Staphylokinase Requires NH\textsubscript{2}-terminal Proteolysis for Plasminogen Activation*

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Staphylokinase (Sak), a single-chain protein comprising 136 amino acids with NH\textsubscript{2}-terminal sequence, forms a complex with plasmin, that is endowed with plasminogen activating properties. Plasmin is presumed to process mature (high molecular weight, HMW) Sak to low molecular weight derivatives (LMW-Sak), primarily by hydrolyzing the Lys\textsuperscript{10}-Lys\textsuperscript{11} peptide bond, but the kinetics of plasminogen activation by HMW-Sak and LMW-Sak are very similar. Here, the requirement of NH\textsubscript{2}-terminal proteolysis of Sak for the induction of plasminogen activating potential was studied by mutagenesis of Lys\textsuperscript{10} and Lys\textsuperscript{11} in combination with NH\textsubscript{2}-terminal microsequence analysis of equimolar mixtures of Sak and plasminogen and determination of kinetic parameters of plasminogen activation by catalytic amounts of Sak. Substitution of Lys\textsuperscript{10} with Arg did not affect processing of the Arg\textsuperscript{10}-Lys\textsuperscript{11} site nor plasminogen activation, whereas substitution with His resulted in cleavage of the Lys\textsuperscript{11}Gly\textsuperscript{12} peptide bond and abolished plasminogen activation. Substitution of Lys\textsuperscript{11} with Arg did not affect Lys\textsuperscript{10}-Arg\textsuperscript{11} processing or plasminogen activation, whereas replacement with His did not prevent Lys\textsuperscript{11}His\textsuperscript{11} hydrolysis but abolished plasminogen activation. Substitution of Lys\textsuperscript{11} with Cys yielded an inactive processed derivative which was fully activated by aminooxylation. Deletion of the 10 NH\textsubscript{2}-terminal amino acids did not affect plasminogen activation, but additional deletion of Lys\textsuperscript{11} eliminated plasminogen activation.

Thus generation of plasminogen activator potential in Sak proceeds via plasmin-mediated removal of the 10 NH\textsubscript{2}-terminal amino acids with exposure of Lys\textsuperscript{11} as the new NH\textsubscript{2} terminus. This provides a structural basis for the hypothesis, derived from kinetic measurements, that plasminogen activation by Sak needs to be primed by plasmin and a mechanism for the high fibrin selectivity of Sak in a plasma milieu.

Staphylokinase (Sak),\textsuperscript{1} a 16-kDa single-chain protein specified by the sak gene encodes a protein of 163 amino acids, with amino acid 28 corresponding to the NH\textsubscript{2}-terminal residue of the mature secreted protein, which consists of 136 amino acids in a single polypeptide chain without disulfide bridges. Three natural variants Sak\textsubscript{C}, Sak42D, and SakSTAR have been identified which differ in only three amino acids; in the mature protein amino acid 34 is Ser in SakSTAR but Gly in Sak\textsubscript{C} and Sak42D, amino acid 36 is Gly in SakSTAR and in Sak\textsubscript{C}, but Arg in Sak42D, and amino acid 43 is His in SakSTAR and in Sak\textsubscript{C}, but Arg in Sak42D (4–6).

During plasminogen activation, mature Sak is converted to lower molecular weight (LMW) derivatives by removal of 10 (primarily) or 6 (secondarily) NH\textsubscript{2}-terminal amino acids (7, 8). These LMW derivatives have the same specific activity (7, 8) and fibrinolytic potential in human plasma (9) as the mature high molecular weight (HMW) protein. Conversion of HMW-Sak to LMW-Sak is presumed to be mediated by plasmin and...
The Sak42D fragment from plasmid Bst was investigated by studying the effect of substitution of lysing of Sak for the induction of plasminogen activator potential. Substitution of codons 5 to 13, substitution of codon 16 by TCA, and insertion of the spacer sequence GAA (encoding Glu) to produce the following construct with unique SfuI and MluI restriction sites.

The synthetic linker cassettes described in Table I were inserted into pMEXsak42D(Δ5–13) to generate genes encoding the Sak42D variants mentioned above. To this end, the pMEXsak42D(Δ5–13) acceptor plasmid was linearized with SfuI, and the 5'-phosphorylated linker pairs were annealed and ligated to the SfuI ends. The ligation mixtures were precipitated with isopropyl alcohol, and the pellets were resuspended in appropriate buffer and digested with MluI. Recombinant linearized plasmids were isolated by the glass milk method, recircularized by T4 DNA ligase, and transformed into E. coli (14). Mutant clones were preselected by SfuI restriction (deletion of the SfuI site) and identified by DNA sequencing using the standard protocol of the Sequenase kit.

### Experimental Procedure

**Materials—**
Enzymes and other reagents used for gene construction experiments were purchased from Boehringer (Mannheim, FRG). Sequenase was supplied by Amersham (Amersham Buchler, Braunschweig, FRG). The protein mixture for molecular weight calibration in SDS-PAGE was supplied by Life Technologies, Inc. (Eggenstein, FRG). The protein mixture used in IEF analysis was purchased from Pharmacia (Freiburg, FRG). Other components were of the highest quality commercially available. Oligonucleotides were synthesized using the phosphoramidite method with a DNA/RNA synthesizer model 394 (Aplicia (Freiburg, FRG)). Other components were of the highest quality commercially available.

Native human plasminogen was purified from human plasma according to Deutsch and Mertz (12). The synthetic plasminogen substrate S-2251 was purchased from Chromogenix (Essen, FRG). Recombinant Glu-plasminogen with the active Ser741 replaced by Ala, rPlg(S741A), was obtained and characterized as described elsewhere (13).

**Construction of Expression Plasmids Encoding Sak42D Variants—**

The present study was carried out with variants of Sak42D (5), derived from the expression plasmid pMEX602Sak42D (14). Variants Sak42DΔN10 and Sak42DΔN11 were generated as described elsewhere (11). The Sak42D gene, truncated at the 5' end was isolated as a BstUI-HindIII (containing codons 14/15 to 136 of the mature gene) fragment from plasmid pMEX602Sak42D. Synthetic oligonucleotides were used to introduce translation start signals and to reconstitute the 5'-coding sequences of the truncated Sak42DΔN10 and Sak42DΔN11, respectively (Table I). The appropriate linker pairs were phosphorylated at the EcoRI end and ligated to the EcoRI linearized expression vector pMEX6 (Medac, Hamburg, FRG). After HindIII digestion, the linearized vector DNA was purified by agarose gel electrophoresis. After recircularization using T4 DNA ligase, the expression plasmid containing the truncated sak gene was inserted into the vectors carrying linker cassettes. Finally the ligation mixtures were transformed into competent Escherichia coli TG1 cells (14).

Sak42DK10H, Sak42DK10R, Sak42DK11H, and Sak42DK11R were constructed by insertion of oligonucleotides into the acceptor plasmid pMEX602Sak42D expression plasmid by deletion of codons 5 to 13, substitution of codon 16 by TCA, and insertion of the spacer sequence GAA (encoding Glu) to produce the following construct with unique SfuI and MluI restriction sites.
of 0.6 m, 0.25 m, and 15 molar excess of bromoethylamine (Sigma, Deisenhofen, FRG) over at room temperature under an atmosphere of argon for 4 h. A 4-fold NAP 5 columns (Pharmacia, Freiburg, FRG) equilibrated with 20 mM finally, the protein moieties were separated from the reagents using immobilization procedure was carried out according to the recommendations of the manufacturer.

$\Delta$-Aminoethylation of Sak42D Variants—Samples of Sak42D and Sak42DK11C were modified by $\Delta$-aminoethylation essentially as described previously (15). Briefly, lyophilized Sak moieties were dissolved in 150 mM guanidinium hydrochloride solution to a protein concentration of 0.2–0.4 mg/ml. For the reduction of the protein samples, Tris, EDTA, and $\beta$-mercaptoethanol were added yielding final concentrations of 0.6 mM, 0.25 mM, and 15 mM, respectively. The probes were incubated at room temperature under an atmosphere of argon for 4 h. A 4-fold molar excess of bromoethylamine (Sigma, Deisenhofen, FRG) over $\beta$-mercaptoethanol was added in three portions at 10-min intervals. The samples were allowed to incubate overnight at room temperature. Finally, the protein moieties were separated from the reagents using NAP 5 columns (Pharmacia, Freiburg, FRG) equilibrated with 20 mM phosphate buffer, pH 6.0, containing 100 mM NaCl.

analytical Methods—Isoelectric focusing was carried out using the PhastSystem™, Dry IEF™ gels, and the IEF protein standard supplied by Pharmacia (Freiburg, FRG). The gels were rehydrated in 8 M urea containing 2.5% Pharmalyte 3–10™ (Pharmacia, Freiburg, FRG) yielding a pH gradient from 3 to 10. The NH$_2$-terminal amino acid sequences of all purified Sak42D variants were determined on the Applied Biosystems model 476A (Applied Biosystems). For the Sak species generated in equimolar (4 $\mu$m) mixtures with plasminogen after 5-min incubation at 37°C, high resolution electrophoresis was carried out with the Mini-Protean II system (Bio-Rad, Munich, FRG) using 16% T, 3% C gels in the discontinuous Tris-Tricine buffer system according to Schagger and von Jagow (16). Samples were applied to the gels after reduction by heating at 100°C for 5 min in the presence of 1% SDS and 0.4% $\beta$-mercaptoethanol. Following gel separation, the proteins were blotted onto polyvinylidene difluoride membranes (Millipore, Eschborn, FRG) and then subjected to amino acid sequencing.

Generation of Amidolytic Activity in Equimolar Mixtures of Sak42D Variants and Plasminogen—Amidolytic activity was quantitated with the chromogenic plasmin substrate S-2251 (final concentration 1 mM) and monitored at 405 nm for up to 12 min using a Spectronic 401 (Milton Bay, Analis Belgium) spectrophotometer or a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Plasminogen (final concentration 3.8 $\mu$m) was incubated with the Sak moieties (final concentration 4 $\mu$m) at 37°C in 100 mM phosphate buffer, pH 7.4, containing 0.01% Tween 80 (activation buffer). Samples were withdrawn at 2-minute intervals and diluted 40-fold in buffer containing chromogenic substrate S-2251, and the change in absorbance at 405 nm ($\Delta$A$_{405}$) was recorded.

Activation of Plasminogen by Catalytic Amounts of Sak42D Variants—The activation of plasminogen (final concentration 1.5 $\mu$m) by Sak42D moieties (final concentration 5 nM) was assayed at 37°C in activation buffer. At different time intervals up to 30 min, generated plasmin was measured with S-2251 substrate.

Kinetic Constants of Plasminogen Activation by Catalytic Amounts of Preformed Equimolar Complexes of Sak42D Variants with Plasmin—The kinetic constants of plasminogen activation by Sak-plasmin complexes were derived from Lineweaver-Burk plots. For this purpose, equimolar mixtures of Sak42D variants and plasminogen were preincubated for 5 min in activation buffer containing 25% glycerol (v/v) and kept on ice. For most mixtures, this preincubation resulted in complete conversion of single-chain plasminogen to two-chain plasmin (see Fig. 1). These preformed activator complexes (final concentration 10–40 nM) were then mixed with plasminogen (final concentration 0.25–10 $\mu$m), and generated plasmin was measured at 37°C from $\Delta$A$_{405}$ with S-2251.

Binding of Sak42D Variants to Plasminogen—Association rate constants ($k_a$) and dissociation rate constants ($k_d$) for the interaction between Glu-Plg or rPlg(S741A) and the Sak variants were determined by real-time biospecific interaction analysis using the BIAcore instrument (Pharmacia) (17). Glu-Plg or rPlg(S741A) were immobilized on the surface of sensor chip CM5 using the amine coupling kit (Pharmacia), as recommended by the manufacturer. This procedure links primary amino groups in the ligand to the carboxymethylated dextran surface of the sensor chip (18). Immobilization was performed from protein solutions with plasminogen.

Table II

| Variant      | NH$_2$-terminal sequence$^a$ | Maximal amidolytic activity (%) | Catalytic efficiency $k_{cat}/K_m$ | Binding to plasminogen $K_a$ (nM) |
|--------------|------------------------------|---------------------------------|-----------------------------------|----------------------------------|
| Sak42D       | SSFDFDKGYKMK/GDDA            | 100                             | 0.08                              | 0.44                             |
| Sak42DK10H   | SSFDFDKGYKMK/GDDA            | 190                             | <0.001                            | 0.39                             |
| Sak42DK11R   | SSFDFDKGYKMK/GDDA            | 120                             | 0.04                              | 0.68                             |
| Sak42DNA10   | MKGDDA                       | 150                             | 0.03                              | 0.64                             |
| Sak42DK11R   | SSFDFDKGYKMK/GDDA            | 150                             | <0.001                            | 0.30                             |
| Sak42DK11R   | SSFDFDKGYKMK/GDDA            | 220                             | 0.18                              | 0.35                             |
| Sak42DK11R   | SSFDFDKGYKMK/GDDA            | 220                             | 0.18                              | 0.35                             |
| Sak42DK11R   | SSFDFDKGYKMK/GDDA            | 240                             | 0.31                              | 0.30                             |
| Sak42DK11F   | SSFDFDKGYKMK/GDDA            | 240                             | 0.31                              | 0.30                             |
| Sak42DK12K   | SSFDFDKGYKMK/GDDA            | 62                              | <0.001                            | 0.71                             |
| Sak42DG12A   | SSFDFDKGYKMK/GDDA            | 130                             | 0.05                              | 0.50                             |

$^a$ The sequences are aligned with Ala$^{26}$ of the mature protein.
$^b$ The mutagenized amino acid is boldface.
$^c$ Sak42DK11E and Sak42DK11P yielded very similar results.

FIG. 1. SDS-PAGE of Sak42D variants and of their equimolar mixtures (4 $\mu$m) with plasminogen. Lane 1, Sak42D; lane 3, Sak42DK10H; lane 5, Sak42DK10R; lane 7, Sak42DK11H; lane 9, Sak42DK11R; lanes 2, 4, 6, 8, and 10, corresponding equimolar mixtures of variant in preceding lane with plasminogen; lane 11, molecular mass calibration; mixtures consisting of a protein ladder with 10-kDa steps.
RESULTS

Production and Characteristics of Sak42D Variants—The Sak42D variants were purified from E. coli TG1 cells with yields of 70 to 500 μg/liter culture, representing average recoveries of 22%. SDS gel electrophoresis (Fig. 1) displayed single bands with relative migrations corresponding to the expected molecular masses. NH$_2$-terminal amino acid sequencing revealed homogeneous sequences as illustrated in Table II, which confirmed that all Sak moieties, except Sak42DΔN10, were correctly processed in the E. coli expression system with removal of the NH$_2$-terminal Met.

Similar purities and expected NH$_2$-terminal sequences were obtained with the variants Sak42DK6H, Sak42DK8H, Sak42DK10Q, Sak42DK11E, Sak42DK11P, Sak42DK11C, Sak42DK11F, Sak42DG12A, Sak42DG12K, Sak42DΔN14, and Sak42DProc (not shown).

Functional Characterization of the Sak42D Variants—SDS gel electrophoresis of equimolar mixtures of plasminogen and Sak variants (Fig. 1) revealed processing of Sak (faster migration) and plasminogen activation (conversion of single-chain plasminogen to two-chain plasmin) with all substitution variants. This was confirmed by NH$_2$-terminal sequencing of the Sak components, as summarized in Table II.

Sak42D was exclusively processed at the Lys$^{10}$-Lys$^{11}$ peptide bond with exposure of Lys$^{11}$ as the new NH$_2$-terminal amino acid. The variants Sak42DK10R, Sak42DK11H, and Sak42DK11R were also hydrolyzed at the Lys$^{10}$-X$^{11}$ peptide bond (Sak42DK10R with some alternative processing at Lys$^8$-Tyr$^9$), whereas Sak42DK10H was hydrolyzed at the Lys$^{11}$-Gly$^{12}$ bond. The variants Sak42DK6H, Sak42DK8H, Sak42DG12A, and Sak42DG12K were also processed at the Lys$^{10}$-Lys$^{11}$ peptide bond, whereas Sak42DK10Q was hydrolyzed at the Lys$^{11}$-Gly$^{12}$ bond (not shown). Sak42DG12K showed a “partial processing” at the Lys$^{10}$-Lys$^{11}$ bond yielding a mixture of two components present in approximately equal amounts: the unprocessed protein and a processed form with two lysines at the NH$_2$-terminal positions.

Generation of maximal amidolytic activity occurred within 4 to 6 min (Fig. 2). The maximal amidolytic activity of equimolar mixtures of plasminogen with variants Sak42DK10H, Sak42DK10Q, Sak42DK11E, Sak42DK11P, Sak42DK11C, Sak42DK11F, Sak42DG12A, Sak42DG12K, Sak42DΔN14, and Sak42DProc was at least equal to and up to 2-fold higher than that of Sak42D.

Sak processing and plasminogen activation with generation of amidolytic activity similar to or higher than that obtained with Sak42D was observed with Sak42DProc, Sak42DK10Q, Sak42DK11F, Sak42DK11C, Sak42DG12A, and Sak42DΔN14. Reduced amidolytic activity (≤70% of Sak42D) was generated with Sak42DK6H, Sak42DK8H, Sak42DK11E, and Sak42DK11P (not shown).
Activation of Plasminogen by Catalytic Amounts of Sak Variants—Catalytic amounts of Sak42D induced progressive activation of plasminogen to plasmin (Fig. 3), with a lag phase of 5 min, reaching a maximal rate (determined from the slope at the inflection point of these curves) after 15 to 20 min. Plasminogen activation was similar to that of Sak42D with Sak42DK10N, Sak42DK10R, and Sak42DK11R, but was virtually absent with Sak42DK10H, Sak42DK11H, and Sak42DK11N. In addition, plasminogen activation was comparable to that of Sak42D with Sak42DK6H, Sak42DK8H, Sak42DProc, and Sak42DG12A, but was virtually absent with Sak42DK10Q, Sak42DK11E, Sak42DK11P, Sak42DK11F, Sak42DK11C, Sak42DG12K, and Sak42DK11N (not shown).

Activation of plasminogen by preformed Sak-plasmin complexes obeyed Michaelis-Menten kinetics, as revealed by linear double-reciprocal plots of the initial activation rate versus the plasminogen concentration (not shown). The catalytic efficiency \( k_{cat}/K_m \) ranged between 0.03 and 0.18 nM\(^{-1}\)s\(^{-1}\) for Sak42D, Sak42DK10R, Sak42DK11R, and Sak42DK11N but was 0.002 nM\(^{-1}\)s\(^{-1}\) for Sak42DK10H, Sak42DK11H, and Sak42DK11N (Table II). Catalytic efficiencies were within the normal range with Sak42DK6H, Sak42DK8H, Sak42DProc, and Sak42DG12A, but were <0.002 nM\(^{-1}\)s\(^{-1}\) with Sak42DK10Q, Sak42DK11E, Sak42DK11P, Sak42DK11F, Sak42DK11C, Sak42DG12K, and Sak42DK11N (not shown).

Binding of Sak Variants to Plasminogen—The apparent affinity equilibrium constants \( K_a \) for binding of Sak42D moieties to native Glu-Plg in the presence of excess plasmin inhibitor or to rPlg(S741A), are summarized in Table II. The \( K_a \) values of all Sak42D variants studied were very similar to those of wild-type Sak42D. The affinity constants for binding to native Glu-Plg were approximately 1000-fold lower than those for binding to rPlg(S741A), as has previously been observed (20).

Effect of S-Aminoethylation of Sak42DK11C on Plasminogen Activation—The extent of plasminogen activation within 30 min by catalytic amounts of Sak42DK11C was unmeasurable (Fig. 4), whereas its catalytic efficiency for plasminogen activation was ≤0.001 nM\(^{-1}\)s\(^{-1}\), both before and after processing on insolubilized plasminogen (Table III). S-Aminoethylation, which introduces a positive charge into the side chain of cysteine, increased the extent of plasminogen activation within 30 min to that observed with Sak42D and raised the catalytic efficiency of Sak42DK11C-plasmin complex to 0.13 nM\(^{-1}\)s\(^{-1}\), whereas wild-type Sak42D was not markedly affected by treatment with the aminoethylating reagent.

Isoelectric focusing (Fig. 5) revealed that the isoelectric point of Sak42D was not affected by S-aminoethylation. Sak42DK11C had a lower isoelectric point than Sak42D which was increased by S-aminoethylation. Processing with insolubilized plasminogen decreased the isoelectric points of Sak42D and Sak42DK11C to a similar extent (Fig. 5).

### Table III

| Plg activation (% wild-type) | \( k_{cat}/K_m \) (nM\(^{-1}\)s\(^{-1}\)) | Binding to plasminogen \( K_a \) (×10\(^{-5}\)) |
|-----------------------------|-----------------|------------------|
| Sak42D | Sak42DK11C | Sak42D | Sak42DK11C | Glu-Plg | rPlg(S741A) |
| Baseline | 100 | 0 | 0.08 | 0.001 | 0.44 | 0.29 |
| Aminoethylated | 65 | 88 | 0.04 | 0.13 | 0.30 | 0.27 |
| Processed | 69 | 0 | 0.11 | <0.001 | ND* | ND* |
| Processed then aminoethylated | ND* | 98 | ND* | 0.08 | ND* | ND* |

*ND, not determined.*

**FIG. 5. Isoelectric focusing (IEF) (pH range 3–10) of Sak42D and Sak42DK11C. Effects of S-aminoethylation (AE) and processing with insolubilized plasminogen (proc). Lane 1, IEF-standard: 1, trypsinogen, 9.30; 2, lentil lectin, 8.65; 3, lentil lectin, 8.15; 4, horse myoglobin, 7.35; 5, horse myoglobin, 6.85; 6, human carbonic anhydrase B, 6.55; 7, β-lactoglobulin, 7.20; 8, soybean trypsin inhibitor, 4.55; 9, amyloglucosidase, 3.50; lane 2, Sak42D; lane 3, Sak42DK11C; lane 4, Sak42DK11C(AE); lane 5, Sak42DK11C; lane 6, Sak42D(AE); lane 7, Sak42DK11C(AE); lane 8, Sak42DK11C(proc); lane 9, Sak42DK11C(proc, AE).**

**DISCUSSION**

The aim of the present study was to investigate the role of NH₂-terminal processing of staphylokinase (Sak) in plasminogen activation. Although conversion of native Sak with NH₂-terminal Ser-Ser- to a proteolytic derivative with NH₂-terminal Lys-Gly-Asp-, by plasmin-mediated removal of the NH₂-terminal 10 amino acids, was found not to be a rate-limiting step (10), deletion or substitution of Lys\(^{11}\) greatly reduced the plasminogen activator properties of the Sak variants (11). The requirement of NH₂-terminal processing of Sak for the induction of plasminogen activating potential was established by site-specific mutagenesis of Lys\(^{10}\) and Lys\(^{11}\) (P₁⁻P₁ positions according to Berger and Schechter (21)). Substitution of either amino acid with Arg did not affect hydrolysis of the P₁⁻P₁ peptide bond nor plasminogen activation, replacement of Lys\(^{10}\) with His resulted in cleavage of the Lys\(^{11}\)-Gly\(^{12}\) peptide bond and loss of plasminogen activating potential, whereas exchange of Lys\(^{11}\) with His produced cleavage of the Lys\(^{11}\)-His\(^{12}\) (P₁⁻P₁) peptide bond with generation of an inactive derivative. Furthermore, deletion of the 10 NH₂-terminal amino acids yielded a fully active Sak derivative, but additional deletion of Lys\(^{11}\) inactivated the molecule.

In aggregate, these data are compatible with the interpretation that Sak requires hydrolysis in the NH₂-terminal region with exposure of a positively charged amino acid at the new NH₂ terminus. This was confirmed using variant Sak42DK11C in combination with S-aminoethylation with 2-bromoethylamine, which introduces a positive charge in the side chain of cysteine converting it to "pseudolysine" (15). As anticipated, hydrolysis of the Lys\(^{10}\)-Cys\(^{11}\) (P₁⁻P₁) peptide bond with insolubilized plasminogen yielded an inactive derivative, which
could, however, be fully activated by S-aminomethylation. Conversely, S-aminomethylation before processing yielded a derivative which was processed to a fully active product.

The apparent discrepancy between the low plasminogen activating potential of catalytic amounts of processed variants lacking a positively charged NH$_2$-terminal amino acid and the full conversion of plasminogen to plasmin in equimolar mixtures can be explained by the fact that the affinity of the Sak42D variants for plasminogen is unaltered and that Sak-plasmin complex is a more potent activator of plasminogen bound to Sak than of free plasminogen. Consequently, even Sak42D variants with very poor plasminogen activating potential will rapidly convert plasminogen to plasmin in equimolar mixtures. The larger than expected variability in maximal amidolytic activity may predominantly contain Sak-plasmin complexes in which the active site of plasmin is not converted into the plasminogen activator configuration with a reduced amidolytic capacity.

Determination of the association equilibrium constants for the binding of the Sak42D variants to plasminogen revealed no significant differences with Sak42D, with respect neither to the previously observed low affinity binding to native Glu-Plg in the presence of excess plasmin inhibitor, nor to the high affinity binding to rPlg(S741A) (20). In the absence of altered binding of Sak42D variants to plasminogen, the mutations that reduce the efficiency of Sak-plasmin complexes to activate plasminogen must affect the conversion of the active site of plasmin into the plasminogen activator configuration.

NH$_2$-processing of the Lys$^{10}$-Lys$^{11}$ (P$_1$-P$_1$) peptide bond with exposure of a positively charged NH$_2$-terminal amino acid is important but neither necessary nor sufficient to endow the Sak molecule with plasminogen activating potential. Indeed Sak42DΔN10, from which the NH$_2$-terminal Met is not removed in the E. coli expression system, has NH$_2$-terminal Met-Lys, but generates a complex with plasmin with (nearly) intact plasminogen activating potential. Alternatively, Sak42DG12K is normally although slowly processed at the Lys$^{10}$-Lys$^{11}$ site, but generates a derivative with NH$_2$-terminal Lys-Lys which has a very poor catalytic efficiency for plasminogen activation. Apparently the presence of an uncharged amino acid in position 12 is also important for the plasminogen activating potential of Sak. Finally, preliminary experiments revealed that Sak42DΔN14, which does not generate a plasminogen activating complex with plasmin, can be “rescued” by substituting its NH$_2$-terminal Ala with Lys yielding an active derivative with NH$_2$-terminal Met-Lys (data not shown).

In the absence of the three-dimensional structures of Sak and plasmin, interpretation of the present results in terms of the submolecular configuration of the active site of the plasminogen activating complex as compared to that of free plasmin, remains speculative. The present observations, however, provide a structural mechanism for the plasmin-mediated priming of plasminogen activation by Sak and the fibrin selectivity of clot lysis with Sak in a plasma milieu (22). In circulating blood, traces of generated plasmin are rapidly inhibited by α$_2$-antiplasmin, whereby Sak cannot be processed to the plasminogen activating derivative. In contrast, at the fibrin surface, plasmin is protected from rapid inactivation by α$_2$-antiplasmin (23) and has an increased affinity for Sak (20), allowing efficient local plasminogen activation. This mechanism may account for the high fibrin specificity and thrombolytic efficacy of Sak in patients with acute myocardial infarction (24) or peripheral arterial occlusion (25).

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