NH$_2$-terminal Structural Motifs in Staphylokinase Required for Plasminogen Activation*

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Staphylokinase (Sak) forms an inactive 1:1 stoichiometric complex with plasminogen which requires both conversion of plasminogen to plasmin and hydrolysis of the Lys$^{10}$-Lys$^{11}$ peptide bond of Sak to become a potent plasminogen activator (Schlott, B., Guhrs, K.-H., Hartmann, M., Rocker, A., and Collen, D. (1997) J. Biol. Chem. 272, 6067–6072). Exposure of a positively charged NH$_2$-terminal amino acid after hydrolysis of Sak is a major determinant of the plasminogen-activating potential, but in itself is neither necessary nor sufficient. Here, the structural motifs of the NH$_2$-terminal region Lys$^{11}$-Gly-Asp-Asp-Ala-Ser$^{16}$-Tyr-Phe-Glu of processed Sak, required for plasminogen activating potential, were studied by deletion and substitution mutagenesis.

Expression in *Escherichia coli* of variants with deletion of 11, 14, 15, or 16 NH$_2$-terminal amino acids yielded correctly processed but inactive molecules. Expression of their homologues with the NH$_2$-terminal amino acid substituted with Lys-generated derivatives from which the NH$_2$-terminal initiation Met was no longer removed, yielding inactive (≤ 10%) Sak42DAN11(M), G12K, active (>50%) Sak42DAN14(M), A15K and Sak42DAN15(M), S16K, and inactive Sak42DAN16(M), Y17K. Lys variants without NH$_2$-terminal Met, generated from fusion proteins in which a His$_8$ tag and a factor Xa recognition sequence were linked to the NH$_2$-terminus of the Sak variants, were indistinguishable from their NH$_2$-terminal Met-containing counterparts. All variants studied had intact affinities for plasminogen as measured by biospecific interaction analysis. The activity of Sak42D-\(\Delta N11(M), G12K\) could be restored by additional substitution of both Asp$^{15}$ and Asp$^{19}$ with Asn, yielding active Sak42DAN11(M), G12K, D13N, D14N, whereas substitution in Sak42DAN16(M), Y17K of Phe$^{18}$ and Glu$^{19}$ with Asn yielded inactive Sak42DAN16(M), Y17K, F18N, E19N.

These data, in combination with the recent finding that the 20 NH$_2$-terminal amino acids of Sak lack secondary structure, suggest that the NH$_2$-terminal region of Sak is not required for binding to plasmin/plasminogen, but that a positively charged amino acid in the ultimate or penultimate NH$_2$-terminal position corresponding to amino acids 11–16 of this flexible region participates in the reconfiguration of the active site of the plasmin molecule to endow it with plasminogen-activating potential.

Staphylokinase, a protein secreted by certain *Staphylococcus aureus* strains, is known to be able to dissolve fibrin clots since the 1940s (1). Following cloning of the gene and isolation of large amounts of recombinant protein (2), its therapeutic potential for thrombolysis was recently demonstrated in patients with acute myocardial infarction (3) or with peripheral arterial occlusion (4). The Sak gene encodes a mature protein of 136 amino acids in a single polypeptide chain without disulfide bridges. Three natural Sak$^1$ variants differ at amino acid positions 34, 36, and 45 (5–7) and in thermal stability (8) but have very similar kinetic parameters of plasminogen activation and fibrin-dissolving potency (2).

Sak is not an enzyme, but a cofactor that initially forms an inactive 1:1 stoichiometric complex with plasminogen, which requires both conversion to Sak-plasmin to expose an active site in plasmin and NH$_2$-terminal processing of Sak to reconfigure this active site for the specific cleavage of single chain plasminogen into active two-chain plasmin (9). The exposure of a positively charged amino acid at the new NH$_2$ terminus after cleavage of the Lys$^{10}$-Lys$^{11}$ (P$_1$-P$_1'$. ) peptide bond is a critical step to unveil the plasminogen-activating potential of Sak, but additional structural motifs may be necessary to endow the Sak molecule with plasminogen-activating potential (9).

The tertiary structure of Sak was recently elucidated by crystallography and x-ray diffraction analysis (10) and the secondary structure independently by NMR (11). In both studies, no secondary structure could be assigned to the NH$_2$-terminal 20 amino acids, which suggests that the NH$_2$-terminal region may be rather flexible.

In the present study, the NH$_2$-terminal structural elements of staphylokinase required to confer plasminogen-activator specificity to its complex with plasmin were studied by deletion and substitution of amino acids extending from Gly$^{12}$ to Glu$^{19}$. These deletion mutants lack plasminogen-activating potential but (some of them) can be functionally rescued by substituting their NH$_2$-terminal amino acid with a positively charged Lys, notwithstanding the fact that the *Escherichia coli* expression system no longer removed the NH$_2$-terminal initiation Met from the Lys-containing variants. The shortest variant with intact plasminogen-activating potential was found to be Sak42DAN15(M), S16K.

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1 The abbreviations used are: Sak, staphylokinase; Sak42D, wild type staphylokinase variant with Gly$^{34}$, Arg$^{36}$, and Arg$^{45}$ and NH$_2$-terminal Ser-Ser-Ser; Sak42DAN, Sak42D with deletion of n NH$_2$-terminal amino acids (n = 11, 14, or 15); Sak42DANXn, (Xn+1)K, Sak42D with deletion of n NH$_2$-terminal amino acids (n = 11, 14, or 15) and substitution of the (n + 1)th amino acid X with Lys; Sak42DANXM, Sak42DANXn with NH$_2$-terminal Met preceding the (n + 1)th amino acid; Sak42DANXn(M)XnB, Sak42DANXn(M) with amino acid X in position # substituted with amino acid B; Glu-Ptg, native human plasminogen with NH$_2$-terminal Glu; pFG7S741A, recombinant plasminogen with the active site Ser$^{174}$ mutagenized to Ala; PAGE, polyacrylamide gel electrophoresis; S-2251, d-Val-Phe-Lys-p-nitroanilide; PCR, polymerase chain reaction; Ni-NTA, nickel-nitrosoacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
 purifying Sak42D variants were determined on the Applied Biosystems model 477A (Applied Biosystems, Foster City, CA). For the Sak species generated in equimolar (4 μM) mixtures with plasminogen after 5 min of incubation at 37 °C, high resolution electrophoresis was carried out with the Mini-Protean II system (Bio-Rad, Munich, Germany) using 16% T (total acrylamide), 3% C (cross-linking agent) gels in the discontinuous Tris-Tricine buffer system according to Schagger and von Jagow (15). Samples were applied to the gels after reduction by heating at 100 °C for 5 min in the presence of 1% SDS and 0.5% β-mercaptoethanol. Following gel separation, the proteins were blotted onto polyvinylidene difluoride membranes (Millipore, Eschborn, Germany) 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 (1 mM final concentration) and monitored at 405 nm for up to 12 min using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Plasminogen (3.6 μM final concentration) was incubated with the Sak moieties (4 μM final concentration) at 37 °C in 100 mM phosphate buffer, pH 7.4, containing 0.01% Tween 80 (activation buffer). Samples were withdrawn at 2-min intervals and diluted 40-fold in buffer containing chromogenic substrate S-2251, and the change in absorbance at 405 nm (∆A405) was recorded.

Activation of Plasminogen by Catalytic Amounts of Sak42D Variants— The activation of plasminogen (1.5 μM final concentration) by Sak42D moieties (5 nM final concentration) was assayed at 37 °C in activation buffer. At different time intervals up to 30 min, generated plasmin was measured with the S-2251 substrate. Sak42D and plasmin-Sak complexes were evaluated at lower temperatures to detect differences in activation energies.

Kinetic Constants of Plasminogen Activation by Catalytic Amounts of Preformed Equimolar Complexes of Sak42D Variants with Plasmin— The kinetic constants of plasminogen activation by plasmin-Sak complexes were derived from Lineeweaver-Burk plots. Therefore, equimolar mixtures of Sak42D variants and freshly prepared plasminogen were incubated in activation buffer containing 25% glycerol (v/v) and kept on ice. These preformed activator complexes (10–40 nM final concentration) were then mixed with plasminogen (0.25–10 μM final concentration), and generated plasmin was measured at 37 °C from the change in absorbance at 405 nm with S-2251. Accurate Lineeweaver-Burk plots could not be constructed with variants displaying catalytic efficiencies <20% of that of wild type. For these variants, catalytic efficiencies were determined from the apparent second order rate constant at a plasminogen concentration of 5 μM.

Binding of Sak42D Variants to Plasminogen— Association rate constants (ka) and dissociation rate constants (kd) for the interaction between Glu-plg or rPlg(ST41A) and the Sak variants were determined by real-time biospecific interaction analysis using the BIAcore instrument (Biacore Inc., Dassel, Germany). Glu-plg or rPlg(ST41A) was immobilized on the surface of sensor chip CM5 using the amine coupling kit (Biacore Inc), with 20% of that of wild type. For these variants, catalytic efficiencies were determined from the apparent second order rate constant at a plasminogen concentration of 5 μM.
or 2–6 μM in 10 mm Heps, 3.4 mm EDTA, 0.15 M NaCl, and 0.005% surfactant P20, pH 7.2, were injected at a flow rate of 20 μl/min for 2 min in the association phase. After equilibrium was reached, sample was replaced by buffer, also at a flow rate of 20 μl/min for 2 min. After each cycle, the surface of the sensor chip was regenerated by injection of 10 μl of 50 mm HCl. Association (ka) and dissociation (kd) rate constants were derived from the observed response curves (resonance/time) as described in detail in the Biacore Evaluation 2.0 Software Handbook (Pharmacia Biosensor AB).

RESULTS

Purification and NH2-terminal Sequence of Sak42D Variants—The Sak42D variants were purified from E. coli TG1 cells transformed with the respective plasmids with yields of 70–500 mg/liter culture volume. SDS-PAGE (data not shown) displayed single bands with electrophoretic mobilities corresponding to the extent of NH2-terminal deletion. Homogeneity of the purified proteins was further revealed by NH2-terminal sequencing as illustrated in Table II. The mutants Sak42DAN11, Sak42DAN14, and Sak42DAN15, like the wild type Sak42D, were correctly processed in the E. coli TG1 expression system with removal of the NH2-terminal initiation Met. The variants with Lys substitution in the NH2-terminal position were expressed with a Met residue at the NH2-terminus, added to initiate expression in E. coli, but which was not removed when followed by Lys.

NH2-terminal Sequences of Sak Mieties in Equimolar Complexes with Plasminogen and Generation of Amidolytic Activities—As expected, wild type Sak42D was processed at the Lys10-Lys11 peptide bond as described earlier (9). The variants with deletion of 11, 14, or 15 amino acids, with or without substitution of the next amino acid with Lys, maintained unaltered NH2-terminal sequences after complex formation. Variants with deletion of 11 amino acids in combination with substitution of Gly12 with Lys and of Asp13 with Ile or Asn were partially processed to derivatives hydrolyzed after the substituted Lys peptide bond (Table II).

In line with previous observations (9), amidolytic activity toward the synthetic plasmin substrate S-2251 was detected in all equimolar mixtures of plasminogen with the Sak variants (not shown). Mutants whose complexes with plasmin did not efficiently activate plasminogen, expressed up to 3-fold higher amidolytic activity than complexes of wild type Sak42D with plasmin. This was not unexpected in view of the known higher specific activity of the active site of plasmin toward the chromogenic substrate, as compared with that of the active site of the plasminogen-activating plasmin/Sak complex (9).

Activation of Plasminogen by Catalytic Amounts of Sak Variants—Catalytic amounts of Sak42D induced activation of plasminogen to plasmin with a lag phase of approximately 5 min, reaching a maximal rate (determined from the slope at the inflection point of these curves) after 5–15 min. The results obtained with active complexes are illustrated in Fig. 1. As described previously (9), the Sak42DAN10(M) and Sak42DProc variants displayed activation curves similar to those of wild type Sak42D (not shown). Sak42DAN14 and Sak42DAN15 were inactive but could be rescued by replacing their respective NH2-terminal amino acid by a lysine residue, yielding functionally active Sak42DAN14(M),A15K and Sak42DAN15(M),S16K (Fig. 1). Sak42DAN11 was also inactive but could only be rescued by substitution of Gly12 with Lys and in addition of both Asp13 and Asp14 with Asn, yielding functionally inactive Sak42DAN11(M),G12K and Sak42DAN11(M),G12K,D13I (not shown) but active Sak42DAN11(M),G12K,D13N,D14N (Fig. 1). Sak42DAN11 was also inactive but could only be rescued by substitution of Gly12 with Lys and in addition of both Asp13 and Asp14 with Asn, yielding functionally inactive Sak42DAN11(M),G12K and Sak42DAN11(M),G12K,D13I (not shown).

Kinetic Constants of Plasminogen Activation by Preformed Plasmin-Sak Complexes—Complexes of plasmin with functionally active Sak variants, identified as described above, activated plasminogen following Michaelis-Menten kinetics with catalytic efficiencies (ka/Km) ranging between 0.044 and 0.059 μM−1 s−1, as determined from Lineweaver-Burk plots (Table II). Catalytic rate constants (ka) and Michaelis constants (Km) comparable with those of wild type Sak42D were obtained for Sak42DAN11(M),G12K,D13N,D14N and Sak42DAN14,M,15K, whereas Sak42DAN14(M),A15K and Sak42DAN15(M),S16K had 10-fold higher ka and Km but similar ka/Km. Sak42DAN11(M),Y17K and Sak42DAN16(M),Y17K,F18N,E19N did not restore plasminogen-activating capacity (not shown).

### Table II

| Variant       | NH2-terminal sequence | Rate of plg activation | Km  | kcat | Catalytic efficiency |
|---------------|-----------------------|------------------------|-----|------|----------------------|
| Sak42D        | SSSFDKGYKGDASYFEP     | % of Sak42D            | 1.0 |      |                      |
| Sak42DAN11    | GDDASYFEP             | 0.0                    | 2.4 | 0.1  | 0.08                 |
| Sak42DAN11(M),G12K |               | 0.0                    | 2.4 | 0.1  | 0.08                 |
| Sak42DAN11,G12K | KDDASYFEP             | 0.0                    | 4.8 |      | 0.002                |
| Sak42DAN11(G12K,D13I) |               | 0.0                    | 4.8 |      | 0.002                |
| Sak42DAN11(M),G12K,D13N,D14N |   | 0.0                    | 4.8 |      | 0.006                |
| Sak42DAN14    | ASYFEP                | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN14(M),A15K |               | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN14,15K | KSYFEP                | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN15    | SYFEP                 | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN15(M),S16K |               | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN16    | KSYFEP                | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN16    | KFEFP                 | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN16(Y17K) |               | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN16(M),Y17K,F18A |   | 0.0                    | 2.3 | 0.1  | 0.054                |
| Sak42DAN16(E19N) |               | 0.0                    | 2.3 | 0.1  | 0.054                |

* The sequences are aligned with Pro32 and mutagenized amino acids are in bold face.

b Sequence not detectable because of degradation of the Sak moiety in the complex.

ND, no Lineweaver-Burk plot constructed.
minogen, whereas the other variants, like their Met-containing counterparts, were inactive.

Temperature Dependence of Functional Properties of Sak Moieties—To evaluate possible differences in activation energies for the induction of plasminogen-activating potential between unprocessed wild type Sak42D and the “preprocessed” deletion mutant with rescued plasminogen-activating potential, Sak42D, the effect of temperature on the activation of plasminogen by catalytic amounts of Sak variant were studied (Fig. 2). At 37 °C, plasmin generation progressed at similar rates, whereas at 10 °C no plasmin generation occurred within 30 min. At intermediate temperatures (15–25 °C), plasmin generation proceeded significantly faster with Sak42D than with wild type Sak42D.

Binding of Sak Variants to Plasmin/Plasminogen—The association and dissociation rate constants ($k_a$ and $k_d$) and the

$$\begin{array}{cccc}
\text{Variant} & \text{Binding to Glu plasminogen} & \text{Binding to recombinant plasminogen (S741A)} \\
& k_a (\times 10^3) & k_d (\times 10^{-3}) & K_a (\times 10^6) & k_a (\times 10^3) & k_d (\times 10^{-3}) & K_a (\times 10^6) \\
\hline
\text{Sak42D} & 9.6 & 16 & 0.60 & 1000 & 3.3 & 300 \\
\text{Sak42D} & 1.7 & 5.8 & 0.30 & 980 & 2.9 & 320 \\
\text{Sak42D} & 11 & 15 & 0.75 & 1000 & 3.2 & 320 \\
\text{Sak42D} & 18 & 22 & 0.80 & 1000 & 3.6 & 280 \\
\text{Sak42D} & 8.3 & 15 & 0.55 & 520 & 2.9 & 180 \\
\text{Sak42D} & 14 & 19 & 0.74 & 1200 & 3.9 & 300 \\
\text{Sak42D} & 8.3 & 11 & 0.75 & 800 & 2.5 & 320 \\
\text{Sak42D} & 5.7 & 14 & 0.41 & 660 & 4.1 & 160 \\
\text{Sak42D} & 11 & 22 & 0.51 & 600 & 4.0 & 150 \\
\text{Sak42D} & 12 & 14 & 0.88 & 620 & 2.6 & 240 \\
\text{Sak42D} & 12 & 14 & 0.88 & 620 & 2.6 & 240 \\
\text{Sak42D} & 4.5 & 15 & 0.30 & 540 & 2.7 & 200 \\
\text{Sak42D} & 4.6 & 22 & 0.21 & 1050 & 3.7 & 280 \\
\text{Sak42D} & 9.4 & 16 & 0.59 & 750 & 3.1 & 240 \\
\text{Sak42D} & 7.0 & 37 & 0.19 & 750 & 3.4 & 220 \\
\text{Sak42D} & 8.6 & 43 & 0.20 & 1100 & 5.8 & 190 \\
\end{array}$$
apparent affinity equilibrium constants \( (K_d) \) for binding of
Sak42D moieties to native Glu-Plg in the presence of excess plasmin inhibitor or to rPlg(S741A) are summarized in Table III. The \( k_a \), \( k_d \), and \( K_d \) 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 a result of 100-fold higher \( k_a \) and 10-fold lower \( k_d \) values.

**DISCUSSION**

Sak is a highly fibrin-selective and potent agent for thrombolytic therapy in patients with thrombembolic arterial disease (3, 4). Fibrin selectivity is due to the fact that Sak has a low affinity for plasminogen in plasma, but a high affinity for plasminogen or for traces of plasmin associated with fibrin clot (17). Furthermore, the plasminogen-Sak complex is inactive and both conversion of plasminogen to plasmin and NH2-termini processing of Sak to Sak42D10 by hydrolysis of the Lys10-Lys11 peptide bond are required to generate active plasmin-Sak complex at the fibrin surface, whereas removal of Lys11 results in loss of plasminogen-activating potential. The aim of the present study was to characterize structural motifs in the NH2-terminal region of Sak possibly required for binding to plasminogen and for reconfiguration of the active site of plasmin in the plasmin-Sak complex from that of fibrin degrading plasmin to that of plasminogen-activating plasmin-Sak complex. This was studied by evaluation of the NH2-terminal processing, the catalytic efficiency for plasminogen activation, and the binding affinity to insulobilized plasminogen of deletion/substitution variants of the Lys13,Gly-Asp-Asp-Ala-Ser16, Tyr-Phe-Glu region of Sak.

The results indicate that removal of K, KGDD, or KGDDA, yielding Sak42DAN11, Sak42DAN14, or Sak42DAN15, did not affect the association and dissociation rate constants of the binding of these variants to plasminogen, but reduced the catalytic efficiency for plasminogen activation by more than 1 order of magnitude. Substitution of the NH2-terminal amino acids by Lys in Sak42DAN14 or Sak42DAN15, yielding Sak42DAN14,A15K and Sak42DAN15,S16K, rescued the plasminogen-activating activity, whereas Sak42DAN16,Y17K and Sak42DAN11,G12K were inactive. Further substitution of Asp13 and Asp14 in Sak42DAN11,G12K with Asn, yielding Sak42DAN11,G12K,D13N,D14N, rescued the plasminogen-activating activity, but a similar substitution of Phe18 and Glu19 in Sak42DAN16,Y17K with Asn, yielding Sak42DAN16,Y17K,F18N,E19N did not endow the latter variant with plasminogen-activating potential. Finally, all variants studied had similar association and dissociation rate constants for low affinity binding to insulobilized native plasminogen and for high affinity binding to insulobilized active site-substituted recombinant plasminogen.

No differences in plasminogen-activating potency were observed among Lys-substituted deletion variants with or without the presence of the NH2-terminal initiation Met. Nevertheless, although the catalytic efficiencies of Sak42DAN14,M, A15K and Sak42DAN15,M,S16K were similar to that of wild type Sak42D, they had 10- to 20-fold higher \( k_{cat} \) and \( K_m \) values. The mechanism underlying these differences remains unclear. It is possible that the exposed NH2-terminal Lys primarily influences the active site configuration via its \( \epsilon \)-amino group and that masking of the NH2-terminal \( \epsilon \)-amino group might yield a more efficient active center.

These findings indicate: 1) that binding to plasmin/plasminogen and reconfiguration of the active site of plasmin are mediated by different structures in the Sak molecule; 2) that a positive amino acid in the NH2-terminal position of Sak, in the region corresponding to positions 11–16 is required for reconfiguration of the active site of plasmin; and 3) that one or two negatively charged amino acids following the positive NH2-terminal amino acid prevent reconfiguration of the active site of plasmin.

The variant Sak42DAN16,Y17K marks the limit for rescuing, by introduction of a Lys at the NH2 terminus, the plasminogen-activating potency of NH2-terminal trimermed Sak molecules. This finding is in agreement with the recent finding (10, 11) that Sak lacks secondary structure in the region spanning the first 20 NH2-terminal amino acids. This region may have a flexible conformation located at the surface of Sak, which after hydrolysis and exposure of the positively charged amino acid may interact with the active site of plasmin to reconfigure its catalytic specificity, converting it from a fibrin-degrading to a plasminogen-activating proteinase.

As reported previously, NH2-terminal processing of wild type Sak42D does not appear to be the rate-limiting step in plasminogen activation by Sak at 37 °C (18). Consequently, no differences in activation efficiency were found at 37 °C between wild type Sak42D, which needs to be processed NH2-terminally, and the “preprocessed” deletion variants. However, at lower temperatures, activation of plasminogen with catalytic amounts of Sak42DAN14,M,A15K proceeded more rapidly compared with that of wild type Sak42D. This finding suggests that the activation energy of the processing of Sak42D is higher than that of the reconfiguration of the active site.

The Lys residue unmasked by cleaving off the NH2-terminal peptide from Sak42D may be directly involved in the reconfiguration of the active site of plasmin. Recent data concerning the molecular interactions modulating the catalytic efficiency and specificity of serine proteinases support this assumption. Thus, a Lys residue in single-chain tissue plasminogen activator stabilizes the active conformation of this enzyme (19). Moreover, in a series of proteases, Lys may substitute His as the general base in the catalytic triad (20–22). Definitive proof of the involvement of Lys residues of Sak in complexes with plasmin that specifically activate plasminogen may require elucidation of the tertiary structure of the complex.

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