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Interactions between Staphylokinase, Plasmin(ogen), and Fibrin

STAPHYLOKINASE DISCRIMINATES BETWEEN FREE PLASMINOGEN AND PLASMINOGEN BOUND TO PARTIALLY DEGRADED FIBRIN*

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Staphylokinase (STA), a protein of bacterial origin, induces highly fibrin-specific thrombolysis both in human plasma *in vitro* and in pilot clinical trials. Using fluorescence microscopy, we investigated the spatial distribution of fluorescein isothiocyanate (FITC)-labeled STA during lysis of a plasma clot and its binding to purified fibrin clots in the presence or in the absence of plasmin(ogen). STA highly accumulated in a thin superficial layer of the lysing plasma clot following the distribution of plasminogen (Pg) during lysis. Experiments with purified fibrin clots revealed that STA binds to Pg bound to partially degraded fibrin but not to Pg bound to intact fibrin. Binding of FITC-labeled STA to various forms of plasmin(ogen) in a buffer solution was studied by measuring fluorescence anisotropy. The binding constant for Glu-Pg was estimated as 7.4 μM and for Lys-Pg as 0.28 μM; for active-site blocked plasmin the binding constant was less than 0.05 μM. The much lower affinity of STA for Glu-Pg compared with that for active site-blocked plasmin was mainly due to a lower association rate constant, as assessed by real time biospecific interaction analysis. Gel filtration of a mixture of STA with a molar excess of Glu-Pg demonstrated that STA migrated as an unbound 18-kDa protein when activation of Pg into plasmin was precluded by inhibitors of plasmin. When gel-filtered under the same conditions with plasmin, STA migrated in complex with plasmin with an apparent molecular mass of 100 kDa. Confocal fluorescence microscopy finally demonstrated that when FITC-labeled STA was added to plasma before clotting, it did not bind to fibrin fibers during the first minutes (lag phase), although Pg bound to the fibers moderately. Then, both Pg and STA started to accumulate on the fibers progressively, followed by complete lysis of the clot. In conclusion, our results imply that, when STA is added to plasma, only a small percentage associates with Pg. In contrast, STA binds strongly to plasmin and to Pg, which is bound to partially degraded fibrin. These findings add a new mechanism to the known explanations for the inefficient Pg activation by STA in plasma and specify the mechanism for fibrin-dependent activation of Pg.

Staphylokinase (STA)[1] was recently demonstrated to induce highly fibrin-specific thrombolysis both in human plasma *in vitro* and in limited clinical trials *in vivo* (1–5). Like streptokinase (SK), STA is a protein of bacterial origin, which is not capable of directly converting plasminogen (Pg) into plasmin (Pl). Both SK and STA form complexes with Pl, which, as a result, exhibit a Pg-activating activity (6–11). Unlike SK, STA is a fibrin-specific agent. The difference in fibrin specificity of the two substances is currently attributed to a number of differences in the interactions of STA-plasmin(ogen) and SK-plasmin(ogen) complexes with α_2-antiplasmin and fibrin (1).

SK forms tight complexes with both Pg and Pl. The serine protease active site is exposed in the SK·Pg complex without conversion of single chain Pg into double chain Pl (7, 8). The SK·Pl complex is almost not susceptible to inhibition by the main plasma inhibitor of Pl, α_2-antiplasmin (12). Thus, SK·Pl exhibits its full activity in plasma in the absence of fibrin, causing depletion of the plasma pool of Pg and α_2-antiplasmin and leading to systemic breakdown of circulating fibrinogen.

The high fibrin specificity of STA is currently attributed to two essential features in which STA differs from SK. First, the active site is not exposed in the STA·Pg complex (11). Second, the STA·Pl complex is effectively inhibited by α_2-antiplasmin in the absence of fibrin (10). Fibrin-bound STA·Pl complex is protected from inhibition by α_2-antiplasmin (13, 14). These properties thus prevent STA-induced activation of Pg in the plasma milieu and make STA an effective indirect activator of Pg within a thrombus. In addition, it has been shown that the binding of α_2-antiplasmin to the STA·Pl complex leads to dissociation of the STA moiety from the complex and to the recycling of STA to other Pg molecules (15). It is believed that this mechanism enhances the Pg activation within thrombi, in which the dissociated STA recycles to fibrin-bound plasmin(ogen). In plasma this mechanism is not effective, since α_2-antiplasmin prevents generation of STA·Pl. It has been suggested that the role of fibrin is not restricted to the protection of STA·Pl from α_2-antiplasmin, but that fibrin also facilitates Pg activation by STA (14). However, the mechanism of such facilitation has not been elucidated.

In the present study, we used fluorescence microscopy to investigate the spatial distribution of STA and the dynamics of its interaction with fibrin fibers during lysis of a plasma clot. STA concentrated near the surface of a lysing clot following the superficial accumulation of Pg, observed in our previous studies (16, 17). A binding study in solution and experiments performed in a purified system with fibrin clots demonstrated that

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[1] The abbreviations used are: STA, staphylokinase; Sak42D and Sak-STAR, variants of staphylokinase; SK, streptokinase; FITC, fluorescein isothiocyanate; Pg, plasminogen; Pl, plasmin; CpB, carboxypeptidase B; TBS, Tris-buffered saline; TAC, Tris acetate buffer; VPLCK, D-Val-Phe-Lys-chloromethylketone; rPg-Ala^{741}, recombinant plasminogen with the active center Ser^{741} mutagenized to Ala; BSA, bovine serum albumin; MAb, monoclonal antibody; KIU, kallikrein-inactivating units.

STA binds to Pl and to Pg, which is bound to partially degraded fibrin, but does not bind appreciably to Glu-Pg in solution. These results add a new element to the explanation for the remarkable stability of Pg in plasma in the presence of STA and give new insights into the mechanism of fibrin stimulation of Pg activation by STA.

EXPERIMENTAL PROCEDURES

Preparations—Recombinant Sak42D or SakSTAR variants of staphylokinase were purified and characterized as described elsewhere (11, 18). The two variants differ in three amino acids but have a similar catalytic efficiency and fibrinolytic potency in a plasma milieu. Unless indicated otherwise, the Sak42D variant was used in the present study. Glu-Pg was a product of Biofine (Leiden, The Netherlands) or was purified from human plasma as described (11). Lys-Pg was a gift from Kabi (Stockholm, Sweden). Recombinant human plasminogen with the active site Ser^{741} residue changed to Ala (rPg-Ala^{741}) was obtained and characterized as described elsewhere (10). The preparations of Pg were stored frozen at a concentration of 50 μM in a buffer containing 10 mM Tris acetate, pH 7.6, and 135 mM sodium acetate (TAC). Two-chain urokinase (Ukidan) was from Serono (Aubonne, Switzerland). Fluorescein isothiocyanate (FITC) and porcine carboxypeptidase B (CpB) were from Sigma. Human fibrinogen (Kabi) was made Pg-free by lysine-Sepharose chromatography. D-Val-Phe-Lys-chloromethylketone (VPLCK) was from Calbiochem. Thrombin was from Leo (Ballerup, Denmark), and aprotinin was from Bayer (Leverkusen, Germany). Pooled, citrated, platelet-poor plasma from healthy volunteers was used in all experiments involving plasma.

MAb40C8, a murine monoclonal antibody raised against SakSTAR, was obtained and characterized as described (19). MAb40C8 at an equimolar concentration did not affect binding of SakSTAR to insolubilized rPg-Ala^{741}, determined as described elsewhere (20).

Labeling of Glu-Pg with FITC was performed as described earlier (16, 17), resulting in a preparation with a molar ratio of fluorescein/Pg of 2. STA was labeled with FITC using essentially the same procedure, yielding a molar ratio of FITC/STA of 0.8. The specific activity of the FITC-labeled STA preparation was compared with that of unlabeled STA by measuring plasma clot lysis time or the kinetics of Pg activation with a chromogenic substrate (*H*-D-Val-Leu-Lys-*p*-nitroanilide) for plasmin. In both assays, no appreciable decrease of the STA activity on labeling was documented.

Plasmin from Kabi was used for the Pl treatment of purified fibrin clots. For the experiments studying the complex formation between STA and Pl, a Pl solution was prepared as follows. Glu-Pg (15 μM) in TAC was incubated with 0.15 μM STA or 500 IU/ml two-chain urokinase for 15 min at 20 °C. The concentrations of STA and urokinase and the time of the incubation were chosen to provide maximal exposure of specific Pl activity (about 15 casein units/mg) as judged by an activity test with the chromogenic Pl substrate (*H*-D-Val-Leu-Lys-*p*-nitroanilide). Both shorter and longer incubations resulted in a lower specific activity of the Pl generated. When indicated, VPLCK (100 μM) was added thereafter to inhibit the Pl enzymatic activity.

Spatial Distribution of FITC-STA during Lysis of a Plasma Clot from the Outside—Human plasma was clotted with thrombin (final concentration, 1.4 NIH units/ml) between two parallel glass slides as described earlier (16, 21). The chamber was perfused with plasma containing 0.33 μM FITC-STA at 37 °C. The perfusing solution flowed freely around the clot; no hydraulic pressure over the clot that could force infiltration of the perfusate into the clot was applied. Consecutive fluorescence photomicrographs of the lysing clots were obtained with a fluorescence microscope (Microphot FXA, Nikon). Perfusions were interrupted for no longer than 2 min for photographing.

Binding of STA and Pg to Fibrin Clots Treated with Pl from the Outside—Purified fibrin clots of approximately 2 mm in diameter were prepared as described earlier (16, 21) by clotting of Pg-free fibrinogen (final concentration, 9.2 μM) with thrombin (1.4 NIH units/ml) between two parallel glass slides in a 10 mM Tris-HCl, pH 7.6, buffer containing 135 mM NaCl (TBS), and 20 mg/ml BSA (TBS-BSA). The remaining volume of the chambers (approximately 25 μl) was filled with TBS-BSA and incubated for 20 min at room temperature. Then the clots were subjected to the action of Pl by replacing the buffer in the chambers with TBS-BSA containing 0.15 casein unit/ml Pl. After a 60-min incubation at 37 °C, the action of Pl was stopped by replacing the Pl-containing buffer with TBS-BSA containing 250 KIU/ml aprotinin. During the treatment with Pl, clot diameters were reduced by approximately 1 mm. When indicated, the clots were treated afterward with CpB (30 μg/ml) in the same buffer for 20 min at room temperature. Then the clots were washed for 1 h with TBS-BSA containing 250 KIU/ml aprotinin.

Fibrin clots, either treated only with Pl or treated consecutively with Pl and CpB, were incubated for 20 h at room temperature in the dark with either FITC-STA or FITC-Pg (both 0.4 μM final concentration) in TBS-BSA with 250 KIU/ml aprotinin. FITC-STA was present in the incubation medium either with a slight molar excess of unlabeled Glu-Pg (0.5 μM) or without Pg. Similarly, FITC-Pg was incubated with the clots either in the presence or in the absence of unlabeled STA (0.5 μM). After incubation, the clots appeared to be completely equilibrated with the labeled substances added to the outer medium. The concentration of fluorescence-labeled substances in the outer medium remained practically unchanged; only a negligible portion was eventually accumulated in the clots. Clots were photographed after the equilibration as described above.

Binding of STA and Pg to Fibrin Clots Treated with Pl from the Inside—Pl (0.1 casein unit/ml) was added to Pg-free fibrinogen (9.2 μM) in TBS-BSA, and the mixture was immediately clotted with thrombin (1.4 NIH units/ml) to form clots with a diameter of 1.0–1.5 mm between two parallel glass slides. After incubation for 10 min at room temperature, TBS-BSA containing 250 KIU/ml aprotinin was added to the outer medium to arrest the action of Pl. Under these conditions the integrity of the clot was not noticeably affected. When indicated, the clots were treated afterward with CpB as described above.

After 1 h of washing with TBS-BSA containing 250 KIU/ml aprotinin, the clots were equilibrated for 20 h with FITC-STA (0.4 μM) or FITC-Pg (0.4 μM) as described in the previous section. Unlabeled Pg or STA were added in a slight molar excess (0.5 μM) when indicated. After the equilibration, the clots were photographed, the negatives were scanned in a scanning densitometer (TLC Scanner CS-910, Shimadzu), and the accumulation of fluorescence inside the clots was determined using a calibration curve, as described earlier (16, 21).

Binding Study with Fluorescence Anisotropy—Steady-state fluorescence data were collected at 22 °C with a spectrofluorometer (Perkin-Elmer LS-3) equipped with a polarization accessory (Perkin-Elmer 5212-3269). The anisotropy (22, 23) was calculated from Equation 1:

$$A = \frac{I_v - G \times I_h}{I_v + 2 \times G \times I_h} \qquad \text{(Eq. 1)}$$

where I_v and I_h are, respectively, the parallel and perpendicular components of the fluorescence light, and G is a correction factor for the different instrument responses to the two polarized components. The G factor was determined using excitation with horizontally polarized light. The excitation wavelength was 492 nm, and the emission wavelength was 520 nm.

FITC-STA was added to a cuvette in TBS containing 2.5 mg/ml BSA and 250 KIU/ml aprotinin to a final concentration of 0.05 μM. The initial anisotropy was 0.167. The content of the cuvette was stirred intensively after the addition of varying amounts of Glu-Pg, Lys-Pg, or Pl, and anisotropy was measured again after a 2-min incubation. Urokinase-activated, VPLCK-inhibited Pl was used in these experiments. For Glu-Pg, the experiments were also performed in TAC buffer under the same conditions. In this case, Cl^- ions were removed from all the reagents used (Pg, FITC-STA, and aprotinin).

The resulting concentration-dependent increase in anisotropy was fitted to the following equation:

$$\Delta A = \frac{\Delta A_{max} \times [P]}{[P] + K_d} \qquad \text{(Eq. 2)}$$

where [P] is the concentration of the plasmin(ogen) variant. K_d and ΔA_{max} were treated as fitting parameters and were calculated by means of nonlinear regression analysis using a Graphpad computer program (ISS Inc., San Diego, CA). In the cases of Glu-Pg and Lys-Pg we could consider the calculated parameter K_d as a true value of the dissociation constant, since the concentration of FITC-STA used in the assay was small compared with the K_d, and the concentration of free Pg could be assumed equal to its total concentration. This assumption was not valid for Pl because of much higher affinity (the calculated value of K_d was very close to the concentration of FITC-STA used). Thus, in the case of Pl we could only conclude that the true dissociation constant was lower than the calculated value.

Determination of Binding Parameters by Biospecific Interaction Analysis—Association rate constants (k_a), dissociation rate constants (k_d), and equilibrium dissociation constants ($K_d = k_d/k_a$) for the interaction of STA with different plasmin(ogen) moieties were determined by real time biospecific interaction analysis using the BIAcore instrument

(Pharmacia Biotech Inc.), essentially as described elsewhere (20). However, to minimize nonspecific interactions, for each determination STA was bound to a monoclonal antibody (MAb40C8) immobilized to the sensor chip. For these experiments, the SakSTAR variant was used, because the available monoclonal antibodies did not have an appropriate affinity for Sak42D allowing measurement of the binding of plasmin(ogen) moieties without significant dissociation of Sak42D from the antibody. Under the conditions used, the half-life of the MAb40C8-SakSTAR complex was about 30 min, suggesting that no extensive dissociation occurred during the measurement (10 min). The binding of each plasmin(ogen) moiety was analyzed using three different concentrations (50–2000 nM) in 10 mM Hepes, 3.4 mM EDTA, 0.15 M NaCl, and 0.005% surfactant P20, pH 7.2, as described (20). Binding experiments with native Glu-Pg were performed in the presence of Pl inhibitors aprotinin (final concentration, 100 KIU/ml) or VPLCK (final concentration, 10 μM).

Complex Formation Study with Gel Filtration—VPLCK (final concentration, 100 μM) was added either to STA-activated Pl or to Glu-Pg (both at the concentration of 15 μM in TAC; the concentration of Pl was calculated from the concentration of Glu-Pg used for preparation of Pl). The two preparations were diluted to a final concentration of 1 μM in 1 ml of TBS containing 250 KIU/ml aprotinin, and FITC-STA was added to a final concentration of 0.5 μM. After a 20-min incubation at 4 °C, the samples were subjected to gel filtration at 4 °C on a Sephacryl S-200 column (1.6 × 55 cm) equilibrated with the same buffer containing aprotinin at a flow rate of 12 ml/h. Fractions of 2.6 ml were collected for measurements of fluorescence at an excitation wavelength of 492 nm and an emission wavelength of 520 nm (Kontron SFM-25 spectrofluorometer).

Similar experiments were performed with unlabeled STA in the absence of aprotinin to exclude the possibility that either the FITC label or aprotinin might affect the complex formation. A mixture of unlabeled STA (0.5 μM) with Glu-Pg (1 μM) was prepared in TBS containing 50 μM VPLCK, as above. The column was rapidly pre-equilibrated for 3 h with TBS containing 40 μM VPLCK immediately prior to the sample application to ensure the presence of unhydrolyzed VPLCK throughout the column during the chromatography. After the chromatography, STA activity in the eluted fractions was detected as follows. Fifteen-microliter aliquots of the fractions were added to microtiter plate wells containing 0.7 μM Glu-Pg, 5 mM ϵ-aminocaproic acid, and 0.5 mM *H*-D-Val-Leu-Lys-*p*-nitroanilide in 135 μl of 50 mM Tris-HCl buffer, pH 7.6, containing 50 mM NaCl and 0.05% Tween 80. Although some VPLCK remained obviously unhydrolyzed by the time of the assay, Pl was generated after a delay in the wells containing STA. After a 30-min incubation at 37 °C, STA-containing fractions were identified by measuring absorbance at 405 nm. A mixture of unlabeled STA (0.5 μM) with STA-activated Pl (1 μM) was gel filtered under similar conditions in the absence of any Pl inhibitors in the sample buffer and in the elution buffer (TBS). The presence of STA in fractions was analyzed using the same activity assay. In this case, the plasmin activity in STA-containing fractions was generated faster due to the absence of VPLCK in the assay wells, and the absorbance at 405 nm was recorded after a 10-min incubation at 37 °C.

Interaction of FITC-STA and FITC-Pg with the Fibrin Network during Lysis of a Plasma Clot from the Inside—Either FITC-STA (0.4 μM) or tracer FITC-Pg (0.3 μM) together with 0.4 μM unlabeled STA was added to plasma, followed immediately by clotting with thrombin (final concentration, 1.4 NIH units/ml) between two parallel glass slides. The interaction of the FITC-labeled components with the fibrin fibers was monitored at room temperature using a confocal laser scanning fluorescence microscope (MRC-600 Laser Sharp, Bio-Rad), as described earlier (17). The design of the system allowed the collection of images periodically during the ongoing lysis. To exclude the disturbing influence of the clot-glass interface, all images were taken at a distance of 50 μm from the surface of the glass.

RESULTS

Lysis of a Plasma Clot with FITC-STA Added to the Surrounding Plasma—Fig. 1 represents the spatial distribution of FITC-labeled STA in a plasma clot during its lysis induced by addition of FITC-STA to the surrounding plasma. Lysis started after an initial lag phase, which lasted for about 10 min. During the lag phase, the size of the clot remained unchanged, and FITC-STA diffused into the clot without a noticeable accumulation at the boundary (not shown). After 10 min, a 100–150-μm layer near the surface of the clot collapsed within 1–2 min (Fig. 1*A*). Then a phase of progressive lysis started (Fig. 1, *B* and *C*), during which the size of the clot continuously decreased, and FITC-STA appeared to be constantly accumulated in a thin superficial layer of the lysing clot. In our previous study (16) we observed a very similar distribution of plasmin(ogen) during lysis of a plasma clot induced by either tissue-type plasminogen activator or two chain urokinase.

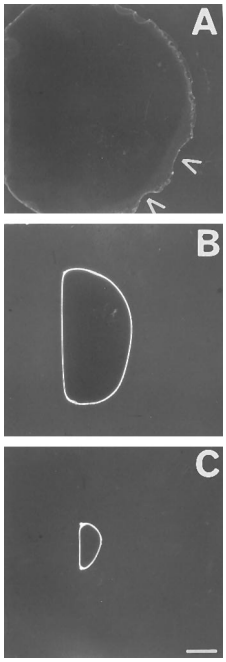

FIG. 1. **Spatial distribution of FITC-STA during lysis of a plasma clot.** FITC-STA (0.33 μM) was present in the perfusing plasma. Fluorescence photomicrographs were taken after 10 (*A*), 30 (*B*) and 45 (*C*) min of perfusion. *Arrows* in *A*, areas where the surface of the clot starts to collapse. *Bar,* 250 μm.

To elucidate the molecular basis for this superficial accumulation of STA, we performed the following experiments in a purified system.

Binding of STA and Pg to Fibrin Clots Treated with Pl from the Outside—Purified fibrin clots were treated with Pl added to the outer medium after clot formation, and then lysis was stopped by the addition of aprotinin. One-half of the clots were treated afterward with CpB to remove C-terminal lysine residues generated at the fibrin surface as a result of the Pl treatment. The clots were equilibrated for 20 h with FITC-STA either in the presence of a slight molar excess of unlabeled Glu-Pg or in the absence of Pg. A high concentration of aprotinin was present in the incubation medium to prevent generation of Pl. The resulting distributions of FITC-STA are presented in Fig. 2, *A–D*. In the presence of Pg, FITC-STA accumulated in a thin superficial layer of the Pl-treated fibrin clot (Fig. 2*A*). No remarkable binding of FITC-STA was observed in this type of clot in the absence of Pg (Fig. 2*B*). CpB-treated clots did not accumulate FITC-STA either in the presence (Fig. 2*C*) or in the absence (Fig. 2*D*) of Pg.

Fig. 2, *E–H,* presents the spatial distributions of FITC-labeled Pg in the two types of clots described above, either in the presence of a slight molar excess of unlabeled STA (Fig. 2, *E* and *G*), or in the absence of STA (Fig. 2, *F* and *H*). In accordance with our earlier results (16), the distribution of FITC-Pg in Pl-treated fibrin clots was seen as a strong superficial accumulation within a thin layer, accompanied by a moderate accumulation throughout the clot (Fig. 2*F*). In the clots treated consecutively with Pl and CpB, the strong superficial accumulation of FITC-Pg was not observed anymore, whereas the

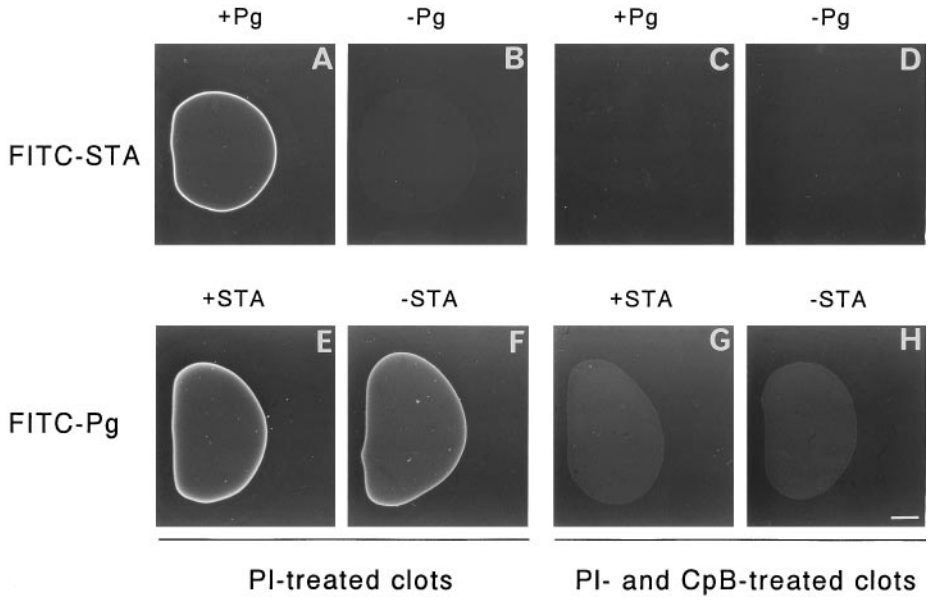

FIG. 2. **Spatial distribution of FITC-STA and FITC-Pg in purified fibrin clots treated with Pl from the outside with or without subsequent CpB treatment.** All the clots were pretreated with Pl from the outside. Clots depicted in *C, D, G,* and *H* were subsequently treated with CpB. *A–D,* distribution of FITC-STA (0.4 μM) in the presence of 0.5 μM unlabeled Pg (*A* and *C*) or in the absence of Pg (*B* and *D*). *E–H,* distribution of FITC-Pg (0.4 μM) in the presence of 0.5 μM unlabeled STA (*E* and *G*) or in the absence of STA (*F* and *H*). *Bar,* 500 μm.

moderate accumulation throughout the whole volume of the clot remained virtually unchanged (Fig. 2*H*). A similar moderate accumulation of FITC-Pg throughout the clots was seen with intact fibrin clots (not shown). Thus, the treatment with CpB destroyed Pg binding sites generated on the surface of the clot by Pl (presumably C-terminal lysines), but did not affect the binding sites present throughout the clot (intrachain lysines rather than C-terminal lysines).

In both types of clots, STA apparently did not influence the spatial distributions of FITC-Pg (Fig. 2, *E* and *G*). Comparison of the spatial distributions of FITC-STA and FITC-Pg, presented in Fig. 2, indicates that in the presence of Pg, STA followed the pattern of the superficial accumulation of Pg in Pl-treated clots (Fig. 2, compare *A* and *F*), but, surprisingly, it did not follow the distribution of Pg in carboxypeptidase-treated clots. Indeed, FITC-STA apparently did not interact with CpB-treated clots in the presence of Pg (Fig. 2*C*), whereas FITC-Pg was moderately accumulated in this type of clot either in the presence or in the absence of STA (Fig. 2, *G* and *H*).

The latter findings imply that STA does not bind or binds with a very low affinity to Pg, which is bound to intact fibrin. The strong accumulation of STA in the superficial layer of a Pl-treated clot in the presence of Pg (Fig. 2*A*) could be explained in two ways: 1) despite of a low affinity for Pg, STA concentrates in the superficial layer due to a very high local concentration of bound Pg (the local concentration of Pg can be 30-fold higher than in the surrounding buffer (17), up to 15 μM under the experimental conditions used); and 2) STA discriminates between two forms of fibrin-bound Pg; it binds strongly to Pg bound to C-terminal lysines, but does not bind or binds weakly to Pg bound to intrachain lysines. The two mechanisms can also operate simultaneously.

The experiments presented in Fig. 3 were performed to check the hypothesis about the differential binding of STA to Pg bound either to intact or partially degraded fibrin.

Binding of STA and Pg to Fibrin Clots Treated with Pl from the Inside—Purified fibrin clots were pretreated with Pl throughout the clots. After equilibration, the accumulation of FITC-labeled molecules was quantitated using scanning densitometry of the photographic film. As demonstrated in Fig. 3, Pg accumulated moderately throughout such clots, to a level about 2.3-fold higher than its concentration in the surrounding buffer. If the clots were treated with CpB after the Pl treatment (or were not treated with Pl at all; not shown), Pg accumulated

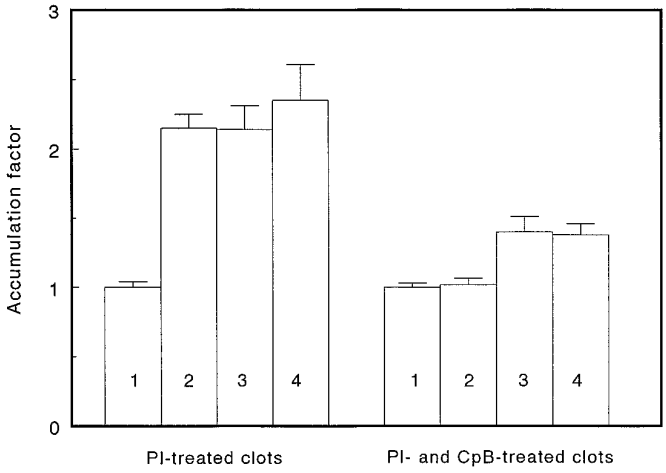

FIG. 3. **Accumulation of FITC-STA and FITC-Pg in purified fibrin clots treated with Pl from the inside with or without subsequent CpB treatment.** The clots were equilibrated with TBS-BSA containing: *1,* 0.4 μM FITC-STA; *2,* 0.4 μM FITC-STA and 0.5 μM Pg; *3,* 0.4 μM FITC-Pg; and *4,* 0.4 μM FITC-Pg and 0.5 μM STA. The accumulation factor (mean ± S.D. (*bars*) of three determinations) was determined as the ratio of fluorescence intensity inside the clots to that in the buffer surrounding the clots after a 20-h equilibration.

about 1.4-fold. Thus, in this setup, the accumulation of Pg was rather moderate and comparable in both types of clots.

FITC-STA did not bind to both types of clots in the absence of Pg. In the presence of equimolar unlabeled Pg, FITC-STA (0.4 μM) accumulated in Pl-treated clots to a similar extent as Pg (about 2.2-fold). In terms of molar concentrations, this means that up to 0.5 μM STA appeared to be bound to Pl-treated fibrin in the presence of Pg under the experimental conditions used. In contrast, FITC-STA did not bind detectably to clots pretreated with Pl and CpB (and to intact clots; not shown), although FITC-Pg accumulated about 1.4-fold in such clots. Thus, FITC-STA essentially followed the distribution of Pg in the clots containing C-terminal lysines (Pl-treated clots), but not in the clots containing only intrachain lysines (Pl and CpB-treated clots as well as intact clots). STA did not influence the degree of accumulation of FITC-Pg inside the clots when added in an equimolar proportion with FITC-Pg. Since the concentrations of fibrin-bound Pg in both types of clots were comparable in this setup, we conclude that STA selectively

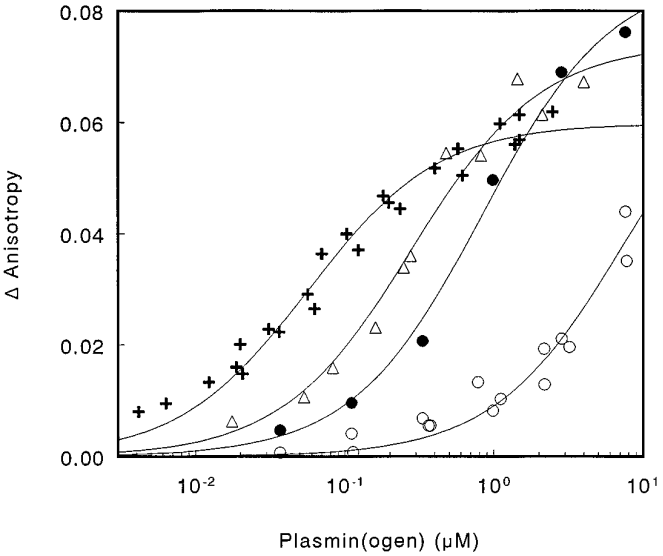

FIG. 4. **Binding of FITC-STA to Pl, Lys-Pg, and Glu-Pg as measured with fluorescence anisotropy.** The fluorescence anisotropy of FITC-STA at a concentration of 0.05 μM was measured in the presence of varying concentrations of Pl (+), Lys-Pg (△), and Glu-Pg (○) in Tris-buffered saline or of Glu-Pg in Tris acetate buffer (●). *Curves*, best theoretical fit to Equation 2.

TABLE I
Binding parameters of different plasmin(ogen) moieties to STA, determined by biospecific interaction analysis

Ligand	k_a ($\times 10^5$)[a]	k_d ($\times 10^{-3}$)[a]	K_d[a]
	$M^{-1}\ s^{-1}$	s^{-1}	μM
Glu-Pg/aprotinin	0.015 ± 0.002	4.9 ± 0.53	3.52 ± 0.74
Glu-Pg/VPLCK	0.016 ± 0.0007	4.6 ± 0.52	2.84 ± 0.43
Pl-VPLCK	4.0 ± 0.64	1.5 ± 0.15	0.0038 ± 0.0006
rPg-Ala^{741}	0.61 ± 0.12	0.71 ± 0.068	0.0123 ± 0.0015

[a] Data are mean ± S.E. of three determinations.

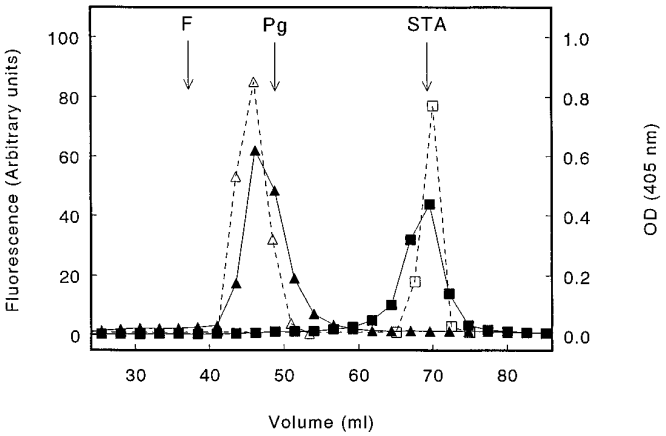

FIG. 5. **Gel filtration of mixtures of FITC-STA or STA with Glu-Pg or Pl on Sephacryl S-200.** *Solid lines,* mixtures of 0.5 μM FITC-labeled STA with 1 μM Glu-Pg (■) or VPLCK-inhibited Pl (▲). The presence of FITC-STA in the fractions was monitored by measuring fluorescence (*left y* axis). *Dashed lines,* mixtures of 0.5 μM unlabeled STA with 1 μM Glu-Pg (□) or Pl (△). STA activity in the fractions was measured in a Pg activation test with the Pl substrate *H*-D-Val-Leu-Lys-*p*-nitroanilide (*right y* axis). *Arrows,* elution positions of Pg and STA as well as the void volume of the column assessed as the elution position of ferritin (*F*).

binds to Pg bound to partially degraded fibrin, compared with Pg bound to intact fibrin.

This finding stimulated us to investigate in more detail binding of STA to different molecular forms of plasmin(ogen).

Binding of FITC-STA to Plasmin(ogen) in Solution Studied with Fluorescence Anisotropy—The binding of Glu-Pg, Lys-Pg, and Pl to FITC-STA in solution was investigated by measuring the fluorescence anisotropy of FITC-STA in the presence of varying concentrations of the plasmin(ogen) variants. This method (22, 23) uses the increase in fluorescence anisotropy on binding of the small FITC-STA molecule (18 kDa) to the relatively large molecule of plasmin(ogen) (about 90 kDa). On the basis of the results presented in Fig. 4, we calculated the following dissociation constants for the binding of FITC-STA: 7.4 μM to Glu-Pg and 0.28 μM to Lys-Pg. For the active center-blocked Pl the value for the K_d (0.057 μM) calculated according to Equation 2 appeared to be very close to the concentration of FITC-STA used in the assay (0.05 μM). Therefore, we concluded (see "Experimental Procedures") that in this case the true value of the dissociation constant was considerably lower than 0.057 μM. Interestingly, the dissociation constant for Glu-Pg dropped about 1 order of magnitude down (to 0.84 μM) if the chloride anions in the buffer were replaced by acetate anions.

Determination of Binding Parameters by Biospecific Interaction Analysis—Table I summarizes the k_a and k_d values for binding of different molecular forms of plasmin(ogen) to SakSTAR immobilized via MAb40C8. Comparison of the calculated K_d values confirms that in the presence of Pl inhibitors, STA has a much lower affinity for Glu-Pg (K_d, approximately 3 μM) than active site-blocked Pl (K_d, approximately 4 nM). This appears to be due mainly to a much lower association rate constant for Glu-Pg. In agreement with previous results, rPg-Ala^{741} has an affinity for binding to STA that is comparable with that of active site-blocked Pl (20).

STA-Plasmin(ogen) Complex Formation Studied by Gel Filtration—To substantiate the finding about differential binding of STA to Pl and Glu-Pg, we performed experiments with gel filtration of mixtures of FITC-STA and a 2-fold molar excess of either Pl or Glu-Pg. In the latter case, generation of Pl was precluded by the addition of Pl inhibitors (see "Experimental Procedures"). The gel filtration profiles presented in Fig. 5 show that FITC-STA eluted in complex with Pl, as was demonstrated earlier (15). When applied in a mixture with Pg in the presence of VPLCK and aprotinin, FITC-STA eluted as a free 18-kDa protein, confirming that FITC-STA did not form a stable complex with Glu-Pg.

We also performed a similar set of gel filtrations with unlabeled STA in the absence of aprotinin, using an activity test for measuring STA in the chromatographic fractions. The results of the gel filtrations with unlabeled STA were the same as those obtained with FITC-labeled STA (Fig. 5), indicating that STA forms stable complexes with Pl, but not with Glu-Pg. Essentially the same results were obtained using the SakSTAR variant (not shown).

Interaction of STA and Pg with the Fibrin Network of a Plasma Clot Studied with Confocal Microscopy—The results described above suggest that although STA in plasma binds Pg only very weakly, STA-induced fibrinolysis should involve the formation of a ternary complex among STA, plasmin(ogen), and partially degraded fibrin. We used confocal fluorescence microscopy to elucidate the dynamics of the interaction of STA with fibrin fibers during STA-induced lysis of a plasma clot (Fig. 6). In parallel experiments, either STA together with a tracer amount of FITC-Pg or FITC-STA was added throughout the clot. During the first 6–7 min after clotting (lag phase), Pg binding to fibers was constant and moderate (Fig. 6, *A* and *B*), as it was in an intact plasma clot (17), whereas STA did not bind to the fibers noticeably (Fig. 6, *E* and *F*). Then, both Pg and STA started to accumulate progressively onto the fibers (Fig. 6, *C* and *G*). A few minutes after the start of the progressive accumulation of STA and Pg on the fibers, the clot was

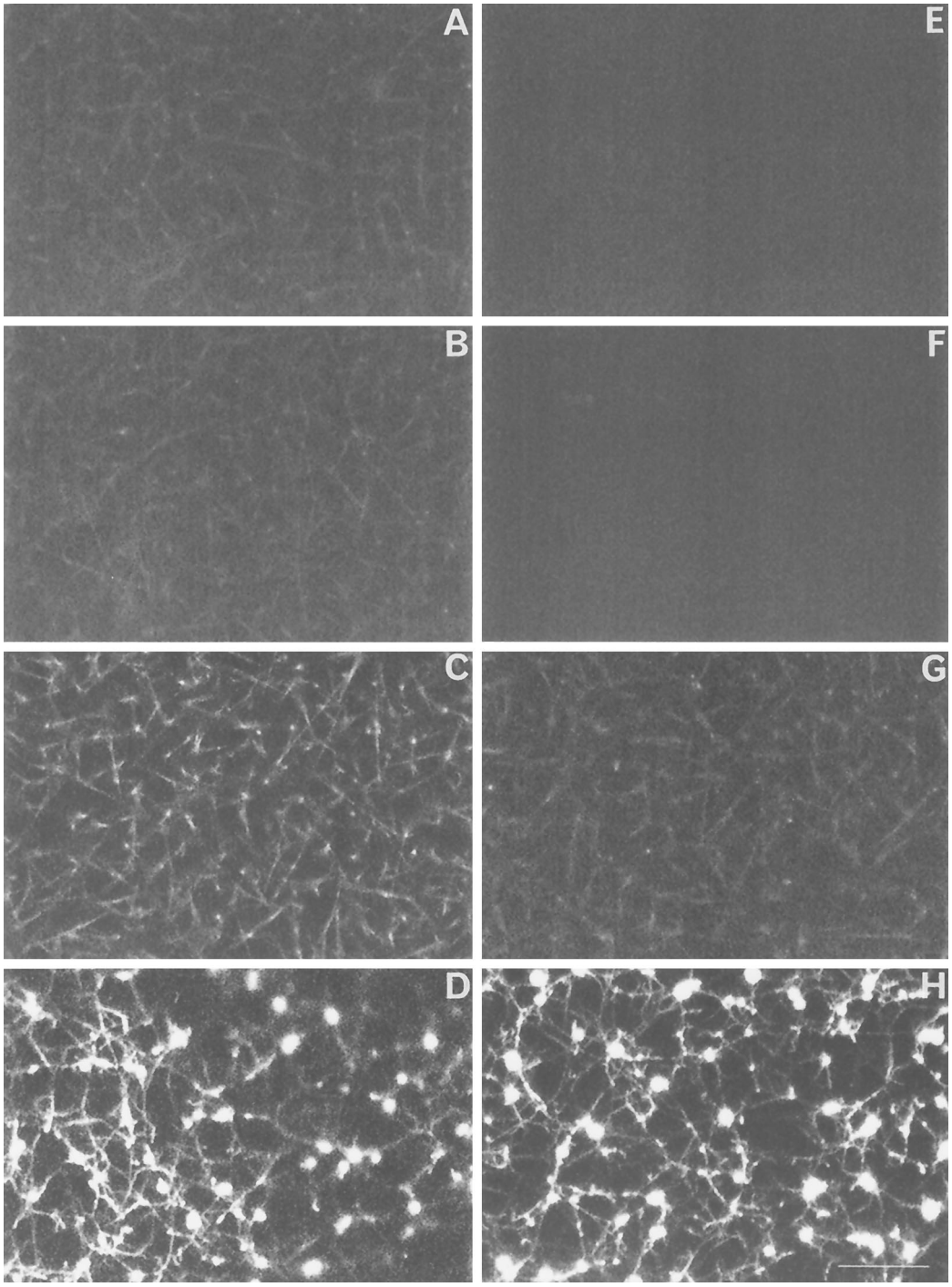

FIG. 6. **Interaction of FITC-Pg and FITC-STA with the fibrin network during lysis of a plasma clot.** Either tracer FITC-Pg (0.3 μM) together with 0.4 μM unlabeled STA (*A–D*) or 0.4 μM FITC-STA (*E–H*) was added to plasma, followed immediately by clotting with thrombin. The images were taken with a confocal laser scanning fluorescence microscope 2 (*A* and *E*), 6 (*B* and *F*), 8 (*C* and *G*), and 11 (*D* and *H*) min after the addition of thrombin. *Bar,* 20 μm.

completely lysed. Fig. 6, *D* and *H*, shows that both Pg and STA were concentrated on disconnected pieces of the fibrin network in the very last moments before its complete dissolution. Bright spots of fluorescence present on these images are probably the result of a very fast collapse of the network, which was not so pronounced during more gradual tissue plasminogen activator-induced lysis documented in our previous study (17).

DISCUSSION

The main finding of this study is the demonstration that STA binding to plasmin(ogen) is largely dependent on the molecular form of plasmin(ogen) and on the association of the latter with a specific form of fibrin (partially Pl-digested fibrin).

The interaction of STA with different molecular forms of Pg in solution was studied with three techniques, including fluorescence anisotropy, gel filtration, and biospecific interaction analysis. The results obtained with all three techniques indicate that the binding of STA to Glu-Pg is very weak (K_d, 7.4 μM, as measured quantitatively by fluorescence anisotropy), whereas the binding of STA to Pl is much stronger (K_d, $<$0.05 μM, which is at least 150-fold lower).

These findings are relevant for the model for Pg activation by STA in a buffer milieu, as proposed previously (1). This model suggests that Pg and STA produce an inactive 1:1 stoichiomet-

ric complex (Pg·STA), which does not activate Pg. The activation reaction would be initiated by trace amounts of Pl, which generate an active Pl·STA complex. Generated Pl·STA may convert Pg·STA to Pl·STA (route 1) and may also activate excess Pg to Pl (route 2) (1, 24). The present data indicating poor complex formation between Pg and STA suggest that the major pathway goes through route 2.

Fluorescence microscopic experiments showed that STA did not bind significantly to Pg associated with intact fibrin (Figs. 2 and 3). In contrast, it bound to Pg associated with fibrin partially degraded by Pl. Such a discrimination between the two types of Pg was in line with the sequence of events observed by means of fluorescence microscopy during STA-induced lysis of a plasma clot. During a lag phase of several minutes, STA did not interact with the fibrin matrix of a plasma clot, either when added from the outside or from the inside of the clot. After the lag phase, STA started to accumulate progressively on the fibrin fibers, in concert with accumulation of plasmin(ogen). During lysis induced from the outside, STA followed the pattern of superficial accumulation observed previously for Pg during tissue-type plasminogen activator- and urokinase-induced clot lysis (16).

The observed difference in affinity of STA for different species of plasmin(ogen) under various conditions can be an indication of its sensitivity to the plasmin(ogen) conformation. STA bound poorly to Glu-Pg in its closed compact conformation, existing in the presence of Cl^- ions (25–28). Glu-Pg in the absence of Cl^- ions (26, 29) as well as Lys-Pg (25, 28, 30), exhibiting an open, relaxed conformation, bound STA more strongly. Kinetic and structural data suggest that Pg bound to partially degraded fibrin also adopts an open conformation (31, 32). Thus, it is likely that the STA binding site of Pg becomes available in an open conformation of Pg.

In the experiments with biospecific interaction analysis, nonspecific interactions were minimized by binding of SakSTAR to the immobilized monoclonal antibody, which does not hinder the interaction with Pg. Using this system, a high affinity of STA for active site-blocked Pl and for rPg-Ala^{741} was observed, as reported previously by direct coupling of STA to the sensor chip (20). However, the present finding that the binding affinity of STA for Glu-Pg in the presence of Pl inhibitors is much lower than that for rPg-Ala^{741} suggests that this active site mutant of Pg may adopt an open conformation that is more similar to active site-blocked Pl than to Glu-Pg.

The results obtained add some new insights into the mechanism of fibrin specificity of the STA-induced fibrinolysis. First, on the basis of the high value of the K_d for STA binding to Glu-Pg in the presence of Cl^- ions, it can be deduced that only a small fraction of STA should be bound to Pg in human plasma. The bulk of STA remains unbound and is apparently excluded from Pg activation, explaining the inefficiency of Pg activation in plasma in the absence of fibrin. Second, binding of STA to Pg associated with partially degraded fibrin can explain the acceleration by fibrin of STA-induced Pg activation (10, 14). The portion of Pg bound to C-terminal lysines is believed to play an important role in the acceleration of fibrinolysis induced by other plasminogen activators. It is activated by two chain urokinase faster than Glu-Pg in solution (33); it is responsible for the acceleration of Pl generation during the second phase of tissue-type plasminogen activator-induced fibrinolysis (34). Liu and Gurewich (35) have postulated the existence of a unique conformation of Glu-Pg bound to C-terminal lysines on fibrin fragment E-2, which plays an essential role in the mechanism of fibrin-specific plasminogen activation by single-chain urokinase-type plasminogen activator (35, 36). Probably the same highly activatable conformation of fibrin-bound Pg mediates binding of STA to partially degraded fibrin observed in the present study. Anyway, the demonstrated specific binding of STA to Pg bound to partially degraded fibrin is a good basis for positive fibrin-dependent feedback, which can be an important part of the mechanism of the fibrin specificity of STA. It has indeed been suggested previously that fibrin not only protects the bound Pl·STA complex from inhibition by α_2-antiplasmin but also facilitates activation of Pg by STA via an unidentified mechanism that involves the lysine binding sites of Pg (14). The data obtained in this study thus provide a detailed molecular mechanism for the role of fibrin in the fibrin specificity of STA.

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