Structure-Function Relationships in Staphylokinase as Revealed by “Clustered Charge to Alanine” Mutagenesis*

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Eighteen mutants of recombinant staphylokinase (SakSTAR) in which clusters of two or three charged residues were converted to alanine (“clustered charge-to-alanine scan”) were characterized. Fifteen of these mutants had specific plasminogen-activating activities of >20% of that of wild-type SakSTAR, whereas three mutants, SakSTAR K11A D13A D14A (SakSTAR13), SakSTAR E46A K50A (SakSTAR48), and SakSTAR E65A D69A (SakSTAR67) had specific activities of 3%. SakSTAR13 had an intact affinity for plasminogen and a normal rate of active site exposure in equimolar mixtures with plasminogen. The plasin-SakSTAR13 complex had a 14-fold reduced catalytic efficiency for plasminogen activation but was 5-fold more efficient for conversion of plasminogen-SakSTAR13 to plasin-SakSTAR13. SakSTAR48 and SakSTAR67 had a 10–20-fold reduced affinity for plasminogen and a markedly reduced active site exposure; their complexes with plasminogen had a more than 20-fold reduced catalytic efficiency toward plasminogen. Thus, plasminogen activation by catalytic amounts of SakSTAR is dependent on complex formation between plasminogen and SakSTAR, which is deficient with SakSTAR48 and SakSTAR67, but also on the induction of a functional active site configuration in the plasin-SakSTAR complex, which is deficient with all three mutants. These findings support a mechanism for the activation of plasminogen by SakSTAR involving formation of an equimolar complex of SakSTAR with traces of plasminogen, which converts plasminogen to plasmin and, more rapidly, inactive plasminogen-SakSTAR to plasin-SakSTAR.

Staphylokinase, produced by certain strains of Staphylococcus aureus, activates the human plasma fibrinolytic system indirectly (1, 2). It forms a 1:1 stoichiometric complex with plasminogen, which activates plasminogen (3, 4). Recombinant staphylokinase (SakSTAR)1 was shown to induce fibrin-specific clot lysis in a human plasma milieu in vitro (5, 6), in animal models of thrombosis (7), and in patients with acute myocardial infarction (8, 9). This fibrin specificity has been explained by specific molecular interactions between SakSTAR, plasminogen, α2-antiplasmin, and fibrin. The plasmin-SakSTAR complex is rapidly inhibited by α2-antiplasmin (6, 10), resulting in dissociation of active SakSTAR from the complex and recycling to other plasminogen molecules (11). The inhibition rate of the complex by α2-antiplasmin (second-order rate constant = 107 M−1 s−1) is, however, reduced more than 100-fold in the presence of fibrin (12). Thus, in the absence of fibrin, α2-antiplasmin inhibits the activation of plasminogen by SakSTAR by preventing generation of active plasin-SakSTAR complex. Fibrin stimulates plasminogen activation by SakSTAR via a mechanism involving the lysine-binding sites of plasminogen, probably by facilitating the generation of plasin-SakSTAR complex and by delaying its inhibition at the surface of a clot (13).

SakSTAR consists of 136 amino acids, of which 45 are charged, in a single polypeptide chain without disulfide bridges; it consists of two widely separated domains of similar size linked by a flexible helix (14). To investigate the structure-function relationships of SakSTAR, which determine its interaction with plasminogen in more detail, charged amino acids were mutagenized to alanine in clusters of 2 or 3 residues (clustered charge to alanine scan) (15). Replacement of clusters of charged amino acids in the SakSTAR regions comprising amino acids 11–14, 46–50, and 65–69 resulted in a markedly reduced plasminogen activating capacity. Investigation of the mechanism of plasminogen activation by wild-type and mutant SakSTAR moieties supported a model involving formation of an equimolar complex of SakSTAR with traces of plasminogen, which converts plasminogen to plasmin and, more rapidly, inactive plasminogen-SakSTAR to active plasmin-SakSTAR.

MATERIALS AND METHODS

Proteins and Reagents—Wild-type mature recombinant staphylokinase (SakSTAR) was produced as described previously (9). Native human plasminogen, low M, plasminogen (consisting of kringle 5 and the serine proteinase domain), plasmin, and α2-antiplasmin were obtained and characterized as described elsewhere (16–19). Recombinant Glu-plasminogen with the active site Ser395 replaced by Ala (rPilg-Ala395) was obtained and characterized as described previously (20). Fibrinogen was prepared from human plasma and depleted in plasminogen by adsorption with lysine-Sepharose. Aprotinin-Sepharose and lysine-Sepharose were prepared by coupling aprotinin (Trasylol®, Bayer, Leverkusen) or lysine to CNBr-activated Sepharose 4B. The chromogenic substrate S-2403 (L-pyroglutamyl-L-phenylalanyl-L-lysine-p-nitroanilide) was purchased from Chromogenix (Antwerp) and p-nitrophenyl-

consisting of triple-loop structure 5 of the plasmin A-chain and an intact B-chain (M, 39,000); kₐ, association rate constant; k₋ₒ, dissociation rate constant; Kₐ, association affinity constant; SakSTAR-Δ10, SakSTAR derivative lacking the NH₂-terminal 10 amino acids.

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p'-guanidinobenzoate (NPGB) from Sigma.

T4 DNA ligase, KloneF Fragment of E. coli DNA polymerase I and alkaline phosphatase were obtained from Boehringer Mannheim. The oligonucleotide-directed mutagenesis system and the pMa/c plasmids were kindly provided by Corvas (Ghent) (22). The expression vector pME602SAKB was constructed as described previously (9). M13K07 helper phage was purchased from Promega (Lüdén). Luria Broth growth medium was purchased from Life Technology (Merelbeke).

Laboratory Techniques—Plasmid DNA was isolated using a QIA-GEN-purification protocol (provided by Westburg N.V., Leusden, The Netherlands). Transformations of Escherichia coli were performed with the calcium phosphate procedure (22). DNA sequencing was performed using the dideoxy chain termination reaction method and Automated Laser Fluorescent A.L.F. 477A (Pharmacia Biotech Inc.).

Protein concentrations were determined according to Bradford (23). SDS-PAGE was performed with the Phast System (Pharmacia) using 10–15% gradient gels and Coomassie Brilliant Blue staining. Reduction of the samples was performed by heating at 100 °C for 3 min in the presence of 3% SDS and 1% dithioerythritol. SakSTAR-related antigen was determined with a specific enzyme-linked immunosorbent assay (24), calibrated with the respective purified SakSTAR moieties. NH2-terminal amino acid sequence analysis was performed on an Applied Biosystems 477A protein sequencer with identification of amino acids by high performance liquid chromatography.

The specific plasminogen-activating activities of SakSTAR moieties were determined with a plasminogen-coated chromogenic substrate assay, and were expressed in home units by comparison with an in-house standard of natural staphylokinase, which was assigned an activity of 100,000 home units/mg of protein as determined by amino acid analysis (25).

Association rate constants (k+), dissociation rate constants (k−), and affinity constants (K = k−/k+) for the interactions between different staphylokinase moieties and pNP-Ala44 or VFK-plasmin were determined by real time biospecific interaction analysis using the BIACore instrument (Pharmacia) as described elsewhere (26). The kinetic parameters for the hydrolysis of S-2403 (final concentration, 0.05–1 mM) by plasmin-SakSTAR complexes (final concentration, 5 and 10 nM) were determined by linear regression analysis (24). STAR complexes (final concentration, 5 nM) were monitored continuously in the presence of S-2403 (final concentration, 0.3 mM), and the apparent second-order inhibition rate constants were calculated as described previously (6).

Construction of SakSTAR Mutants—In total, 20 SakSTAR mutants were constructed in which clusters of two or three charged residues were converted to alanine (Fig. 1). Eleven SakSTAR mutants (Table I) were constructed at the Center for Molecular and Vascular Biology in Leuven, Belgium, by site-directed mutagenesis, using the pMa vector and the repair-deficient E. coli strain WKG/MutS. Therefore, a 453-base pair EcoRI-HindIII fragment containing the entire coding region of SakSTAR was cut out of the plasmid pME602SAKB and cloned into the XmnI site of pMc-SakSTAR, which lacks the promoter. The COOH-terminal SakSTAR-encoding fragment was generated by polymerase chain reaction in pMEX6 using the l- and u-primers listed in Table I. The l- and u-primers were used to generate the NH2-terminal SakSTAR-encoding fragment (N-fragment). The d-primer carrying the coding sequence for the charge to Ala mutation and the r-primer were used to generate the modified COOH-terminal SakSTAR-encoding fragment. The fragments were trimmed by appropriate restriction enzymes and cloned into the EcoRI-HindIII-digested expression vector pME6X.

To construct mutants SakSTAR118 and SakSTAR120, the NH2-terminal SakSTAR-encoding fragments were generated by polymerase chain reaction in pME6X using the l- and u-primers listed in Table I. The COOH-terminal SakSTAR-encoding fragments were generated by annealing the complementary oligonucleotides (I-1 and I-2), encoding the charge to Ala modification and extending upstream to a prodruding end compatible with the Xmal site induced in the codon for Pro118 with the u-primer and downstream to the StyI site of SakSTAR. The EcoRI-Xmal digested NH2-terminal SakSTAR-encoding fragment and the COOH-terminal SakSTAR-encoding oligonucleotides were ligated in EcoRI-Styl-digested pME602SAKB. Mutant SakSTAR135 was generated by digestion of pME602SAKB with StyI and PstI, and ligation of the annealed complementary oligonucleotides I-1 and I-2 (Table I), with protruding StyI and PstI compatible ends, into the linearized vector.

Expression and Purification of SakSTAR Mutants—The mutated HindIII-EcoRI fragment was ligated back into the pME602SAKB expression vector with the pTaq promoter. The mutant proteins were produced intracellularly in soluble form in E. coli WKG cells transformed with this vector. SakSTAR mutants, as well as wild-type protein, were purified from the sonicated bacterial extracts by cation-exchange chromatography on Sephadex SP-50 and hydrophobic interaction chromatography on phenyl-Sepharose (9). Alternatively, mutants with a significantly decreased specific activity as compared with wild-type SakSTAR were also prepared with the freeze-thawing method, as described previously (27).

Complex Formation of SakSTAR Mutants with Plasminogen—The time course of active site generation in equimolar complexes of plasminogen with SakSTAR was monitored by titration with NPGB at room temperature, as described elsewhere (4, 28, 29). Concentrated stock

FIG. 1. Schematic representation of the primary structure of SakSTAR mutants. The clusters of charged amino acids that were mutated to Ala are identified by their mean residue number.
The clusters of charged amino acids are identified by their mean residue number; amino acids that were mutagenized to Ala are underlined. The nucleotide sequence, in antisense direction from 3' to 5' end, represents the oligonucleotide used for site-directed mutagenesis. New restriction sites are represented in boldface and are indicated by the dash. u, d, upward or downward polymerase chain reaction primer in the left flanking region of the pMEX6 vectors outside of the Sak gene.

For kinetic analysis of plasminogen activation, equimolar plasminogen solution (final concentration, 20 nM) by SakSTAR (final concentration, 5 μM) was monitored at 37°C in 0.1M phosphate buffer, pH 7.4, and generated plasmin was measured at different time intervals (0–4 min) with S-2403. Initial activation rates were obtained from linear plots of the concentration of generated plasmin versus time. Kinetic parameters (Km and kcat) were determined from Lineweaver-Burk plots by linear regression analysis. With wild-type SakSTAR, SakSTAR13, SakSTAR48, and SakSTAR67, a similar kinetic analysis was performed with low M, plasminogen.

In separate experiments, the rate of the conversion of single chain rPlg-Ala342 to two chain rPl-Ala442 either free or in an equimolar complex with SakSTAR13 (final concentration, 3 μM) by addition of preformed plasmin-SakSTAR13 complex (final concentration, 20 nM) was monitored as a function of time. Therefore, samples were removed from the incubation mixtures at different time points (0–35 min) and subjected to SDS-PAGE under reducing conditions followed by quantitation of generated rPl-Ala442 by densitometric scanning.

**RESULTS**

**Production of SakSTAR Mutants**

Mutant SakSTAR moieties were purified from E. coli WK6 cell culture medium with yields of 2–250 mg/l (Table I), recovering 17–88% of SakSTAR-related antigen. Wild-type SakSTAR was obtained in the same way with a yield of 83 mg/l, representing a recovery of 15%. SakSTAR100a could not be obtained because of a very low expression level, and SakSTAR100b could not be purified because of apparent instability of the expressed protein, as shown by SDS-PAGE of crude cell extracts. Therefore, samples were removed from the incubation mixtures at different time points (0–35 min) and subjected to SDS-PAGE under reducing conditions followed by quantitation of generated rPl-Ala442 by densitometric scanning.

**TABLE I**

| Mutant | Mean residue number | Oligonucleotide | Restriction site | Yield (mg/l) |
|--------|---------------------|-----------------|-----------------|-------------|
| D3SA K6A | 5 | GTTACAGTTCAGAATGCGCGCCCTTTTATATTTTTTGCC | Sadv1 | 13 |
| K8A K10A | 9 | TTATTTTGCAGGGGCGCGCCCGTGAAT | Narl1 | 4 |
| K11A D13A D14A | 13 | CAATATCAGGTCACGGTTTTTTTTTGC | Sadv1 | 47 |
| K3SA E38A | 37 | TAATACGACGAGCTGAGTTTTTTTTTGCC | Nsil | 130 |
| E46A K50A | 48 | AATTACGAGCTGACGAGTTTTTTTTTGCC | Smgl | 40 |
| E61A E65A | 63 | AAATATAGTGGCTACGGTTTTTTTTTGCC | Sadv2 | 36 |
| E65A D69A | 67 | AATTATAGTGGCTACGGTTTTTTTTTGCC | Narl2 | 17 |
| K74A E75A R77A | 75 | TATATATCTCAGTGGCTACGGTTTTTTTTTGCC | Nsil2 | 33 |
| E80A D82A | 78 | TATATATCTCAGTGGCTACGGTTTTTTTTTGCC | Narl3 | 25 |
| K86A E88A | 87 | TATATATCTCAGTGGCTACGGTTTTTTTTTGCC | Xmnl | b |
| E99A E100A K102A | 100b | TATATATCTCAGTGGCTACGGTTTTTTTTTGCC | (removed) | |

**Notes:**

*a* Concentration of the purified proteins was determined according to Bradford (23), and yields are expressed in mg/liter of culture medium.

*b* This mutant could not be obtained because of apparent protein instability as shown by SDS-PAGE of crude cell extracts.

*c* This mutant could not be obtained because of a very low expression level.

solutions of plasminogen and SakSTAR were diluted in 0.1 M Veronal buffer, pH 8.3, containing 0.1 M arginine, to final concentrations of 4.0 and 3.6 μM, respectively. At different time points after mixing of plasminogen and SakSTAR (0–60 min), NPGB was added to a final concentration of 100 μM.

The stability of equimolar plasmin-SakSTAR complexes was analyzed by incubation of 0.5 ml of a 3 μM solution in 0.1 M phosphate buffer, pH 7.4, for 1 h at 4°C with 75 mg of suction-dried lysine-Sepharose gel. After excessive washing, the gel was eluted with 50 ml of 6-aminohexanoic acid in 0.1 M phosphate buffer, pH 7.4, and the distribution of SakSTAR between the unbound fraction and the eluate was monitored by enzyme-linked immunosorbent assay and SDS-PAGE.

**Activation of Plasminogen by SakSTAR Mutants**—Activation of plasminogen (final concentration, 1 μM) by SakSTAR (final concentration, 5 nM) was monitored at 37°C in 0.1 M phosphate buffer, pH 7.4, containing 0.01% Tween 80, by measurement of the generated plasmin concentration at different time intervals (0–50 min) with S-2403 (final concentration, 0.3 μM), as described previously (6). With SakSTAR13, SakSTAR48, and SakSTAR67, plasminogen activation was also monitored after addition of traces of plasmin (0–3% (mol/mol)) to the plasminogen solution.

For kinetic analysis of plasminogen activation, equimolar plasminogen-SakSTAR complexes (final concentration, 2 μM) were prepared by incubation of plasminogen with the SakSTAR mutants at 37°C for 5–30 min in 0.1 M phosphate buffer, pH 7.4, containing 25% glycerol; the mixtures were then stored on ice. Plasmin-SakSTAR complex (final concentration, 10–20 μM) was incubated with plasminogen (1–33 μM) at 37°C in 0.1 M phosphate buffer, pH 7.4, and generated plasmin was measured at different time intervals (0–4 min) with S-2403. Initial activation rates were obtained from linear plots of the concentration of generated plasmin versus time. Kinetic parameters (Km and kcat) were determined from Lineweaver-Burk plots by linear regression analysis. With wild-type SakSTAR, SakSTAR13, SakSTAR48, and SakSTAR67, a similar kinetic analysis was performed with low M, plasminogen.

In separate experiments, the rate of the conversion of single chain rPlg-Ala342 to two chain rPl-Ala442 either free or in an equimolar complex with SakSTAR13 (final concentration, 3 μM) by addition of preformed plasmin-SakSTAR13 complex (final concentration, 20 nM) was monitored as a function of time. Therefore, samples were removed from the incubation mixtures at different time points (0–35 min) and subjected to SDS-PAGE under reducing conditions followed by quantitation of generated rPl-Ala442 by densitometric scanning.

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Mutant SakSTAR moieties were purified from E. coli WK6 cell culture medium with yields of 2–250 mg/l (Table I), recovering 17–88% of SakSTAR-related antigen. Wild-type SakSTAR was obtained in the same way with a yield of 83 mg/l, representing a recovery of 15%. SakSTAR100a could not be obtained because of a very low expression level, and SakSTAR100b could not be purified because of apparent instability of the expressed protein, as shown by SDS-PAGE of crude cell extracts (data not shown). NH2-terminal amino acid analysis (data not shown) and sequencing of crude cell extracts (data not shown) revealed one homogeneous sequence (with yields in parentheses) corresponding to Ser(184)-Ser(169)-Ser(158)-Ser(142)-Ser(126)-Ser(110)-Ser(94)-Ser(78)-Ser(62)-Ser(46)-Ser(30)-Ser(14)-Ser(2) (data not shown).
Clustered Charge to Alanine Scan of Staphylokinase

The specific activities and affinity for binding to plasminogen of SakSTAR mutants are summarized in Table II. Two mutants (SakSTAR9 and SakSTAR97) had a specific activity of 20–25% of that of wild-type SakSTAR and of 18 mutants, determined with a plasminogen-coupled chromogenic substrate assay, are summarized in Table II. The data represent mean ± S.D. of three determinations.

### Table II

| SakSTAR Mutant | Specific Activityb | Binding to plasminogenb | Ka (×10⁵ M⁻¹) | kd (×10⁻⁶ s⁻¹) | Kd (×10⁶ M⁻¹) |
|----------------|--------------------|-------------------------|---------------|----------------|---------------|
| Wild-type      | 120 ± 5            | 120 ± 24                | 0.49 ± 0.25   | 2.4 ± 1         |
| SakSTAR5       | 150 ± 14           | 410 ± 10                | 0.68 ± 0      | 5.9 ± 0.1       |
| SakSTAR9       | 27 ± 2             | 140 ± 7                 | 0.28 ± 0      | 4.9 ± 0.2       |
| SakSTAR13      | 3.8 ± 0.7          | 130 ± 1                 | 0.58 ± 0.05   | 2.2 ± 0.2       |
| SakSTAR34      | 94 ± 29            | 505 ± 9                 | 0.46 ± 0.02   | 1.08 ± 0.1      |
| SakSTAR37      | 110 ± 7            | 90 ± 10                 | 0.31 ± 0.02   | 0.40 ± 0.40     |
| SakSTAR48      | 11.1 ± 0.2         | 15 ± 3                  | 1.5 ± 0.9     | 0.11 ± 0.02     |
| SakSTAR58      | 94 ± 3             | 120 ± 10                | 0.40 ± 0.00   | 2.9 ± 0.3       |
| SakSTAR63      | 130 ± 24           | 120 ± 2                 | 0.34 ± 0      | 3.5 ± 0         |
| SakSTAR67      | 0.2 ± 0.02         | 29 ± 6                  | 1.4 ± 0.11    | 20 ± 0.03       |
| SakSTAR75      | 93 ± 13            | 100 ± 7                 | 0.20 ± 0.01   | 5.0 ± 0.40      |
| SakSTAR81      | 120 ± 5            | 100 ± 10                | 0.41 ± 0.02   | 2.5 ± 0.10      |
| SakSTAR87      | 95 ± 9             | 120 ± 9                 | 0.37 ± 0.01   | 3.5 ± 0.30      |
| SakSTAR94      | 100 ± 14           | 79 ± 8                  | 0.87 ± 0.06   | 0.92 ± 0.07     |
| SakSTAR97      | 29 ± 2             | 120 ± 6                 | 0.45 ± 0      | 2.7 ± 0.10      |
| SakSTAR109     | 70 ± 8             | 130 ± 20                | 0.28 ± 0.02   | 4.5 ± 0.30      |
| SakSTAR118     | 52 ± 5             | 170 ± 0.8               | 0.24 ± 0.01   | 2.1 ± 0.30      |
| SakSTAR120     | 100 ± 12           | 160 ± 10                | 0.66 ± 0.03   | 2.4 ± 0.20      |
| SakSTAR135     | 120 ± 8            | 140 ± 10                | 0.66 ± 0.01   | 1.9 ± 0.10      |

a Determined with the plasminogen-coupled chromogenic substrate assay.

b Association rate constants (ka), dissociation rate constants (kd), and affinity constants (Kd) for binding to rPlg-Ala741 were determined by biospecific interaction analysis.

Functional Characterization of SakSTAR Mutants

**Specific Activity**—The specific activities of wild-type SakSTAR and of 18 mutants, determined with a plasminogen-coupled chromogenic substrate assay, are summarized in Table II. Two mutants (SakSTAR9 and SakSTAR97) had a specific activity of 20–25% of that of wild-type SakSTAR and three mutants (SakSTAR13, SakSTAR48, and SakSTAR67) had a specific activity of ≈3% of that of wild-type, whereas the specific activities of the remaining 13 mutants were less than 2.5-fold different from the wild-type molecule.

**Affinity for Binding to Plasminogen**—Association and dissociation rate constants (ka and kd) and apparent affinity constants (Kd) for binding of SakSTAR moieties to rPlg-Ala741, as measured by biospecific interaction analysis, are summarized in Table II. The Kd value of SakSTAR48 and SakSTAR67 was, respectively, 22- and 12-fold lower than that of wild-type SakSTAR, as a result of a more than 4-fold lower ka and an approximately 3-fold higher kd. Kd values of the other mutants ranged between 1 × 10⁸ and 6 × 10⁹ M⁻¹, as compared with 2.4 × 10⁸ M⁻¹ for wild-type SakSTAR, SakSTAR13, with a low specific activity, had an intact affinity for plasminogen (Kd = 2.2 × 10⁸ M⁻¹).

Binding of SakSTAR13 or SakSTAR67 to VFK-plasmin occurred with a 3.5- or a 2.3-fold lower affinity than binding of wild-type SakSTAR. Binding of SakSTAR13 was characterized by (mean ± S.D.; n = 3) ka = 4.4 ± 0.12 × 10⁸ M⁻¹s⁻¹ and kd = 19 ± 0.9 × 10⁻⁴ s⁻¹, yielding Kd = 2.4 ± 0.2 × 10⁸ M⁻¹, as compared with ka = 2.1 ± 4.3 × 10⁸ M⁻¹s⁻¹ and kd = 58 ± 1.2 × 10⁻⁴ s⁻¹, resulting in Kd = 3.6 ± 0.67 × 10⁻⁶ M⁻¹ for SakSTAR67. For wild-type SakSTAR, ka = 3.4 ± 0.42 × 10⁸ M⁻¹s⁻¹, kd = 4.3 ± 1.2 × 10⁻⁴ s⁻¹, and Kd = 8.4 ± 1.6 × 10⁸ M⁻¹. Binding of SakSTAR48 to VFK-plasmin was characterized by a 9-fold lower Kd (0.93 ± 0.37 × 10⁸ M⁻¹) as compared with binding of wild-type SakSTAR, mainly as a result of a higher ka value (130 ± 4.0 × 10⁻⁶ s⁻¹) with a comparable ka value (12 ± 4.4 × 10⁻⁶ M⁻¹s⁻¹).

**Active Site Exposure in Equimolar Mixtures with Plasminogen**—In equimolar mixtures of plasminogen and SakSTAR, the active site, as monitored by titration with NPGB, was rapidly exposed with wild-type SakSTAR and all its mutants, with the exception of SakSTAR48 and SakSTAR67 (Fig. 2). Indeed, active site exposure in mixtures of plasminogen (final concentration, 3.6 μM) and wild-type SakSTAR (final concentration, 4 μM) occurred with a lag phase (determined as the abscissa intercept of the linear phase of plots of generated plasminogen vs. time) of 7 min, and with a rate (determined from the maximal slope) of 42 nm⁻¹s⁻¹, resulting in quantitative active site exposure. With SakSTAR13, corresponding values were 1 min for the lag phase and 21 nm⁻¹s⁻¹ for the rate, resulting in 97% active site exposure. SakSTAR48 showed a delayed active site exposure, with a lag phase of 20 min and a rate of 15 nm⁻¹s⁻¹, resulting in quantitative active site exposure. In contrast, with SakSTAR67, no significant complex formation was observed after 60 min (Fig. 2). All other mutants were comparable with wild-type SakSTAR (data not shown).

**Stability of Plasmin-SakSTAR Complexes**—Adsorption of preformed equimolar mixtures of plasminogen and wild-type SakSTAR, SakSTAR13, SakSTAR48, or SakSTAR67 to lysine-Sepharose followed by elution with 6-aminohexanoic acid re-
Activation of Plasminogen—Catalytic amounts of wild-type SakSTAR induced rapid activation of plasminogen to plasmin (Fig. 3), with a lag phase (determined as the abscissa intercept of the linear phase of plots of generated plasmin concentration versus time) of 2.5 min and a rate (determined from the slope of the linear phase of these plots) of 1.4 nmol s⁻¹, resulting in approximately 80% plasminogen activation within 15 min. In contrast, SakSTAR13, SakSTAR48, and SakSTAR67 did not induce measurable plasmin generation within 50 min (detection limit ≤ 0.03 nmol s⁻¹). Additional traces of plasmin to the plasminogen solution prior to the addition of SakSTAR, SakSTAR13, SakSTAR48, or SakSTAR67 resulted in an enhanced activation rate of plasminogen, comparable or somewhat more rapid than that obtained by the addition of equimolar preformed plasmin-SakSTAR complexes (data not shown). All other SakSTAR mutants induced extensive plasminogen activation (71–108% of maximal), with lag phases ranging between 1.5 and 17 min and rates ranging between 0.77 and 2.3 nmol s⁻¹ (data not shown). SDS-PAGE under reducing conditions (Fig. 3, inset) confirmed quantitative conversion of plasminogen to plasmin with wild-type SakSTAR, but no conversion with SakSTAR13, SakSTAR48, or SakSTAR67.

Activation of plasminogen by preformed plasmin-SakSTAR complexes obeyed Michaelis-Menten kinetics, as revealed by linear double-reciprocal plots of the initial activation rate versus the concentration of generated plasmin was quantitated as a function of time with S-2403. The data represent mean ± S.D. of three experiments. The inset shows SDS-PAGE (10–15% gradient gels) under reducing conditions of samples taken at time 0 (lane 1) and 20 min (lane 2) for wild-type SakSTAR and at 50 min for SakSTAR13 (lane 3), SakSTAR48 (lane 4) and SakSTAR67 (lane 5). Lane 6 represents a protein calibration mixture, as in Fig. 2. Plg, plasminogen; PliA, plasmin A-chain; PliB, plasmin B-chain.

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Activation rate of plasminogen (final concentration, 1 μM) by wild-type SakSTAR (●), SakSTAR13 (▲), SakSTAR48 (■), and SakSTAR67 (◇) (final concentration, 5 nm each). The concentration of generated plasmin was quantitated as a function of time with S-2403. The data represent mean ± S.D. of three experiments. The inset shows SDS-PAGE (10–15% gradient gels) under reducing conditions of samples taken at time 0 (lane 1) and 20 min (lane 2) for wild-type SakSTAR and at 50 min for SakSTAR13 (lane 3), SakSTAR48 (lane 4) and SakSTAR67 (lane 5). Lane 6 represents a protein calibration mixture, as in Fig. 2. Plg, plasminogen; PliA, plasmin A-chain; PliB, plasmin B-chain.

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Catalytic efficiency of preformed plasmin-SakSTAR complexes for the activation of plasminogen. The values obtained with the mutants (identified by the mean residue number) are expressed as a ratio with wild-type SakSTAR.

Conversion of rPlg-Ala⁷⁴¹ (final concentration, 3 μM) to rPli-Ala⁷⁴¹ by catalytic amounts (20 nm) of preformed plasmin-SakSTAR13 complex occurred with an initial rate of 0.37 nmol s⁻¹ and reached a maximum of 270 nmol after 12 min. In contrast, conversion to Pli-Ala⁷⁴¹ in the rPlg-Ala⁷⁴¹-SakSTAR13 complex was much more efficient, occurring with an initial rate of 1.4 nmol s⁻¹ to reach a maximum of 1,700 nmol after 20 min. Plasmin did not significantly convert rPlg-Ala⁷⁴¹ to rPli-Ala⁷⁴¹ (Fig. 5).

Activation of low M₄ plasminogen by preformed plasmin-SakSTAR complexes also obeyed Michaelis-Menten kinetics. The kinetic parameters of the plasmin-SakSTAR13 complex (Kₘ = 4.0 μM and kₘ = 0.08 s⁻¹) and of the wild-type plasmin-SakSTAR complex (Kₘ = 5.3 μM and kₘ = 1.6 s⁻¹) for activation of low M₄ plasminogen were comparable with those for the activation of intact plasminogen, as reported above. In contrast, activation of low M₄-plasminogen by the plasmin-SakSTAR48 and plasmin-SakSTAR67 complexes was characterized by higher kₘ values (25 μM and >60 μM, respectively) and comparable or higher kₘ values (0.2 s⁻¹ and >0.2 s⁻¹) than those for activation of intact plasminogen. Thus, the catalytic efficiencies for activation of low M₄ plasminogen were about 2-fold higher for wild-type plasmin-SakSTAR, plasmin-SakSTAR13, and plasmin-SakSTAR48 and about 2-fold lower for plasmin-SakSTAR67, as compared with those for activation of intact plasminogen.

The activity of preformed plasmin-SakSTAR complexes toward S-2403, as determined by Lineweaver-Burk analysis, was...
Clustered Charge to Alanine Scan of Staphylokinase

The finding that mutations in the regions of amino acids 11–14, 46–50, and 65–69 affected the interaction with plasminogen in different aspects suggested that mutants SakSTAR13, SakSTAR48, and SakSTAR67 may be useful to study different steps in the mechanism of the interaction of SakSTAR with plasminogen. The following kinetic model has been proposed for the activation of plasminogen by SakSTAR (4).

\[
(P + S \rightleftharpoons p + S) \\
\text{p} + S \rightleftharpoons \text{p} \cdot S \\
\text{p} \cdot S \rightleftharpoons \text{p} + \text{S}
\]

Plasminogen (P) and SakSTAR (S) produce an inactive 1:1 stoichiometric complex (P-S), which does not activate plasminogen, as demonstrated by titration with NPGB. The activation reaction appears to be initiated by trace amounts of contaminating plasmin (p), which generates pS, which converts P to p and PS to pS. According to this model, SakSTAR48 and SakSTAR67, with a 10–20-fold reduced affinity for binding to plasminogen, have an impaired formation of pS and PS complexes, and thus of subsequent conversion of P to p and of PS to pS. This is confirmed by our findings that no active site exposure occurred in equimolar mixtures of plasminogen with SakSTAR67, whereas with SakSTAR48, active site exposure was markedly delayed as compared with wild-type SakSTAR (Fig. 2). With SakSTAR13, which had a normal affinity for plasminogen, formation of pS and of PS was apparently normal, as was conversion of PS to pS, but conversion of P to p was markedly impaired.

Preformed complexes of SakSTAR13, SakSTAR48, or SakSTAR67 with plasmin (pS) had a comparable affinity for the plasminogen substrate as wild-type SakSTAR (K_m values for plasminogen activation of 5–12 μM) but a much lower catalytic rate constant for plasminogen activation (k_cat of 0.03–0.11 s⁻¹), as compared with 1.7 s⁻¹, resulting in 14–28-fold lower catalytic efficiencies. These findings explain why catalytic amounts of SakSTAR13 (normal formation of pS complex, but low enzymatic activity) or of SakSTAR48 and SakSTAR67 (delayed pS formation) do not induce measurable plasminogen activation.

The finding of rapid and quantitative formation of pS in equimolar mixtures of plasminogen and SakSTAR13, despite the low catalytic efficiency of the pS complex of this mutant for activation of P, can be explained by the observation that the conversion of P-S to pS by catalytic amounts of preformed pS is much more efficient than conversion of P to p. The finding that the rate of plasminogen activation by catalytic amounts of SakSTAR48 and SakSTAR67 is enhanced by the addition of traces of plasmin, from undetectable to a rate similar to that observed with their preformed complexes with plasmin, suggests that, under these conditions, active pS is formed, not as a result of conversion of P-S to pS but of direct formation of the pS complex between added plasmin and SakSTAR. However, the rate of plasmin generation is still much lower than that observed in control experiments with wild-type SakSTAR because of the low catalytic efficiency of the mutant pS complexes. The affinities of SakSTAR48 and SakSTAR67 for VFK-plasmin are 10–20-fold higher than for plasminogen, which is confirmed by the observation that preformed complexes of plasmin with SakSTAR13, SakSTAR48, and SakSTAR67 are stable following adsorption onto lysine-Sepharose.

Taken together, these data further demonstrate that the running concentration of active pS determines the rate of conversion.

Fig. 5. Conversion of rPlg-Ala44 (final concentration, 3 μM) either free (2) or in the rPlg-Ala44-SakSTAR13 complex (2) to rPli-Ala741 by plasmin-SakSTAR13 complex (final concentration 20 nM) monitored as a function of time. A control experiment with addition of plasmin to rPli-Ala741 is also shown (4). The data were obtained by densitometric scanning of SDS-PAGE (10–15% gradient gels) under reducing conditions, as illustrated in the inset for samples taken at 16 min from the mixture of rPlg-Ala44 and plasmin-SakSTAR13 (lane 1), the mixture of rPlg-Ala44-SakSTAR13 complex, and plasmin-SakSTAR13 (lane 2), or the mixture of rPli-Ala741 and plasmin (lane 3). Lane 4 represents a protein calibration mixture, as in Fig. 2. rPlg, rPlg-Ala44; PliA, A-chain of rPli-Ala741; PliB, B-chain of rPli-Ala741.

comparable for wild-type SakSTAR and all the mutants. K_m values for the mutants ranged between 120 and 390 μM, as compared with 240 μM for wild-type SakSTAR, and k_cat values ranged between 50 and 100 s⁻¹, as compared with 63 s⁻¹ for wild-type SakSTAR, yielding catalytic efficiencies of 0.16–0.62 μM⁻¹ s⁻¹ for the mutants, as compared with 0.26 μM⁻¹ s⁻¹ for wild-type SakSTAR (data not shown).

Inhibition of Plasmin-SakSTAR Complexes by α2-Antiplasmin—The second-order inhibition rate constants (k_inh) for the inhibition of preformed mutant plasmin-SakSTAR complexes by α2-antiplasmin ranged between (mean ± S.D.; n = 3 or 4) 1.6 ± 0.05 × 10⁶ and 2.6 ± 0.08 × 10⁶ M⁻¹ s⁻¹, as compared with 2.0 ± 0.01 × 10⁶ M⁻¹ s⁻¹ for the wild-type plasmin-SakSTAR complex (data not shown).

DISCUSSION

In the present study, structure-function relationships in SakSTAR, which determine its interaction with plasminogen, were investigated by construction of mutants in which clusters of two or three charged amino acids were mutated to alanine. A clustered charge to alanine scan approach has previously been used to study structure-function relationships in other plasminogen activators, e.g., tissue-type plasminogen activator (30) and urokinase-type plasminogen activator (31).

Twenty mutants were designed, two of which could not be obtained because of a very low expression level or of protein instability (SakSTAR100a with E99A and E100A substitution, and SakSTAR100b with E99A, E100A, and K102A substitution). Out of 18 mutants that were studied in detail, only three (SakSTAR13, SakSTAR48, and SakSTAR67) were markedly different from wild-type SakSTAR with respect to their interaction with plasminogen. This was revealed by a specific activity of ≥3% of that of wild-type, the absence of measurable plasminogen activation by catalytic amounts of the mutants, and a 10–20-fold lower catalytic efficiency of preformed complexes with plasmin for the activation of plasminogen. Furthermore, two of these mutants (SakSTAR48 and SakSTAR67) had a 10–20-fold reduced affinity for binding to plasminogen as compared with wild-type SakSTAR, and one mutant (SakSTAR67) did not induce active site exposure in equimolar mixtures with plasminogen.

The finding that mutations in the regions of amino acids 11–14, 46–50, and 65–69 affected the interaction with plasminogen in different aspects suggested that mutants SakSTAR13, SakSTAR48, and SakSTAR67 may be useful to study different steps in the mechanism of the interaction of SakSTAR with plasminogen. The following kinetic model has been proposed for the activation of plasminogen by SakSTAR (4).

\[
(P + S \rightleftharpoons p + S) \\
\text{p} + S \rightleftharpoons \text{p} \cdot S \\
\text{p} \cdot S \rightleftharpoons \text{p} + \text{S}
\]
conversion of P to p and of P-S to pS. In the presence of excess p-inhibitor (e.g. NPGB), P-S and P cannot be converted because of lack of active pS. In the absence of inhibitor, the reaction most likely is initiated by trace amounts of p that form p-S that convert both P to p and, more rapidly, P-S to pS. Indeed, a contamination of 30 ppm of p-S is sufficient to explain the kinetics of activation of P by SakSTAR (4).

In summary, mutagenesis in the regions 11–14, 46–50, or 65–69 of SakSTAR resulted in impairment of its interaction with plasminogen. These mutants have allowed to identify different steps of the interaction between plasminogen and SakSTAR in functional isolation.

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