen binding was rapid, achieving a maximum plateau by 2 min which was stable for at least 30 min at 21 °C.

Microtiter Plate Binding Assays. The binding of ^{125}I-Glu-plasminogen to various recombinant SK fragments was also studied by direct solid-phase binding assays. Wells of a microtiter plate were coated with 25 μL of purified SK fragment in a concentration of 10 μg/mL for 3 h at room temperature. Control wells were coated with purified MBP (0.5 μg/mL) or no antigen. The wells were washed, and nonspecific protein binding sites were blocked with 200 μL of 1% BSA (Sigma, St. Louis, MO) for 1 h. The wells were washed again, and ^{125}I-Glu-plasminogen (1−200 000 cpm) was added to each well for 1 h. The unbound plasminogen was removed, the wells were washed, and the amount of bound plasminogen was determined by γ counting. The competitive binding to plasminogen of various recombinant SK fragments was also studied. Wells of a microtiter plate were coated with natural SK (25 μL, 1 μg/mL, 1 h) or, in separate assays, with MBP−SK 244−352 (25 μL, 20 μg/mL, 2 h). Nonspecific protein binding sites were blocked by adding 200 μL of 1% BSA for 1 h. Then 25 μL of various concentrations of recombinant SK fragment (ranging from 0 to 0.835 mg/mL) or no SK (all diluted in 1% BSA in Tris-buffered saline) was added to wells in duplicate. Immediately thereafter, 25 μL of ^{125}I-Glu-plasminogen (50 000 cpm) was added to each well. After 1 h of incubation, the wells were washed, and bound ^{125}I-Glu-plasminogen was counted in a γ counter. Wells coated with 1% BSA and no SK were used as negative controls. The percentage of plasminogen binding was determined by computing the fractional binding of ^{125}I-Glu-plasminogen to SK in the presence of a given inhibitor in comparison with that occurring in the absence of an inhibitor, after correcting for nonspecific binding.

Active Site Titration. The number of active sites generated by SK 1−414 in Glu-plasminogen was determined by active site titration (Chase & Shaw, 1969) at 25 °C as described for SK by McClintock and Bell (1971) using the fluorogenic substrate 4-methylumbelliferyl *p*-guanidinobenzoate (Sigma) in a Hitachi 2000 fluorescence spectrophotometer.

Assay for the Formation of an Amidolytic Active Site in the SK−Plasminogen Complex. Purified SK fragments (75 nM) were separately mixed with Glu-plasminogen (50 nM) in 900 μL of assay buffer (50 mM Tris, 0.1 M NaCl, pH 7.4) for 10 min at 21 °C in a quartz cuvette. Then 100 μL of S2251 substrate (0.5 mM; H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; Chromogenix, Sweden) was added, and the rate of cleavage of the substrate was monitored by recording the absorption at 404 nm every 20 s for 7 min in a Hewlett Packard 8451A spectrophotometer. Samples containing Glu-plasminogen and S2251 but no SK were also tested as controls.

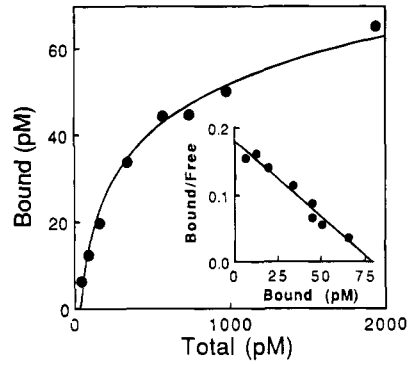

FIGURE 1: Saturation binding of Glu-plasminogen and recombinant full-length SK in solution. Binding studies were performed as described under Experimental Procedures analyzed by the Ligand program (Munson & Rodbard, 1980). The ordinate shows the amount of ^{125}I-Glu-plasminogen specifically bound to recombinant full-length SK as a function of the total amount added (abscissa). Inset: Scatchard transformation of the binding isotherm.

Competition assays were performed to determine the effects of SK 244−352 on the creation of an active site in plasminogen by full-length SK. Various concentrations of MBP−SK 244−352 (0, 25 nM, 250 nM, and 2 μM) or MBP−SK 1−127 (0 or 2 μM) were incubated with human Glu-plasminogen (20 nM; American Diagnostica Inc., Greenwich, CT) in assay buffer at 21 °C for 15 min. Then MBP−SK 1−414 (25 nM) was added. After 15 min incubation, the mixture was transferred to a quartz cuvette containing assay buffer and 0.5 mM S2251 (0.5 mM) in a final volume of 600 μL. The absorption was measured continuously at 404 nm for 15 min. Samples containing Glu-plasminogen and S2251 but no SK proteins were also tested as controls.

RESULTS

Saturation binding assays were performed to study the binding of SK to ^{125}I-Glu-plasminogen in solution. Figure 1 shows the data from a typical binding assay analyzed with the Ligand program (Munson & Rodbard, 1980). The binding of recombinant, full-length SK to ^{125}I-Glu-plasminogen was saturable and could be inhibited by an excess of cold plasminogen. The Scatchard transformation of the data (Figure 1, inset) showed that SK bound to a single class of high-affinity binding sites with a dissociation constant (K_D) of 4.2×10^{-10} M.

Recombinant SK fragments were generated to identify regions of the SK molecule which bound to plasminogen. Figure 2A shows a Coomassie blue-stained gel with bacterial lysates containing the induced MBP−SK proteins. The expected molecular masses for the purified MBP−SK proteins were as follows: MBP−SK 1−414 (full length), 89 kDa; MBP−SK 1−352, 82 kDa; MBP−SK 1−253, 70 kDa; MBP−SK 1−127, 56 kDa; MBP−SK 120−352, 69 kDa; MBP−SK 244−414, 62 kDa; and MBP (MBP−Laczα without SK), 53 kDa. All the expressed proteins were of the expected molecular masses. To determine which MBP−SK fragments bound to human plasminogen, we performed

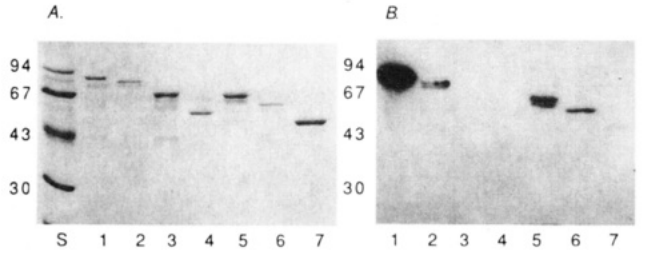

FIGURE 2: Panel A shows Coomassie blue-stained 10% SDS−polyacrylamide gels of bacterial lysates containing recombinant MBP−SK proteins and MBP alone. Panel B shows the same samples electrophoretically transferred to poly(vinylidene difluoride) membranes and probed with ^{125}I-plasminogen. The membranes were then subjected to autoradiography to detect bound plasminogen. The lanes contained (S) molecular mass standards, (1) SK 1−414, (2) SK 1−352, (3) SK 1−253, (4) SK 1−127, (5) SK 244−414, (6) SK 120−352, and (7) MBP. The molecular masses of standards (in kDa) are indicated on the left.

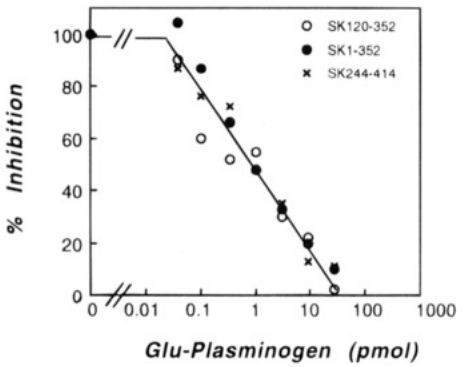

FIGURE 3: Binding of recombinant MBP−SK fragments to plasminogen in solution. The binding of SK 1−352, 120−352, and 244−414 to ^{125}I-Glu-plasminogen was measured using a "cold inhibition" assay (Munson & Rodbard, 1980) as described under Experimental Procedures. The ordinate shows the percent of inhibition of binding of ^{125}I-Glu-plasminogen to the SK fragments as a function of increasing concentrations of unlabeled Glu-plasminogen (abscissa). The data were analyzed with the aid of the Ligand program.

ligand blotting studies. MBP−SK fragments were electrophoresed on 10% SDS−polyacrylamide gels under reducing conditions and transferred to poly(vinylidene difluoride) membranes. The membranes were then probed with ^{125}I-human plasminogen. Figure 2B shows that (under these denaturing conditions) full-length SK (SK 1−414) bound plasminogen, as did the fragments SK 1−352, SK 244−414, and SK 120−352. Fragments SK 1−127 and SK 1−253, and MBP alone, did not show detectable binding to plasminogen.

To determine whether the binding of MBP−SK fragments SK 1−127 or SK 1−253 was affected by the presence of the MBP moiety or the denaturing conditions of the ligand blot, we cleaved the fusion proteins with factor Xa. After purification, the binding of the cleaved SK fragments (i.e., without MBP) was studied in a solid-phase microtiter plate assay. In these assays, full-length SK 1−414 and SK fragments 1−352, 120−352, and 244−414 all showed significant binding to plasminogen, but SK 1−127 and 1−253 still did not (data not shown). Solution binding assays (cold inhibition type) were performed to measure the binding affinities of the SK fragments which bound to Glu-plasminogen (Figure 3). All three fragments (SK 120−352, SK 1−352, SK 244−414) completely inhibited the binding of ^{125}I-Glu-plasminogen to full-length SK, suggesting that

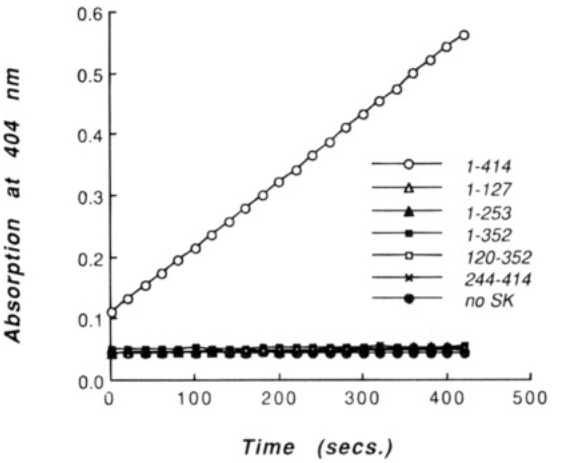

FIGURE 4: Generation of an active site in plasminogen by various recombinant MBP−SK fragments. Purified recombinant MBP−SK fragments (75 nM) or no SK were mixed with human Glu-plasminogen (50 nM). The generation of an amidolytic active site in the mixture was detected by continuously monitoring the cleavage of S2251 (0.5 mM final) at 404 nm as a function of time in a Hewlett Packard diode array spectrophotometer.

each fragment contained a binding region. The calculated dissociation constants were similar for each fragment but lower than for full-length SK 1−414: SK 120−352, K_d = 8.3 (±0.7) × 10^{-8} M; SK 1−352, K_d = 2.9 (±0.9) × 10^{-8} M; and SK 244−414, K_d = 4.3 (±1.3) × 10^{-8} M.

Enzymatic assays were performed to determine whether the fragments that bound to plasminogen could generate a functional active site in the SK−plasminogen complex. The recombinant SK fragments were preincubated with human plasminogen, and the appearance of an active site in the SK−plasminogen complex was monitored by recording the rate of cleavage of a chromogenic substrate. In this assay, full-length SK 1−414 generated an amidolytic active site in plasminogen (Figure 4). Active site titration studies showed that 71.9% of the SK 1−414 molecules were functional. However, the SK fragments that bound to plasminogen, SK 1−352, SK 244−414, and SK 120−352, showed no discernible ability to generate an amidolytic active site upon interaction with plasminogen (Figure 4).

Since all three SK fragments bound with roughly comparable affinities to plasminogen and contained overlapping SK sequences, we reasoned that the two smallest SK fragments, SK 244−414 and SK 120−352, might both contain the same plasminogen binding region(s). To test this hypothesis, we examined whether SK 120−352 could completely inhibit the binding of plasminogen to SK 244−414 in a solid-phase microtiter plate assay. Figure 5 shows that SK 120−352, like full-length SK 1−414, completely inhibited the binding of plasminogen to SK 244−414. This suggested that the region shared by these two fragments, SK 244−352, contained most of the residues necessary for plasminogen binding in these assays. To investigate this possibility, we expressed the SK 244−352 fragment using the same expression system. Figure 6 compares the binding of SK 244−352, SK 120−352, or no antigen to ^{125}I-plasminogen in a solid-phase microtiter plate assay. In comparison with control wells, wells containing SK 244−352 and SK 120−352 showed significant binding to plasminogen, confirming that the smaller fragment contained the plasminogen binding site(s). To determine if the binding of wild-type SK to plasminogen via the 244−352 region was

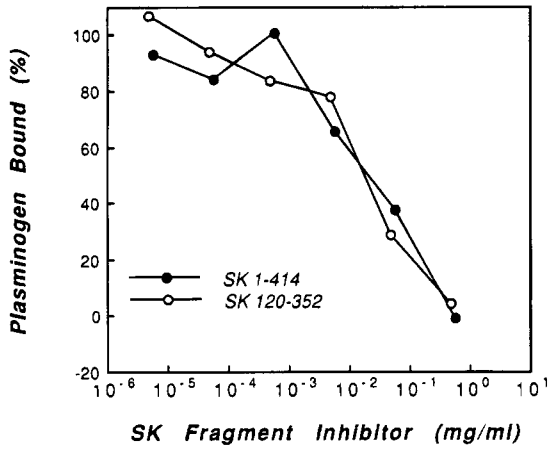

FIGURE 5: Competitive binding of recombinant MBP−SK fragments to plasminogen. Wells of a microtiter plate were coated with SK 244−414. Then the overlapping fragment SK 120−352, or full-length SK 1−414 (control), was added to the wells as an inhibitor in various concentrations. Subsequently, ^{125}I-plasminogen was added to the wells for 1 h. The wells were washed, and the amount of bound plasminogen was determined by γ counting. The percentage of plasminogen binding was determined by computing the fractional binding of ^{125}I-plasminogen to SK in the presence of a given inhibitor in comparison with that occurring in the absence of an inhibitor, after correcting for nonspecific binding.

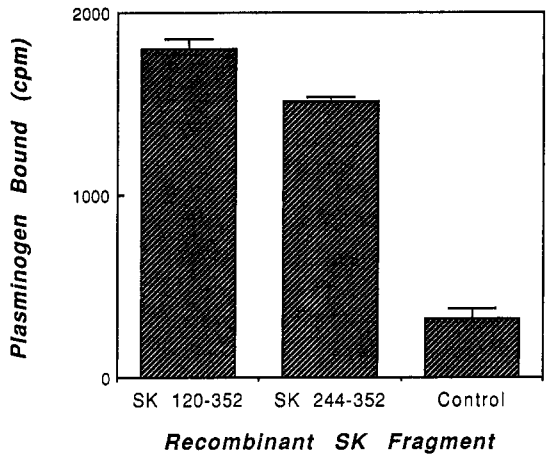

FIGURE 6: Binding of plasminogen to SK fragments. Recombinant MBP−SK fragments were immobilized in wells of a microtiter plate. After nonspecific binding sites had been blocked with bovine serum albumin, ^{125}I-plasminogen was added for 1 h. The wells were then washed and γ-counted to detect bound plasminogen. Control wells contained bovine serum albumin only. The data represent the means ± SD of duplicate observations.

necessary for the formation of a functional SK−PAC, inhibition studies were performed. Figure 7 shows that increasing amounts of the recombinant SK 244−352 fragment progressively inhibited the generation of an active site by recombinant full-length SK and Glu-plasminogen with almost complete suppression seen at a 2 μM concentration. In contrast, similar experiments showed that a 2 μM concentration of the nonbinding fragment SK 1−127 had no inhibitory effect on active site generation.

DISCUSSION

Surprisingly little is known about the binding of streptokinase to plasminogen although it is the necessary first step in the generation of a functional SK−plasminogen activator complex. The saturation binding experiments in this study indicate that SK and Glu-plasminogen bind to each other in

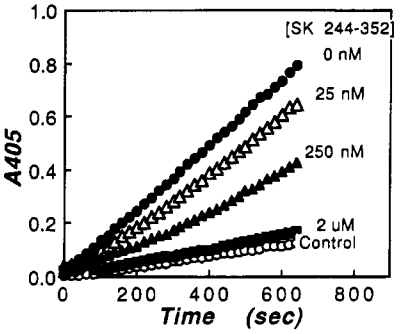

FIGURE 7: Effect of SK 244−352 on the generation of an active site in plasminogen by full-length SK. Various concentrations of SK 244−352 (0, 25 nM, 250 nM, 2 μM) were premixed with 50 nM Glu-plasminogen and S2251. Then full-length recombinant SK was added, and the generation of an active site was monitored by recording the change in absorption at 404 nm in a spectrophotometer. A control reaction containing Glu-plasminogen without any SK is also shown.

a saturable and specific manner. The Scatchard transformation of the data is most consistent with a single class of high-affinity binding sites with a $K_d = 4.2 \times 10^{-10}$ M. Although little is known about the region of SK which binds to plasminogen, our previous studies showed that a monoclonal antibody directed against SK 120−352 was the best inhibitor of this interaction (Reed et al., 1993). The present experiments show that SK fragments spanning 1−352, 120−352, and 244−414 bound to plasminogen with comparable dissociation constants in the range of about 10^{-8} M. Since these fragments completely inhibited the binding of wild-type SK to plasminogen and competed with each other, it appeared that the region of overlap of these fragments, 244−352, contained residues which interact with plasminogen to effect binding. Fragment 244−352 was found to bind to plasminogen and to competitively inhibit the formation of an active site in plasminogen by full-length SK. However, fragments which contained this binding region were not capable of generating an active site in plasminogen. This suggests that SK−plasminogen interactions at other sites outside of the 244−352 region are likely to be important for the construction of a functional activator complex. Alternatively, residues outside of this region, missing in these fragments, may play important indirect roles by providing the underlying structure necessary for the 244−352 region to fold into the conformation needed for activation.

These are the first studies systematically investigating the binding of different SK regions to plasminogen and their effects in generating a functional plasminogen activator. Cederholm-Williams and colleagues (1979), using kinetic methods, reported that SK bound to human *plasmin* with a K_d of 0.5×10^{-10} M. While their estimates may have been distorted by the use of a simplified kinetic model [compare Wohl et al. (1980)], the confounding effects of SK binding to bovine plasminogen,[2] and the fragmentation of SK by plasmin (e.g., Summaria et al., 1974), their results are roughly comparable to our estimates of K_d of 4.2×10^{-10} M for the binding of SK to *Glu-plasminogen*. A few other studies have examined the activity of proteolytic fragments of SK. For example, Klessen et al. (1988) showed that deletion of the first 15 amino acid residues in SK did not affect its activity. Similarly, Brockway and Castellino (1974) noted that SK

[2] Reed et al., 1994, unpublished observations.

lacking the first 59 amino acids was still a functional plasminogen activator. Jackson et al. (1986) demonstrated that deletion of the carboxyl-terminal amino acids (beyond residue 383) did not abolish function. Following up on these observations, Shi et al. (1994) recently studied proteolytic fragments of SK derived by reaction with immobilized plasmin. They showed that the fragment spanning amino acids 60−387 of SK had about one-sixth of the activity of the whole SK in plasminogen activation assays and, because it required guanidine for dissociation, appeared to bind tightly to immobilized *plasmin*. A smaller fragment spanning 60−333 of SK had less than 1% catalytic activity and, because it could be washed away by phosphate buffer, appeared "weakly associated" with immobilized plasmin. Other SK fragments as small as 26 kDa have been found bound to plasminogen after cleavage by different mammalian plasmins (Summaria et al., 1974), but their identity is uncertain and they do not appear to be active.

Although these experiments have identified a functionally important binding region for plasminogen in SK, they have also shown that the mere binding of plasminogen to SK via this region is not sufficient for the construction of a functional SK−plasminogen activator complex. Further studies are needed to define the intra- and intermolecular interactions of SK and plasminogen which generate the enzyme site of the SK−plasminogen activator complex and facilitate the binding of substrate plasminogen molecules.

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