On the Mechanism of Fibrin-specific Plasminogen Activation by Staphylokinase*

H. Roger Lijnen†, Berthe Van Hoef, Frans De Cock, Kiyotaka Okadag, Shigeru Ueshima, Osamu Matsumo, and Désiré Collen

From the Center for Thrombosis and Vascular Research, University of Leuven, B-3000 Leuven, Belgium and the Department of Physiology, Kinki University School of Medicine, Osaka 589, Japan

The mechanism of plasminogen activation by recombinant staphylokinase was studied both in the absence and in the presence of fibrin, in purified systems, and in human plasma. Staphylokinase, like streptokinase, forms a stoichiometric complex with plasminogen that activates plasminogen following Michaelis-Menten kinetics with $K_m = 7.0 \mu M$ and $k_2 = 1.5 \, s^{-1}$. In purified systems, $\alpha_2$-antiplasmin inhibits the plasminogen-staphylokinase complex with $k_{1(app)} = 2.7 \pm 0.30 \times 10^6 \, M^{-1} \, s^{-1}$ (mean $\pm$ S.D., $n = 12$), but not the plasminogen-streptokinase complex. Addition of 6-aminohexanoic acid induces a concentration-dependent reduction of $k_{1(app)}$ to $2.0 \pm 0.17 \times 10^6 \, M^{-1} \, s^{-1}$ (mean $\pm$ S.D., $n = 5$) at concentrations $\geq 30 \, mM$, with a 50% reduction at a 6-aminohexanoic acid concentration of 60 $\mu M$. Staphylokinase does not bind to fibrin, and fibrin stimulates the initial rate of plasminogen activation by staphylokinase only 4-fold.

Staphylokinase induces a dose-dependent lysis of a 0.12-ml $^{125}$I-fibrin-labeled human plasma clot submerged in 0.5 ml of citrated human plasma; 50% lysis in 2 h is obtained with 17 $nM$ staphylokinase and is associated with only 5% plasma fibrinogen degradation. Corresponding values for streptokinase are 68 $nM$ and more than 90% fibrinogen degradation. In the absence of a fibrin clot, 90% fibrinogen degradation in human plasma in 2 h requires 750 $nM$ staphylokinase, but only 4.4 $nM$ streptokinase.

These results suggest the following mechanism for relatively fibrin-specific clot lysis with staphylokinase in a plasma milieu. In plasma in the absence of fibrin, the plasminogen-staphylokinase complex is rapidly neutralized by $\alpha_2$-antiplasmin, thus preventing systemic plasminogen activation. In the presence of fibrin, the lysine-binding sites of the plasminogen-staphylokinase complex are occupied and inhibition by $\alpha_2$-antiplasmin is retarded, thus allowing preferential plasminogen activation at the fibrin surface.

Plasminogen activators convert plasminogen, the inactive proenzyme of the fibrinolytic system in blood, to the proteolytic enzyme plasmin. Plasmin dissolves the fibrin of a blood clot, but may also degrade normal components of the hemostatic system and induce the so-called lytic state. Physiologically, fibrinolysis, however, is fibrin-oriented as a result of specific molecular interactions between tissue-type plasminogen activator, fibrin, plasminogen, and $\alpha_2$-antiplasmin (1). Streptokinase, an $M$, 45,000 protein secreted by $\beta$-hemolytic streptococci, is used in thrombolytic therapy, but its administration is associated with extensive systemic fibrinogenolysis (2). Staphylokinase, an $M$, 15,500 protein produced by Staphylococcus aureus (3), was shown to have profibrinolytic properties more than 4 decades ago (3, 4). Limited availability of the bacterial protein has, however, precluded a detailed investigation of its plasminogen-activating properties. The gene coding for the bacterial protein has now been cloned and expressed in Escherichia coli (5, 6) and Bacillus subtilis (7). The nucleotide sequence of the staphylokinase gene and the deduced amino acid sequence are not related to those of streptokinase (8–10).

It has been suggested that, like streptokinase, staphylokinase forms a stoichiometric complex with plasminogen, which subsequently converts plasminogen to plasmin (11–13). Recently, it was shown that recombinant staphylokinase is a more potent and more fibrin-specific fibrinolytic agent than streptokinase in human plasma in vitro (14). It was suggested that plasminogen activation by staphylokinase is inhibited by $\alpha_2$-antiplasmin in circulating plasma, but not at the fibrin surface (15).

In the present study, the mechanism of plasminogen activation by staphylokinase was investigated in more detail, using quantitative studies of the interactions between plasminogen, staphylokinase, fibrin, and $\alpha_2$-antiplasmin. In addition, the mechanism of fibrin-specific clot lysis in human plasma in vitro was evaluated.

MATERIALS AND METHODS

Proteins and Reagents—Staphylokinase was produced in transformed E. coli, purified from cell culture medium, and characterized as described elsewhere (6). Streptokinase, devoid of albumin, was obtained from Boehringer Mannheim; for experiments in plasma, Streptase® (Hoechst, Brussels) was used.

Native human plasminogen was purified from plasma and characterized as described elsewhere (16, 17). Recombinant plasminogen with the active site Ser$^{122}$ mutated to Ala ($r$Plg-Ala$^{122}$) was obtained by expression in Chinese hamster ovary cells of the plasmid PLG 251a/219b (18) using the vector Zem 229 (19). These materials were kindly provided by ZymoGenetics. $r$Plg-Ala$^{122}$ was purified from conditioned cell culture medium and characterized as described elsewhere (20, 21). Human $\alpha_2$-antiplasmin was purified from plasma and its activity determined by titration with plasmin (22). Fibrinogen was prepared from human plasma and depleted in plasminogen by addition of fibrin.

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1 The abbreviations used are: rPlg-Ala$^{122}$, recombinant plasminogen with the active site Ser$^{122}$ mutated to Ala; 6-AHA, 6-aminohexanoic acid; S-2251, d-valyl-leucyl-lysine-p-nitroanilide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; des-AAlb, fibrinogen with the two fibrinopeptides A removed, fibrin 1.

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Plasminogen was prepared as described elsewhere (23), and solubilized des(AA)Fibrinogen (Desfib) was obtained from Biopool (Umeå, Sweden). The chromogenic substrate D-valyl-leucyl-lysine-p-nitroanilide (S-2251) was purchased from Kabi Vitrum. The synthetic thrombin inhibitor D-Ile-Pro-Arg-ChlCl (24) was custom-synthesized at University College London. Staphylokinase (Staphaclone, Enterozyme, BRL) and plasminogen was purchased from Amesham. Plasma was pooled human plasma obtained from at least five healthy blood donors. α2-Antiplasmin-depleted plasma was obtained from normal human plasma by immunoadsorption on an insolubilized monoclonal antibody (MA-34F7) directed against α2-antiplasmin. After depletion, this plasma contained about 1% residual α2-antiplasmin, as determined by enzyme-linked immunosorbent assay (25), whereas fibrinogen and plasminogen levels remained within the normal range.

Techniques—Protein concentrations were determined according to Bradford (26). Staphylokinase and plasminogen-streptokinase complexes were labeled with 125I using the IODO-GEN method (27) to specific activities of 15 x 10^6 cpm/μg, respectively. SDS-PAGE was performed with the PHAST System (Pharmacia) using 10–15% gradient gels and Coomassie staining. Reduction of samples was performed by heating at 100°C for 3 min in the presence of 1% SDS and 10% dithiothreitol.

Complex Formation with Plasminogen—The generation of an active site in complexes of plasminogen with staphylokinase or streptokinase was monitored as follows. Plasminogen (final concentration, 1 μM) was incubated with staphylokinase or streptokinase (final concentration, 5 nM) at 37°C for 10 min, and aliquots were removed at different time intervals (0–10 min), incubated with 0.1 M phosphate buffer, pH 7.4, containing 25% glycerol; the mixture was then stored on ice. For kinetic analysis, this plasma contained approximately 5% residual α2-antiplasmin, as determined by enzyme-linked immunosorbent assay (25), whereas fibrinogen and plasminogen levels remained within the normal range.

Kinetics of Plasmin Generation—The effect of 6-aminohexanoic acid (final concentration, 0–500 μM) on the inhibition rate of the plasminogen-staphylokinase complex by α2-antiplasmin was determined in the same way. At each concentration of 6-aminohexanoic acid, Km was expressed in percentage of the value in the absence of ligand. The effect of saturating concentrations of 6-aminohexanoic acid (final concentration, 30 μM) on the inhibition rate of the plasminogen-staphylokinase complex by α2-antiplasmin (5-fold molar excess) was determined as described above for the plasminogen-streptokinase complex. Under the conditions used, 6-AHA does not interfere with the hydrolysis of S-2251 by the plasminogen-staphylokinase complex.

The interactions between plasminogen, staphylokinase, and α2-antiplasmin or between plasminogen, streptokinase, and α2-antiplasmin were also monitored by SDS-PAGE under nonreducing and reducing conditions. Plasminogen (final concentration, 1.5 μM) was mixed with staphylokinase (final concentration, 150 nM) at 37°C for 30 min, followed by the addition of α2-antiplasmin (final concentration, 4.5 μM). Samples were removed before the addition of α2-antiplasmin and 1 min and 30 min after its addition.

Effect of CNBr-digested Fibrinogen and DesAaFibrinogen on the Kinetics of Plasmin Generation—Plasminogen (final concentration, 1.5 μM) in 0.1 M phosphate buffer, pH 7.4, was activated at 37°C with equimolar plasminogen-streptokinase or plasminogen-staphylokinase prepared as described above (final concentration, 2 nM) in the presence of different concentrations of CNBr-digested fibrinogen or desAaFibrinogen (final concentration, 0–1 μM). At different time points (0–5 min), samples were removed from the incubation mixtures, and 50 μl of plasmin was quantitated with S-2251 (final concentration, 1 μM) after 25-fold dilution.

Binding to Fibrin—Purified plasminogen-free human fibrinogen (0–0.3 mg/ml) in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.038 M NaCl, 0.01% Tween 80, and 1 mg/ml bovine serum albumin was clotted by the addition of thrombin (final concentration, 10 NIH units/ml) in the presence of 125I-labeled staphylokinase or [125I]labeled streptokinase (50,000 cpm/ml). After incubation at 37°C for 1 min, thrombin was inactivated by the addition of D-Ile-Pro-Arg-ChlCl (final concentration, 10 μM), and the clots were removed by centrifugation and washed extensively, and the radioactivity associated with the fibrin clots was quantitated. In addition, normal human plasma was clotted by the addition of CaCl2 (final concentration, 45 mM) and thrombin (final concentration, 2 NIH units/ml) in the presence of the 125I-labeled staphylokinase or 125I-labeled streptokinase (50,000 cpm/ml), and binding was quantitated as described above.

Fibrinolytic Properties in Purified Systems—[125I]Labeled clots

The fibrinolytic activity was determined as described above.
puriﬁed human ﬁbrin were prepared by the addition of CaCl₂ (ﬁnal concentration, 50 mM) and thrombin (ﬁnal concentration, 3.5 NIH units/ml) to puriﬁed ﬁbrinogen (ﬁnal concentration, 3 mg/ml, containing approximately 250,000 cpm/ml of ¹²⁵I-labeled ﬁbrinogen) in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 0.038 M NaCl. Alternatively, clots were prepared in the same way but in the presence of plasminogen (ﬁnal concentration, 150 μg/ml). After incubation at 37°C for 60 min in silicon tubing (internal diameter, 4 mm), pieces about 1.0 cm in length were cut off and the ﬁbrin clots (volume, 0.12 ml) were extensively washed in 0.15 M NaCl. Puriﬁed ¹²⁵I-labeled ﬁbrin clots were then incubated at 37°C in 1 ml of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80, 0.038 M NaCl, 1 kallikrein-inactivating unit/ml aprotinin and 1.5 μM Glu-Arg amidolytic activity is measured with S-2251 (ﬁnal concentration, 1 mM) after 50-fold dilution of samples. The data represent the mean ± S.D. of three determinations.

Fibrinolytic Properties in Human Plasma in Vitro—Puriﬁed plasma clots were prepared from normal human plasma or from α₂-antiplasmin-depleted plasma as described above, following addition of 500,000 cpm/ml of ¹²⁵I-labeled ﬁbrinogen and coagulation with CaCl₂ (ﬁnal concentration, 25 mM) and thrombin (ﬁnal concentration, 2 NIH units/ml). Lysis of ¹²⁵I-labeled plasma clots (volume, 0.12 ml) by addition of different concentrations of staphylokinase (ﬁnal concentration, 2.1–133 nM) or streptokinase (ﬁnal concentration, 1.4–355 nM) in 0.5 ml of normal human plasma or α₂-antiplasmin-depleted plasma was measured over 4 h as previously described (31). Residual ﬁbrinogen levels were monitored with a clotting rate assay (32). The concentration of plasminogen activator required to obtain 50% lysis in 1 h was determined from plots of percent lysis (in percent) versus the concentration of the plasminogen activators. Residual α₂-antiplasmin and/or plasminogen levels in the solutions were monitored with chromogenic substrate assays (29, 30).

RESULTS

Complex Formation with Plasminogen—Fig. 1 shows that, in mixtures of plasminogen with a 5-fold molar excess of either staphylokinase or streptokinase, the active site, as monitored with the chromogenic substrate S-2251, is rapidly exposed. Under the experimental conditions used, plasminogen, staphylokinase, or streptokinase alone did not react with S-2251. Preincubation of plasminogen with α₂-antiplasmin (5-fold molar excess over plasminogen) completely abolishes exposure of an active site following addition of staphylokinase but only slightly affects the generation of an active site following the addition of streptokinase.

The 2.5-fold lower amidolytic activity observed with the plasminogen-staphylokinase complex than with the plasminogen-streptokinase complex is due to a lower reactivity of the former with S-2251. Lineweaver-Burk analysis of the hydrolysis of S-2251 by plasminogen-staphylokinase, plasminogen-streptokinase, or plasmin revealed that the Kₘ values are comparable (0.57, 0.55, and 0.48 mM, respectively), whereas kₐ₋ₑ is 2-fold lower for plasminogen-staphylokinase (17 s⁻¹)

FIG. 1. Generation of amidolytic activity in mixtures of plasminogen with a 5-fold molar excess of staphylokinase ( []), or streptokinase ( [] ) in the absence (open symbols) or the presence (closed symbols) of α₂-antiplasmin at a 5-fold molar excess over plasminogen. Amidolytic activity is measured with S-2251 (ﬁnal concentration, 1 mM) after 50-fold dilution of samples. The data represent the mean ± S.D. of three determinations.

FIG. 2. Activation of natural plasminogen (panels I) or rPlg-Ala⁷⁴⁰ (panels II) (ﬁnal concentration, 1.5 μM) with staphylokinase (A) or streptokinase (B) (ﬁnal concentration, 50 nM). Samples for reduced SDS-PAGE were taken at times 0 (lane 2), 2 min (lane 3), 5 min (lane 4), 10 min (lane 5), 15 min (lane 6), and 20 min (lane 7) for natural plasminogen (panels I) or at times 0 (lane 2), 15 min (lane 3), 30 min (lane 4), 45 min (lane 5), 60 min (lane 6), and 90 min (lane 7) for rPlg-Ala⁷⁴⁰ (panels II). The protein calibration mixture (lanes 1) consists of phosphorylase b (M, 97,000), albumin (M, 67,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 30,000), trypsin inhibitor (M, 20,100), and α-lactalbumin (M, 14,400), than for plasminogen-streptokinase or plasmin (35 s⁻¹) (data not shown).

Both staphylokinase and streptokinase convert natural plasminogen to two-chain plasmin, as revealed by reduced SDS-PAGE, whereas active-site mutated plasminogen, rPlg-Ala⁷⁴⁰ is not converted to a two-chain derivative by either staphylokinase or streptokinase (Fig. 2). Under the same experimental conditions, urokinase completely converts rPlg-Ala⁷⁴⁰ to plasmin (not shown). Fig. 3 shows that both the plasminogen-staphylokinase and the plasminogen-streptokinase complexes convert rPlg-Ala⁷⁴⁰ to a two-chain plasmin molecule, whereas an equivalent amount of plasmin does not.

Kinetics of Plasminogen Activation—Kinetic analysis revealed that plasminogen is activated to plasmin by both plasminogen-staphylokinase and plasminogen-streptokinase, following Michaelis-Menten kinetics, as shown by linear dou-
or by plasminogen-streptokinase complex (A) or by plasminogen-streptokinase complex (B) (20 nm each) as monitored on reduced SDS-PAGE. Samples are taken at times 0 (lanes 1), 10 min (lanes 2), and 60 min (lanes 3). In panel C, lane 4 represents rPig-Ala (1.5 μM) treated with plasmin (20 nm) for 60 min at 37 °C and lane 5 represents plasminogen fully converted with plasminogen-staphylokinase to plasmin.

**FIG. 3.** Conversion at 37 °C of rPig-Ala (1.5 μM) to a two-chain derivative by plasminogen-staphylokinase complex (A) or by plasminogen-streptokinase complex (B) (20 nm each) as monitored on reduced SDS-PAGE. Samples are taken at times 0 (lanes 1), 10 min (lanes 2), and 60 min (lanes 3). In panel C, lane 4 represents rPig-Ala (1.5 μM) treated with plasmin (20 nm) for 60 min at 37 °C and lane 5 represents plasminogen fully converted with plasminogen-staphylokinase to plasmin.

**FIG. 4.** Activation of plasminogen (final concentration, 1.5 μM) by staphylokinase or streptokinase (final concentration, 5 nm) in the absence or the presence of α₂-antiplasmin (final concentration, 3 μM). A, activation of plasminogen (in percent) by staphylokinase (C, ■) or streptokinase (C, ●) as a function of time, in the absence (open symbols) or in the presence of α₂-antiplasmin (closed symbols). The data represent mean ± S.D. of four determinations. B, SDS-PAGE under nonreducing (I) or reducing (II) conditions of samples taken after 30 min from the experiments represented in panel A. Lane 1, protein calibration mixture consisting of phosphorylase b (M, 97,000), albumin (M, 67,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 30,000), trypsin inhibitor (M, 20,100) and α-lactalbumin (M, 14,400); lane 2, plasminogen (PLG) plus staphylokinase in the presence of α₂-antiplasmin (α₂AP); lane 3, plasminogen plus streptokinase in the presence of α₂-antiplasmin; lane 4, plasmin-α₂-antiplasmin complex (P-α₂AP), B-α₂AP, complex of plasmin B-chain with α₂-antiplasmin; A, plasmin A-chain.

ble-reciprocal plots of the initial rate of activation versus the plasminogen concentration (not shown). The kinetic constants, obtained by linear regression analysis, are Kₐ = 7.0 μM and kₐ = 1.5 s⁻¹ for plasminogen-staphylokinase (mean of two independent determinations with r > 0.99) and Kₐ = 0.65 μM and kₐ = 0.52 s⁻¹ for plasminogen-streptokinase (mean of two independent determinations with r > 0.99). The catalytic efficiency (kₐ/Kₐ) of plasminogen-streptokinase thus is about 4-fold higher than that of plasminogen-staphylokinase (0.80 and 0.21 μM⁻¹ s⁻¹, respectively).

**Effect of α₂-Antiplasmin on Plasminogen Activation.—**Fig. 4A shows a very similar time-dependent activation of plasminogen (final concentration, 1.5 μM) by staphylokinase or streptokinase (final concentration, 5 nM) as monitored by quantitation of generated plasmin with S-2251. Under the conditions used, about 50% of the plasminogen is activated in 15 min, followed by a progressive decrease in plasmin activity due to its instability at 37 °C. Preincubation with α₂-antiplasmin (final concentration, 3 μM) does not markedly affect plasminogen activation by streptokinase, but virtually abolishes the activation by staphylokinase, as shown by quantitation of residual plasminogen concentrations. SDS-PAGE under nonreducing conditions of samples from mixtures with α₂-antiplasmin (Fig. 4B) reveals the presence of plasmin-α₂-antiplasmin complexes in incubation mixtures with streptokinase but not in mixtures with staphylokinase. Under reducing conditions, the plasmin-α₂-antiplasmin complex dissociates in the plasmin B-chain-α₂-antiplasmin complex and the plasmin A-chain.

**Inhibition of Plasminogen-Staphylokinase or Plasminogen-Streptokinase Complexes by α₂-Antiplasmin.—**Semilogarithmic plots of residual complex as a function of time, following incubation of preformed plasminogen-staphylokinase or plasminogen-streptokinase complexes with α₂-antiplasmin under pseudo first-order kinetic conditions, were linear (not shown). The apparent second-order rate constant (kₐ[app]) for the inhibition of 5 nM plasminogen-staphylokinase by 25 nM α₂-antiplasmin was 2.7 ± 0.30 × 10⁻⁴ M⁻¹ s⁻¹ (mean ± S.D., n = 12). In the presence of 5 μM α₂-antiplasmin, the t½ of the 1 μM plasminogen-streptokinase complex was 29 min, corresponding to a kₐ[app] value (corrected for spontaneous neutralization) of 3.7 ± 6 M⁻¹ s⁻¹ (mean ± S.D., n = 4). Upon prolonged incubation at 37 °C in the absence of α₂-antiplasmin, the amidolytic activity of the plasminogen-streptokinase complex in buffer containing 25% glycerol slowly decreased with a t½ of about 150 min, whereas that of the plasminogen-staphylokinase complex remained constant.

SDS-PAGE under nonreducing conditions (Fig. 5, panel I) of preincubated (3 min at 37 °C) mixtures of 1.5 μM plasminogen with 4.5 μM staphylokinase or streptokinase showed that the addition of 4.5 μM α₂-antiplasmin resulted in quantitative plasmin-α₂-antiplasmin complex formation within 1 min in the mixture with plasminogen-staphylokinase (lane 6), whereas in the mixture with plasminogen-streptokinase, no complex formation was observed (lane 5). After 30 min, some plasmin-α₂-antiplasmin complex is also generated in the plasminogen-staphylokinase complex.
min-streptokinase sample (lane 7), as shown by an M, of about 140,000 under nonreducing conditions. SDS-PAGE under reducing conditions (Fig. 5, panel II) shows that the single-chain plasminogen moiety is converted to a two-chain plasmin derivative in both the complex with staphylokinase (lane 4) and that with streptokinase (lane 3). The plasmin moiety in the staphylokinase complex reacts with α2-antiplasmin, whereas that in the streptokinase complex is protected from α2-antiplasmin. The mixture of plasminogen-staphylokinase complex and α2-antiplasmin dissociated into three components with M, 65,000, 15,000, and 80,000, corresponding to the plasmin A-chain and excess α2-antiplasmin, staphylokinase, and the plasmin B-chain-α2-antiplasmin complex (Fig. 5, panel II, lane 6). The mixture of plasminogen-streptokinase and α2-antiplasmin displays three components with M, 65,000, 45,000, and 25,000, corresponding to the plasmin A-chain and excess α2-antiplasmin, streptokinase, and the uncomplexed plasmin B-chain (Fig. 5, panel II, lane 5).

Addition of 6-AHA to mixtures of plasminogen-staphylokinase and α2-antiplasmin induced a concentration-dependent reduction of the inhibition rate of the plasminogen-staphylokinase complex (5 nM) by α2-antiplasmin (25 nM) (Fig. 6). A 50% reduction of k1(app) was obtained at a 6-AHA concentration of 60 μM. Saturation of the lysine-binding sites of the plasminogen moiety in the plasminogen-staphylokinase (0.5 μM) complex with 6-AHA (30 mM) reduced k1(app) for the inhibition by α2-antiplasmin (2.5 μM) to 2.0 ± 0.17 × 10^4 M^-1 s^-1 (mean ± S.D., n = 5).

Effect of CNBr-digested Fibrinogen and DesAAFibrin on Plasminogen Activation—Addition of CNBr-digested fibrinogen or desAAFibrin resulted in a concentration-dependent increase of the initial activation rate of plasminogen by plasminogen-streptokinase or plasminogen-staphylokinase (Fig. 7). At saturating concentration of fibrin-like stimulator, simulation of the initial activation rate of plasminogen was less then 2-fold for plasminogen-streptokinase and 3-4-fold for plasminogen-staphylokinase.

Binding to Fibrin—In purified systems (0–3.4 mg/ml fibrin), binding of 125I-labeled streptokinase to fibrin ranged between 18 ± 2% (mean ± S.D., n = 3) at a fibrin concentration of 0.025 mg/ml and 41 ± 2% at a fibrin concentration of 3.4 mg/ml (data not shown). In contrast, no binding of 125I-labeled staphylokinase was observed under the same conditions (0% at 0.025 mg/ml and 5 ± 0.6% at 3.4 mg/ml fibrin, respectively). When human plasma was clotted in the presence of 125I-labeled streptokinase or staphylokinase, the observed binding (mean ± S.D., n = 6) was 4.6 ± 0.9% and 1.5 ± 1.0%, respectively (not shown).

**Fibrinolytic Properties in Purified Systems**—Both staphylokinase and streptokinase induced a time- and concentration-dependent lysis of a purified 125I-labeled fibrin clot (prepared either in the presence or the absence of plasminogen) immersed in a Glu-plasminogen solution, as quantitated by the release of 125I-labeled degradation products (not shown). Fifty percent lysis of plasminogen-free clots in 1 h was obtained with 0.5 nM staphylokinase or with 0.4 nM streptokinase (Table I). In the presence of α2-antiplasmin (final concentration, 1 μM), 50% lysis of a labeled plasminogen-free fibrin clot immersed in Glu-plasminogen (final concentration, 1.5 μM) required 23 nM staphylokinase or 5 nM streptokinase. Compared with staphylokinase, streptokinase caused more extensive plasminogen activation (45% activation versus 10% with staphylokinase) and more extensive α2-antiplasmin consumption (68 versus 15% with staphylokinase). In control experiments with the addition of preformed equimolar plasminogen-staphylokinase or plasminogen-staphylokinase complex, 50% lysis of purified fibrin clots in buffer without the addition of plasminogen or α2-antiplasmin required ≥10 nM of both complexes.

Fibrin clots prepared in the presence of plasminogen were more sensitive to subsequent lysis in a plasminogen solution (with or without α2-antiplasmin), with 50% lysis in 1 h, requiring 0.3 nM staphylokinase in the absence and 8 nM in the presence of α2-antiplasmin; corresponding values for streptokinase were 0.1 and 3 nM, respectively (Table I).

**Fibrinolytic Properties in Human Plasma in Vitro**—Dose-dependent lysis of 125I-fibrin-labeled plasma clots immersed in human plasma was obtained in all experiments with both staphylokinase and streptokinase. Fifty percent lysis in 2 h of a normal plasma clot in normal plasma was obtained with 18 nM staphylokinase or with 68 nM streptokinase, whereas the equipotent doses in α2-antiplasmin-depleted plasma were 23 and 40 nM, respectively (Table II). Fifty percent lysis of an α2-antiplasmin-depleted plasma clot immersed in normal plasma required 12 nM staphylokinase or 17 nM streptokinase; corresponding values in α2-antiplasmin-depleted plasma were 17 or 9 nM. In the experiments with staphylokinase, only moderate fibrinogenolysis occurred at C50, whereas with streptokinase at C50, fibrinogen was virtually depleted in all conditions.

**Fibrinogenolytic Properties in Human Plasma in Vitro**—In the absence of fibrin, 50% activation of plasminogen in 2 h was obtained with 4–7 nM streptokinase, both in normal human plasma and in α2-antiplasmin-depleted plasma (Table III). Equi-effective plasminogen activation (50% in 2 h) required 730 nM staphylokinase in normal plasma and about 3-
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Comparative fibrinolytic properties of staphylokinase and streptokinase in purified systems

The data represent the concentration of plasminogen activator (nM) required to obtain 50% lysis within 1 h (C_{50}) of purified fibrin clots (prepared in the absence or the presence of plasminogen) submersed in buffer containing 1.5 μM Glu-plasminogen in the absence or the presence of 1.0 μM α₂-antiplasmin. The data are mean ± S.E. of three to five independent determinations.

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<th>Incubation milieu</th>
<th>Staphylokinase (C_{50})</th>
<th>Streptokinase (C_{50})</th>
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<td>Glu-plasminogen and α₂-antiplasmin solution</td>
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Comparative fibrinolytic properties of staphylokinase and streptokinase in human plasma in vitro

The data represent the concentration of plasminogen activator (nM) required to obtain 50% lysis within 2 h (C_{50}) of plasma clots (normal or α₂-antiplasmin-depleted) in plasma (normal or α₂-antiplasmin-depleted). The residual fibrinogen levels after 2 h, at C_{50}, are expressed as percent of the baseline value. The data are mean ± S.E. of two to five independent determinations.

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<th>Incubation milieu</th>
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<th>Streptokinase</th>
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<td>α₂-Antiplasmin-depleted plasma clot/whole plasma</td>
<td>12 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>α₂-Antiplasmin-depleted plasma clot/α₂-antiplasmin-depleted plasma</td>
<td>17 ± 5</td>
<td>92 ± 2</td>
</tr>
</tbody>
</table>

Comparative systemic activation of the fibrinolytic system by staphylokinase or streptokinase in human plasma in vitro, in the absence of fibrin

The data represent the concentration of plasminogen activator (nM) required to reduce the fibrinogen or plasminogen levels to 50% of their baseline value, within 2 h. The data are mean values of three independent experiments.

<table>
<thead>
<tr>
<th>Incubation milieu</th>
<th>Staphylokinase</th>
<th>Streptokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibrinogen breakdown</td>
<td>Plasminogen activation</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>790</td>
<td>4.4</td>
</tr>
<tr>
<td>α₂-Antiplasmin-depleted plasma</td>
<td>730</td>
<td>4.0</td>
</tr>
</tbody>
</table>

DISCUSSION

Recently, a plasminogen activator secreted by S. aureus has been cloned by recombinant DNA technology and expressed in E. coli (5, 6) or B. subtilis (7). Recombinant staphylokinase was found to be a more potent and more fibrin-specific fibrinolytic agent than streptokinase in human plasma in vitro (14). Initial experiments have suggested that plasminogen activation by staphylokinase may be inhibited by α₂-antiplasmin in circulating plasma, but not at the fibrin surface (15).

In the present study, the interactions between plasminogen, staphylokinase, fibrin, and α₂-antiplasmin were studied in more detail. The results indicate that, like streptokinase, staphylokinase is not an enzyme. It does not directly convert plasminogen to plasmin, but forms a stoichiometric complex with plasminogen, which then activates other plasminogen molecules. This is evidenced by our finding that active-site tagged plasminogen (rPlg-Ala^{146}) is converted to a two-chain derivative by the plasminogen-staphylokinase complex but not by staphylokinase alone. Plasminogen activation obeys Michaelis-Menten kinetics with a catalytic efficiency of the plasminogen-staphylokinase complex for plasminogen activation that is about 4-fold lower than that of the plasminogen-streptokinase complex. Staphylokinase does not bind to fibrin, whereas fibrin-like stimulators enhance the initial rate of plasminogen activation by staphylokinase 4-fold, probably due to a somewhat more readily activatable plasminogen conformation in the presence of fibrin. In the presence of α₂-antiplasmin, activation of plasminogen by staphylokinase is completely abolished, whereas activation by streptokinase is not affected. This is due to rapid inhibition of the plasminogen-staphylokinase complex by α₂-antiplasmin, whereas the plasminogen-streptokinase complex is virtually not inhibited. These findings suggest that in human plasma, in the absence of fibrin, the plasminogen-staphylokinase complex, when formed, may be rapidly neutralized by α₂-antiplasmin, whereas the plasminogen-streptokinase complex is not neutralized. In addition, the affinity of the plasminogen-staphylokinase complex for plasminogen is about 10-fold lower than that of the plasminogen-streptokinase complex. Consistent with these observations, we have found that 50% activation...
of plasminogen and 50% fibrinogen degradation in human plasma in vitro require 180-fold (on a molar basis) more staphylokinase than streptokinase. Removal of α2-antiplasmin from the plasma results in a 3-4-fold enhanced sensitivity to staphylokinase, whereas the sensitivity to streptokinase is not altered. The residual high resistance of α2-antiplasmin-depleted plasma to staphylokinase may be due to inhibition of the plasminogen-staphylokinase complex by other plasma protease inhibitors or may reflect a low generation rate of such complexes in plasma.

Addition of 6-AHA to concentrations that saturate the lysine-binding sites of the plasmin(ogen) moiety in the plasminogen-staphylokinase complex reduces the inhibition rate by α2-antiplasmin in purified systems by 130-fold. A 50% reduction of the inhibition rate is obtained with 60 μM 6-AHA, comparable with the concentration of 20 μM required to reduce the inhibition rate of plasmin by α2-antiplasmin to 50% (33). These findings suggest that fibrin-bound plasminogen-staphylokinase complex may be protected from inhibition by α2-antiplasmin. This hypothesis is supported by our findings that equipotent concentrations (50% fibrin clot lysis in 2 h in human plasma in vitro) for staphylokinase are 4-fold lower than for streptokinase and that no fibrinolysis is observed with staphylokinase, whereas fibrinogen is virtually depleted with streptokinase. Because it is known that α2-antiplasmin is cross-linked to fibrin when plasma is clotted in the presence of activated factor XIII and calcium (34, 35), we have performed additional clot lysis experiments with α2-antiplasmin-depleted plasma clots in normal or in α2-antiplasmin-depleted plasma (Table II). These results indicate that for staphylokinase the C50 values in normal plasma for a whole plasma clot (18 ± 2 nM) or an α2-antiplasmin-depleted plasma clot (12 ± 1 nM) are not significantly different (p = 0.12), whereas for streptokinase the differences appear statistically significant (68 ± 2 versus 17 ± 1 nM, p = 0.002). Similarly, for staphylokinase the C50 values in α2-antiplasmin-depleted plasma for a whole plasma clot (23 ± 5 nM) or an α2-antiplasmin-depleted plasma clot (17 ± 5 nM) are not different (p = 0.42), whereas for streptokinase the C50 values are different (40 ± 6 versus 9 ± 2 nM, p = 0.003). These results suggest that α2-antiplasmin cross-linking to fibrin does not play an important role in the fibrinolytic potency of staphylokinase, at least in this static in vitro system.

These results suggest the following mechanism for fibrin-specific plasminogen activation by staphylokinase. In plasma in the absence of fibrin the plasminogen-staphylokinase complex, when formed, is rapidly neutralized by α2-antiplasmin, thus preventing systemic plasminogen activation. In the presence of fibrin, some of the lysine-binding sites of the plasminogen-staphylokinase complex are occupied, and inhibition by α2-antiplasmin is impaired, thus allowing efficient plasminogen activation.

This mechanism is somewhat reminiscent of that of the fibrin specificity of tissue-type plasminogen activator. The low affinity of tissue-type plasminogen activator for plasminogen in the absence of fibrin precludes generation of plasmin in the circulation, whereas fibrin-bound plasminogen is activated much more efficiently by tissue-type plasminogen activator and plasmin generated at the fibrin surface is protected from rapid inhibition by α2-antiplasmin (1, 36). For staphylokinase, systemic plasminogen activation is impaired mainly by rapid inhibition of the plasminogen-staphylokinase complex by α2-antiplasmin and possibly also to some extent by the low affinity of the complex for plasminogen (Km = 7 μM). No significant stimulation of plasminogen activation by fibrin is observed, but in the presence of fibrin the plasminogen-staphylokinase complex may be protected from inhibition by α2-antiplasmin.

It remains to be investigated if the superior fibrinolytic potency and fibrin specificity of staphylokinase over streptokinase observed in vitro is maintained in vivo.

REFERENCES