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

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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 μM and k_2 = 1.5 s^{-1}. In purified systems, α_2-antiplasmin inhibits the plasminogen-staphylokinase complex with $k_{1(app)} = 2.7 \pm 0.30 \times 10^6$ M^{-1} s^{-1} (mean ± 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 $2.0 \pm 0.17 \times 10^4$ M^{-1} s^{-1} (mean ± S.D., n = 5) at concentrations ≥30 mM, with a 50% reduction at a 6-aminohexanoic acid concentration of 60 μM. 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 submersed 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, 50% fibrinogen degradation in human plasma in 2 h requires 790 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 α_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 α_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. Physiological fibrinolysis, however, is fibrin-oriented as a result of specific molecular interactions between tissue-type plasminogen activator, fibrin, plasmin(ogen), and α_2-antiplasmin (1).

Streptokinase, an M_r 45,000 protein secreted by β-hemolytic streptococci, is used in thrombolytic therapy, but its administration is associated with extensive systemic fibrinogenolysis (2). Staphylokinase, an M_r 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 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 α_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 α_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^R (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^{740} mutagenized to Ala (rPlg-Ala^{740})[1] 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. rPlg-Ala^{740} was purified from conditioned cell culture medium and characterized as described elsewhere (20, 21). Human α_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 ad-

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[1] The abbreviations used are: rPlg-Ala^{740}, recombinant plasminogen with the active-site Ser^{740} mutagenized to Ala; 6-AHA, 6-aminohexanoic acid; S-2251, D-valyl-leucyl-lysine-*p*-nitroanilide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; des-AAfibrin, fibrinogen with the two fibrinopeptides A removed, fibrin I.

sorption with lysine-Sepharose, as described elsewhere (23). CNBr-digested fibrinogen was prepared as described elsewhere (23), and solubilized desAAfibrin (Desafib) was obtained from Biopool (Umeå, Sweden). The chromogenic substrate D-valyl-leucyl-lysine-*p*-nitroanilide (S-2251) was purchased from KabiVitrum. The synthetic thrombin inhibitor D-Ile-Pro-Arg-CH_2Cl (24) was custom-synthesized at Union Chimique Belge (Brussels). ^{125}I-Labeled fibrinogen was purchased from Amersham. Plasma was pooled human plasma obtained from at least five healthy blood donors. α_2-Antiplasmin-depleted plasma was obtained from normal human plasma by immunoadsorption on an insolubilized monoclonal antibody (MA-34F7) directed against α_2-antiplasmin. After depletion, this plasma contained about 1% residual α_2-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). Streptokinase and staphylokinase were labeled with ^{125}I using the IODO-GEN method (27) to specific activities of 15×10^6 and 19×10^6 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 1% dithioerythritol.

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 μM) at 37 °C in 0.1 M phosphate buffer, pH 7.4. At different time intervals (0–10 min), generation of an active site in the plasminogen-staphylokinase or plasminogen-streptokinase complexes was measured with S-2251 (final concentration, 1 mM) after 50-fold dilution of samples in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.038 M NaCl and 0.01% Tween 80. Alternatively, plasminogen (final concentration, 1 μM) was incubated with α_2-antiplasmin (final concentration, 5 μM) at 37 °C in 0.1 M phosphate buffer, pH 7.4, for 1 min before addition of staphylokinase or streptokinase (final concentration, 5 μM). Generation of active sites as a function of time was monitored as described above. The kinetic parameters for the hydrolysis of S-2251 (0.1–1.0 mM) by plasmin, plasminogen-staphylokinase, or plasminogen-streptokinase (10 nM each) were determined by Lineweaver-Burk analysis.

Natural plasminogen or rPlg-Ala^{740} (final concentration, 1.5 μM) was incubated at 37 °C in 0.1 M phosphate buffer, pH 7.4, with staphylokinase or streptokinase (final concentration, 50 nM). At different time intervals (0–90 min), samples were removed from the incubation mixtures, reduced immediately, and subjected to SDS-PAGE. Alternatively, rPlg-Ala^{740} (final concentration, 1.5 μM) was incubated in 0.1 M phosphate buffer, pH 7.4, with 20 nM plasminogen-streptokinase, plasminogen-staphylokinase (preformed by incubation of 1.5 μM plasminogen with 4.5 μM staphylokinase or streptokinase for 3 min at 37 °C), or with 20 nM plasmin. Samples were removed at different time points (0–60 min), immediately reduced, and subjected to SDS-PAGE.

Kinetics of Plasminogen Activation—Equimolar plasminogen-staphylokinase and plasminogen-streptokinase complexes (final concentration, approximately 5 μM each, with a 5–10% excess of plasminogen) were prepared by incubation of plasminogen with staphylokinase or streptokinase at 37 °C for 3 min in 0.1 M phosphate buffer, pH 7.4, containing 25% glycerol; the mixture was then stored on ice. For kinetic analysis, plasminogen-staphylokinase or plasminogen-streptokinase (final concentration, 2 nM) was incubated with plasminogen (final concentration, 0.1–2 μM for plasminogen-streptokinase and 1.6–20 μM for plasminogen-staphylokinase) at 37 °C in 0.1 M phosphate buffer, pH 7.4. Generated plasmin was measured at different time intervals (0–5 min) with S-2251 (final concentration, 1 mM) after 20-fold dilution of the sample. Initial activation rates were obtained from plots of the concentration of generated plasmin *versus* time.

Effect of α_2-Antiplasmin on Plasminogen Activation—Activation of plasminogen (final concentration, 1.5 μM) at 37 °C in 0.1 M phosphate buffer, pH 7.4, by staphylokinase or streptokinase (final concentration, 5 nM) was measured in the absence or the presence of α_2-antiplasmin (final concentration, 3 μM). Therefore, at different time intervals (0–50 min), 10-μl samples were removed from the incubation mixtures and incubated for 10 min at 37 °C in 300 μl of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.038 M NaCl, 0.01% Tween 80, and 1,000 IU of streptokinase (5,000-fold molar excess of streptokinase over the amount of streptokinase or staphylokinase present in the sample). Under these conditions, plasmin is rapidly and quantitatively complexed by α_2-antiplasmin and the plasminogen-streptokinase complex is measured with S-2251 (final concentration, 1 mM). The amount of plasminogen converted to plasmin at each time point is then expressed as a percentage by subtracting the residual plasminogen concentration from the initial concentration. In the experiments without α_2-antiplasmin, plasmin is directly quantitated with S-2251 (final concentration, 1 mM). The molecular species in the incubation mixtures at 30 min in the presence of α_2-antiplasmin were identified by SDS-PAGE.

Inhibition of Plasminogen-Staphylokinase or Plasminogen-Streptokinase Complexes by α_2-Antiplasmin—Plasminogen (final concentration, 1 μM) was complexed with staphylokinase or streptokinase (final concentration, 5 μM) by incubation for 5 min at 37 °C in 0.1 M phosphate buffer, pH 7.4. Inhibition of plasminogen-streptokinase (final concentration, 1 μM) by α_2-antiplasmin (final concentration, 5–7 μM) was monitored as follows. Samples were removed from incubation mixtures, and residual plasminogen-streptokinase at different time points (0–60 min) was quantitated with S-2251 (final concentration, 0.3 mM) after 50-fold dilution of the samples. The half-life time ($t^{1/2}$) of the plasminogen-streptokinase complex and the apparent second-order rate constant ($k_{1(app)}$) were calculated ($k_{1(app)} = \ln 2/t_{1/2} \cdot [I]$, where $[I] = [\alpha_2\text{-antiplasmin}]$).

Inhibition of the plasminogen-staphylokinase complex (final concentration, 5 nM) by α_2-antiplasmin (final concentration, 25 nM) was monitored continuously in the presence of S-2251 (final concentration, 1.0 mM). The residual complex was determined at different time intervals (0–5 min), and $k_{1(app)}$ was calculated as described above after correction for the effect of substrate on the inhibition reaction (28), using the formula $[E] = T[E]_0/bT_0$, where $b = (1 + [S]/K_m)$, $[E]$ = enzyme concentration at time t, $[E]_0$ = initial enzyme concentration, T = the slope of the absorbance *versus* time curve at time t, T_0 = the initial slope, [S] = the substrate concentration, and K_m = the Michaelis constant of S-2251 for plasminogen-staphylokinase complex. The effect of 6-aminohexanoic acid (final concentration, 0–500 μM) on the inhibition rate of the plasminogen-staphylokinase complex by α_2-antiplasmin was determined in the same way. At each concentration of 6-aminohexanoic acid, $k_{1(app)}$ was expressed in percentage of the value in the absence of ligand. The effect of saturating concentrations of 6-aminohexanoic acid (final concentration, 30 mM) on the inhibition rate of the plasminogen-staphylokinase complex by α_2-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 α_2-antiplasmin or between plasminogen, streptokinase, and α_2-antiplasmin were also monitored by SDS-PAGE under nonreducing and reducing conditions. Plasminogen (final concentration, 1.5 μM) was mixed with staphylokinase or streptokinase (final concentration, 4.5 μM) at 37 °C for 3 min, followed by the addition of α_2-antiplasmin (final concentration, 4.5 μM). Samples were removed before the addition of α_2-antiplasmin and 1 min and 30 min after its addition.

Effect of CNBr-digested Fibrinogen and DesAAfibrin on Plasminogen Activation—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 desAAfibrin (final concentration, 0–1 μM). At different time points (0–5 min), samples were removed from the incubation mixtures, and generated plasmin was quantitated with S-2251 (final concentration, 1 mM) after 25-fold dilution.

Binding to Fibrin—Purified plasminogen-free human fibrinogen (0–3.3 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 ^{125}I-labeled staphylokinase or ^{125}I-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-CH_2Cl (final concentration, 10^{-4} M), 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 Ca^{2+} (final concentration, 45 mM) and thrombin (final concentration, 2 NIH units/ml) in the presence of ^{125}I-labeled staphylokinase or ^{125}I-labeled streptokinase (50,000 cpm/ml), and binding was quantitated as described above.

Fibrinolytic Properties in Purified Systems—^{125}I-Labeled clots of

purified human fibrin were prepared by the addition of $CaCl_2$ (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 ^{125}I-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 ^{125}I-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-plasminogen. Lysis of fibrin clots by addition of different concentrations of staphylokinase (final concentration, 1.1–67 nM) or streptokinase (final concentration, 0.1–2.8 nM) was monitored for 1 h and quantitated by the release of radioactivity from the clot into the surrounding liquid. Alternatively, the same experiments were performed with the addition of purified human α_2-antiplasmin to a final concentration of 1 μM, and clot lysis was monitored after the addition of staphylokinase (final concentration, 2–130 nM) or streptokinase (final concentration, 0.7–44 nM). The concentration of plasminogen activator yielding 50% lysis in 1 h was determined from plots of the extent of clot lysis (in percent) *versus* the concentration of the plasminogen activators. Residual α_2-antiplasmin and/or plasminogen levels in the solutions were monitored with chromogenic substrate assays (29, 30).

Fibrinolytic Properties in Human Plasma in Vitro—^{125}I-Fibrin-labeled plasma clots were prepared from normal human plasma or from α_2-antiplasmin-depleted plasma as described above, following addition of 500,000 cpm/ml of ^{125}I-labeled fibrinogen and coagulation with $CaCl_2$ (final concentration, 25 mM) and thrombin (final concentration, 2 NIH units/ml). Lysis of ^{125}I-fibrin-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 α_2-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% clot lysis in 2 h (C_{50}), was determined from plots of percent lysis *versus* the concentration of plasminogen activator. Residual fibrinogen levels at C_{50} were determined from plots of residual fibrinogen at 2 h *versus* the concentration of plasminogen activator.

Fibrinogenolytic Properties in Human Plasma in Vitro—Systemic activation of the fibrinolytic system by staphylokinase or streptokinase in normal human plasma or in α_2-antiplasmin-depleted plasma (final concentration, 17–4,300 nM for staphylokinase or 0.35–89 nM for streptokinase) in the absence of fibrin was monitored over 4 h at hourly intervals, as described above. The concentration of plasminogen activator required to obtain 50% plasminogen activation and 50% fibrinogen degradation within 2 h, was determined graphically from dose-response curves.

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 α_2-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 K_M values are comparable (0.57, 0.55, and 0.48 mM, respectively), whereas k_{cat} is 2-fold lower for plasminogen-staphylokinase (17 s^{-1}) than for plasminogen-streptokinase or plasmin (35 s^{-1}) (data not shown).

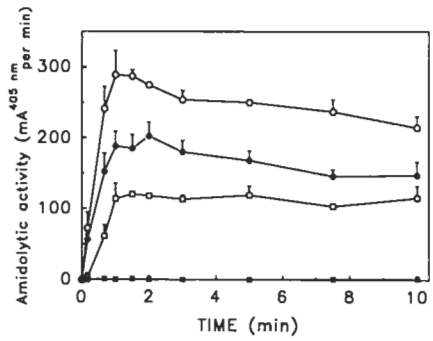

FIG. 1. **Generation of amidolytic activity in mixtures of plasminogen with a 5-fold molar excess of staphylokinase (□, ■) or streptokinase (○, ●) in the absence (*open symbols*) or the presence (*closed symbols*) of α_2-antiplasmin at a 5-fold molar excess over plasminogen.** Amidolytic activity is measured with S-2251 (final concentration, 1 mM) after 50-fold dilution of samples. The data represent the mean ± S.D. of three determinations.

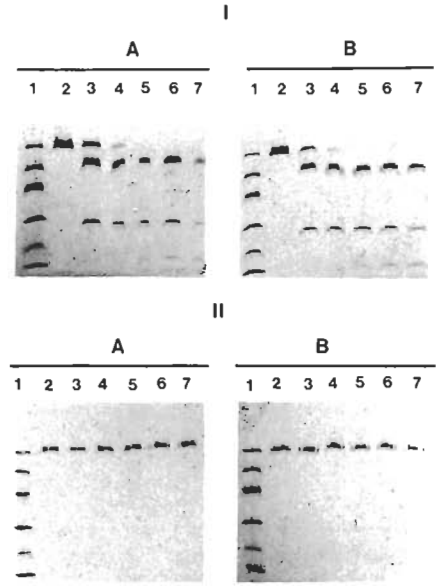

FIG. 2. **Activation of natural plasminogen (*panels I*) or rPlg-Ala^{740} (*panels II*) (final concentration, 1.5 μM) with staphylokinase (*A*) or streptokinase (*B*) (final concentration, 50 nM).** Samples for reduced SDS-PAGE were taken at times 0 (*lane 2*), 2 min (*lane 3*), 5 min (*lane 4*), 10 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-Ala^{740} (*panels II*). The protein calibration mixture (*lanes 1*) consists of phosphorylase *b* (M_r 97,000), albumin (M_r 67,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400).

Both staphylokinase and streptokinase convert natural plasminogen to two-chain plasmin, as revealed by reduced SDS-PAGE, whereas active-site mutagenized plasminogen, rPlg-Ala^{740}, is not converted to a two-chain derivative by either staphylokinase or streptokinase (Fig. 2). Under the same experimental conditions, urokinase completely converts rPlg-Ala^{740} to plasmin (not shown). Fig. 3 shows that both the plasminogen-staphylokinase and the plasminogen-streptokinase complexes convert rPlg-Ala^{740} 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-

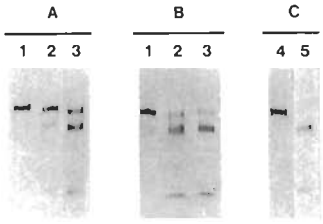

FIG. 3. **Conversion at 37 °C of rPlg-Ala^{740} (1.5 μM) to a two-chain derivative by plasminogen-staphylokinase complex (*A*) or by plasminogen-streptokinase complex (*B*) (20 nM each) as monitored on reduced SDS-PAGE.** Samples are taken at times 0 (*lanes 1*), 10 min (*lanes 2*), and 60 min (*lanes 3*). In *panel C*, *lane 4* represents rPlg-Ala^{740} (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.

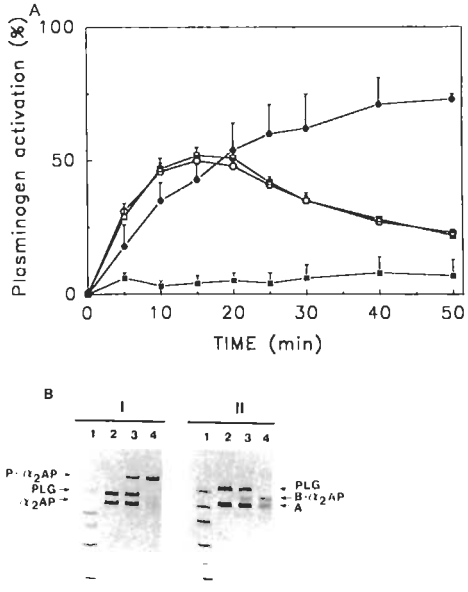

FIG. 4. **Activation of plasminogen (final concentration, 1.5 μM) by staphylokinase or streptokinase (final concentration, 5 nM) in the absence or the presence of α_2-antiplasmin (final concentration, 3 μM).** *A*, activation of plasminogen (in percent) by staphylokinase (□, ■) or streptokinase (○, ●) as a function of time, in the absence (*open symbols*) or in the presence of α_2-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_r 97,000), albumin (M_r 67,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100) and α-lactalbumin (M_r 14,400); *lane 2*, plasminogen (*PLG*) plus staphylokinase in the presence of α_2-antiplasmin (*α_2AP*); *lane 3*, plasminogen plus streptokinase in the presence of α_2-antiplasmin; *lane 4*, plasmin-α_2-antiplasmin complex (*P-α_2AP*). *B-α_2AP*, complex of plasmin B-chain with α_2-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 K_m = 7.0 μM and k_2 = 1.5 s^{-1} for plasminogen-staphylokinase (mean of two independent determinations with $r > 0.99$) and K_m = 0.65 μM and k_2 = 0.52 s^{-1} for plasminogen-streptokinase (mean of two independent determinations with $r > 0.99$). The catalytic efficiency (k_2/K_m) 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 α_2-Antiplasmin on Plasminogen Activation—Fig. 4*A* 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 α_2-antiplasmin (final concentration, 3 μM) does not markedly affect plasminogen activation by streptokinase, but virtually abolishes the activation by staphylokinase, as shown by quantiation of residual plasminogen concentrations. SDS-PAGE under nonreducing conditions of samples from mixtures with α_2-antiplasmin (Fig. 4*B*) reveals the presence of plasmin-α_2-antiplasmin complexes in incubation mixtures with streptokinase but not in mixtures with staphylokinase. Under reducing conditions, the plasmin-α_2-antiplasmin complex dissociates in the plasmin B-chain-α_2-antiplasmin complex and the plasmin A-chain.

Inhibition of Plasminogen-Staphylokinase or Plasminogen-Streptokinase Complexes by α_2-Antiplasmin—Semilogarithmic plots of residual complex as a function of time, following incubation of preformed plasminogen-staphylokinase or plasminogen-streptokinase complexes with α_2-antiplasmin under pseudo first-order kinetic conditions, were linear (not shown). The apparent second-order rate constant ($k_{1(app)}$) for the inhibition of 5 nM plasminogen-staphylokinase by 25 nM α_2-antiplasmin was 2.7 ± 0.30 × 10^6 M^{-1} s^{-1} (mean ± S.D., n = 12). In the presence of 5 μM α_2-antiplasmin, the $t_{1/2}$ of the 1 μM plasminogen-streptokinase complex was 23 min, corresponding to a $k_{1(app)}$ value (corrected for spontaneous neutralization) of 37 ± 6 M^{-1} s^{-1} (mean ± S.D., n = 4). Upon prolonged incubation at 37 °C in the absence of α_2-antiplasmin, the amidolytic activity of the plasminogen-streptokinase complex in buffer containing 25% glycerol slowly decreased with a $t_{1/2}$ 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 α_2-antiplasmin resulted in quantitative plasmin-α_2-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-α_2-antiplasmin complex is also generated in the plas-

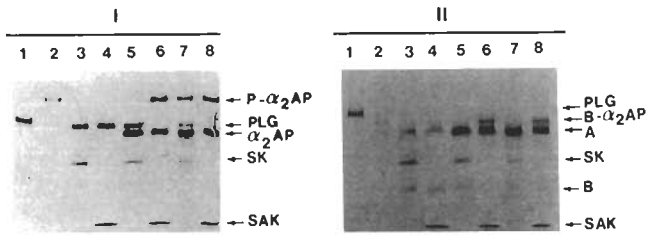

FIG. 5. **SDS-PAGE under nonreducing (*I*) or reducing (*II*) conditions of mixtures of plasminogen-staphylokinase or plasminogen-streptokinase and 4.5 μM α_2-antiplasmin.** The plasminogen-staphylokinase and plasminogen-streptokinase complexes were generated by mixing 1.5 μM plasminogen and 4.5 μM staphylokinase or streptokinase. *Lane 1*, plasminogen (*PLG*); *lane 2*, plasmin-α_2-antiplasmin complex (*P-α_2-AP*); *lane 3*, plasminogen-streptokinase complex; *lane 4*, plasminogen-staphylokinase complex; *lane 5*, plasminogen-streptokinase complex after a 1-min incubation with α_2-antiplasmin (*α_2AP*), *lane 6*, plasminogen-staphylokinase complex after a 1-min incubation with α_2-antiplasmin; *lane 7*, plasminogen-streptokinase complex after a 30-min incubation with α_2-antiplasmin; *lane 8*, plasminogen-staphylokinase complex after a 30-min incubation with α_2-antiplasmin. *SK*, streptokinase; *SAK*, staphylokinase; *A*, plasmin A-chain; *B*, plasmin B-chain; *B-α_2AP*, complex of plasmin B-chain with α_2-antiplasmin.

min-streptokinase sample (*lane 7*), as shown by an M_r 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 α_2-antiplasmin, whereas that in the streptokinase complex is protected from α_2-antiplasmin. The mixture of plasminogen-staphylokinase complex and α_2-antiplasmin dissociated into three components with M_r 65,000, 15,000, and 80,000, corresponding to the plasmin A-chain and excess α_2-antiplasmin, staphylokinase, and the plasmin B-chain-α_2-antiplasmin complex (Fig. 5, *panel II, lane 6*). The mixture of plasminogen-streptokinase and α_2-antiplasmin displays three components with M_r 65,000, 45,000, and 25,000, corresponding to the plasmin A-chain and excess α_2-antiplasmin, streptokinase, and the uncomplexed plasmin B-chain (Fig. 5, *panel II, lane 5*).

Addition of 6-AHA to mixtures of plasminogen-staphylokinase and α_2-antiplasmin induced a concentration-dependent reduction of the inhibition rate of the plasminogen-staphylokinase complex (5 nM) by α_2-antiplasmin (25 nM) (Fig. 6). A 50% reduction of $k_{1(app)}$ 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 $k_{1(app)}$ for the inhibition by α_2-antiplasmin (2.5 μM) to $2.0 \pm 0.17 \times 10^4$ 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, stimulation 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 ^{125}I-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 ^{125}I-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 ^{125}I-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).

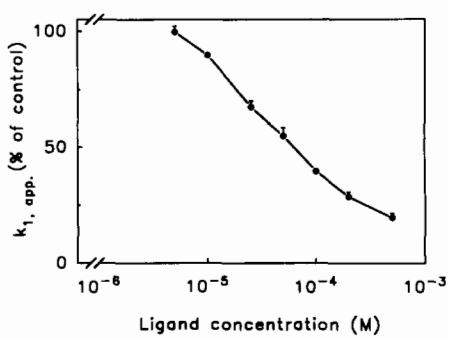

FIG. 6. **Influence of 6-aminohexanoic acid on the apparent second-order rate constant ($k_{1(app)}$) of the inhibition of plasminogen-staphylokinase complex by α_2-antiplasmin.** The $k_{1(app)}$ values, determined in the presence of different ligand concentrations, are each expressed as a percent of the value obtained in the absence of ligand. The data represent the mean ± S.D. of three determinations.

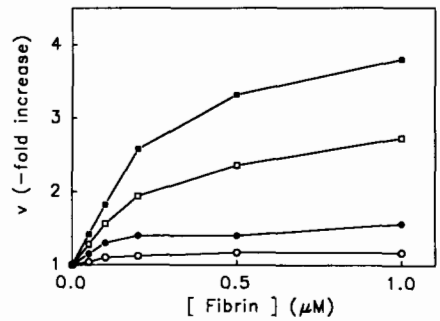

FIG. 7. **Effect of CNBr-digested fibrinogen and desAAfibrin on plasminogen activation by staphylokinase (□, ■) or streptokinase (○, ●).** The increase in the initial activation rate of plasminogen (v) is plotted *versus* the concentration of desAAfibrin (*open symbols*) or CNBr-digested fibrinogen (*closed symbols*).

Fibrinolytic Properties in Purified Systems—Both staphylokinase and streptokinase induced a time- and concentration-dependent lysis of a purified ^{125}I-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 ^{125}I-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 α_2-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 α_2-antiplasmin consumption (68 *versus* 15% with staphylokinase). In control experiments with the addition of preformed equimolar plasminogen-staphylokinase or plasminogen-streptokinase complex, 50% lysis of purified fibrin clots in buffer without the addition of plasminogen or α_2-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 α_2-antiplasmin), with 50% lysis in 1 h, requiring 0.3 nM staphylokinase in the absence and 8 nM in the presence of α_2-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 ^{125}I-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 α_2-antiplasmin-depleted plasma were 23 and 40 nM, respectively (Table II). Fifty percent lysis of an α_2-antiplasmin-depleted plasma clot immersed in normal plasma required 12 nM staphylokinase or 17 nM streptokinase; corresponding values in α_2-antiplasmin-depleted plasma were 17 or 9 nM. In the experiments with staphylokinase, only moderate fibrinogenolysis occurred at C_{50}, whereas with streptokinase at C_{50}, 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 α_2-antiplasmin-depleted plasma (Table III). Equi-effective plasminogen activation (50% in 2 h) required 730 nM staphylokinase in normal plasma and about 3-

TABLE I

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 α_2-antiplasmin. The data are mean ± S.E. of three to five independent determinations.

Incubation milieu	Staphylokinase (C_{50})		Streptokinase (C_{50})	
	Fibrin clot		Fibrin clot	
	−Plasminogen	+Plasminogen	−Plasminogen	+Plasminogen
	nM		*nM*	
Glu-plasminogen solution	0.5 ± 0.1	0.3 ± 0.03	0.4 ± 0.1	0.1 ± 0.01
Glu-plasminogen and α_2-antiplasmin solution	23 ± 1	8 ± 1	5 ± 1	3 ± 0.3

TABLE II

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 α_2-antiplasmin-depleted) in plasma (normal or α_2-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.

Incubation milieu	Staphylokinase		Streptokinase	
	C_{50}	Fibrinogen	C_{50}	Fibrinogen
	nM	%	*nM*	%
Plasma clot/whole plasma	18 ± 2	94 ± 1	68 ± 2	<10
Plasma clot/α_2-antiplasmin-depleted plasma	23 ± 5	71 ± 8	40 ± 6	<10
α_2-Antiplasmin-depleted plasma clot/whole plasma	12 ± 1	95 ± 0	17 ± 1	<10
α_2-Antiplasmin-depleted plasma clot/α_2-antiplasmin-depleted plasma	17 ± 5	82 ± 4	92 ± 2	<10

TABLE III

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.

Incubation milieu	Staphylokinase		Streptokinase	
	Fibrinogen breakdown	Plasminogen activation	Fibrinogen breakdown	Plasminogen activation
	nM		*nM*	
Normal plasma	790	730	4.4	4.0
α_2-Antiplasmin-depleted plasma	220	230	3.3	6.6

fold less in α_2-antiplasmin-depleted plasma. Similarly, 50% fibrinogen degradation in 2 h occurred with only 3–4 nM streptokinase, both in normal plasma and in α_2-antiplasmin-depleted plasma, whereas corresponding values were 790 and 220 nM for staphylokinase.

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 α_2-antiplasmin in circulating plasma, but not at the fibrin surface (15).

In the present study, the interactions between plasminogen, staphylokinase, fibrin, and α_2-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 that then activates other plasminogen molecules. This is evidenced by our finding that active-site mutagenized plasminogen (rPlg-Ala^{740}) 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 α_2-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 α_2-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 α_2-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 α_2-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 α_2-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 α_2-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 α_2-antiplasmin to 50% (33). These findings suggest that fibrin-bound plasminogen-staphylokinase complex may be protected from inhibition by α_2-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 fibrinogenolysis is observed with staphylokinase, whereas fibrinogen is virtually depleted with streptokinase. Because it is known that α_2-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 α_2-antiplasmin-depleted plasma clots in normal or in α_2-antiplasmin-depleted plasma (Table II). These results indicate that for staphylokinase the C_{50} values in normal plasma for a whole plasma clot (18 ± 2 nM) or an α_2-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 C_{50} values in α_2-antiplasmin-depleted plasma for a whole plasma clot (23 ± 5 nM) or an α_2-antiplasmin-depleted plasma clot (17 ± 5 nM) are not different (p = 0.42), whereas for streptokinase the C_{50} values are different (40 ± 6 *versus* 9 ± 2 nM, p = 0.003). These results suggest that α_2-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 α_2-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 α_2-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 α_2-antiplasmin (1, 36). For staphylokinase, systemic plasminogen activation is impaired mainly by rapid inhibition of the plasminogen-staphylokinase complex by α_2-antiplasmin and possibly also to some extent by the low affinity of the complex for plasminogen (K_m = 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 α_2-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*.

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