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Role of the Kringle Domain in Plasminogen Activation with Staphylokinase[1]

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We have evaluated the effect of lysine binding sites in krigle structures on the activation of plasminogen with plasmin and staphylokinase (SAK) complex and on the binding of plasminogen to SAK. Activation of native plasminogen (Glu-plasminogen) by a catalytic amount of plasmin-SAK complex increased in the presence of ε-amino-*n*-caproic acid (EACA) and then decreased with higher concentrations of EACA. By contrast, activation of modified plasminogen (Lys-plasminogen) decreased in an EACA-concentration-dependent manner. This decrease was explained by a more than 10-fold higher K_m for activation of Lys-plasminogen with a catalytic amount of plasmin-SAK complex in the presence of EACA. EACA was a competitive inhibitor with K_i 0.23 mM. In addition, the K_m for activation of mini-plasminogen, which lacks first four kringle structures (K1+2+3+4), was at least 3.5-fold higher than that for the activation of Lys-plasminogen. Furthermore, EACA showed a negligible inhibitory effect on the activation of mini-plasminogen by the plasmin-SAK complex. We observed a similar biphasic effect of EACA on the binding of Glu-plasminogen to SAK and a dose-dependent effect on the Lys-plasminogen binding to SAK by gel filtration methods. Since EACA binds to plasminogen *via* lysine binding sites in the kringle structure, we propose that the lysine binding site in K1+2+3+4 domain plays a role in the activation of plasminogen by plasmin-SAK complex, and in the binding of plasminogen to SAK.

Key words: ε-amino-*n*-caproic acid, kringle domain, lysine binding site, plasminogen, staphylokinase.

Staphylokinase is a 136-amino-acid bacterial protein produced by *Staphylococcus aureus*. Thirty percent of the amino acids in SAK are charged and these amino acids, containing 20 lysine residues, are critical for plasminogen activating capacity (*1*). SAK itself is not an enzyme but it forms a 1 : 1 stable stoichiometric complex with plasmin (*2*). This bimolecular complex activates other plasminogen molecules, following Michaelis-Menten kinetics (*2, 3*).

Glu-plasminogen with NH_2-terminal glutamic acid is a single-chain 92-kDa glycoprotein of 791 amino acids with five kringle domains that contain lysine binding sites with different dissociation constants for EACA (*4, 5*). Glu-plasminogen exhibits a very tight spiral structure (*6*) which has been suggested to attenuate the interaction of the kringle domains with fibrin (*7*). By limited proteolysis with plasmin, Glu-plasminogen is converted to open-form Lys-plasminogen with NH_2-terminal lysine, valine or methionine (*8, 9*). In addition, by binding to Glu-plasminogen, EACA induces a conformational transition in plasminogen from the Glu-form to Lys-plasminogen-like forms in a dose-dependent manner without NH_2-terminal peptide cleavage (*10*). Following digestion of plasminogen with elastase, mini-plasminogen, consisting of kringle 5 (K5) and the protease domain, fragments containing the first three kringle structures (K1+2+3), and the fourth kringle domain (K4) can be isolated (*4*).

Although the plasminogen-streptokinase complex itself has plasminogen activator activity, the plasminogen-SAK complex, formation of which is not definitively proven yet, does not show plasminogen activator activity before conversion of the plasminogen moiety to the two-chain form, plasmin (*1*). Formation of a stable complex between plasmin and SAK requires high-affinity binding of SAK ($K_a = 1 \times 10^8$ M^{-1}) to the serine protease domain of plasmin (*11*). Lijnen *et al.* have asserted that K1+2+3+4 of plasminogen do not contribute significantly to plasminogen binding to SAK because the K1+2+3 peptide and K4 peptide bound to SAK with much lower affinity ($K_a = 1.2 \times 10^5$ M^{-1} and 2.9×10^5 M^{-1}, respectively) (*11*) and EACA does not dissociate the plasmin-SAK complex (*12*). How-

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Abbreviations: SAK, staphylokinase; α_2-PI, α_2-plasmin inhibitor; EACA, ε-amino-*n*-caproic acid; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; p-APMSF, (*p*-aminophenyl)methanesulfonyl fluoride hydrochloride; GBM, Gabexate mesilate[ethyl-4-(6-guanidinohexanoyloxy)benzoate]methane sulfonate.

ever, in the previous binding studies almost all plasminogen molecules in the complex were converted into two-chain plasmin (*11, 13*). Therefore, they observed the effect of K1+2+3+4 peptide and EACA on the binding of plasmin and SAK.

In this study, we used EACA to explore the binding of plasminogen to SAK and the interaction of plasmin-SAK with a lysine binding site of plasminogen.

MATERIALS AND METHODS

The following materials were purchased: Tween 80, *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB) and bovine serum albumin (essentially fatty acid-free, globulin-free) (Sigma, St. Louis, MO); (*p*-aminophenyl)methanesulfonyl fluoride hydrochloride (p-APMSF) (K_i of plasmin: 1.54×10^{-6} M), dithiothreitol and silver staining kit (Wako Pure Chemical Industries, Osaka); EACA (Nakarai Chemicals, Kyoto); S-2251 (H-D-Val-Leu-Lys-pNA), S-2288 (H-D-Ile-Pro-Arg pNA), and S-2444 (L-pyroglutamyl-Gly-Arg-pNA) (Kabi Vitrum AB, Stockholm, Sweden); tricine (BioRad, Hercule, CA); protein calibration mixture, CNBr-activated Sepharose 4B, Lys-Sepharose 4B, Sephadex G-75, and Superdex 75 prep grade (Pharmacia Biotech, Uppsala, Sweden); elastase (porcine pancreas) (Calbiochem-Novabiochem Corporation, San Diego, CA) and $Na^{125}I$ (720 GBq/mg) (New England Nuclear, Boston, MA).

The protease inhibitor, Gabexate mesilate[ethyl-4 (6-guanidino hexanoyloxy)benzoate]methane sulfonate (GBM) (K_i for plasmin: 1.6×10^{-6} M) (*14, 15*), was a gift from Ono Pharmaceutical (Osaka). Aprotinin (K_i for plasmin: 2.3×10^{-10} M) and urokinase (UK) were gifts from Mochida Pharmaceutical (Tokyo).

Recombinant SAK was produced in transformed *Escherichia coli*, purified from the cultured cells and characterized according to the method reported by Sako (*16*). The purity of the SAK was more than 99% as judged by amino-terminal sequence analysis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of highly purified SAK was calculated from its amino acid composition (*17*). SAK concentration was determined with BCA Protein Assay Reagent (Pierce, Rockfold, IL) using purified SAK as the standard. Concentrations of SAK in mixed samples were measured by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies as described (*18*). In this assay system, the concentrations of SAK were not affected by the presence or absence of plasmin(ogen). Monoclonal antibodies against SAK were obtained from the Yakult Central Institute for Microbiological Research (Tokyo).

Human Glu-plasminogen was purified from fresh-frozen plasma using Lys-Sepharose and Ultrogel ACA 44 (IBF Biotechnics, Villeneuve-la-Garenne, France) as described (*19*). Lys-plasminogen was generated by limited proteolysis of Glu-plasminogen by plasmin immobilized on Sepharose 4B (*20*). Both plasminogens were considered to be more than 99% pure as assessed by SDS-PAGE and by amino terminal sequence analysis. NH_2-terminal sequence analysis revealed only glutamic acid for Glu-plasminogen and lysine, valine, or methionine for Lys-plasminogen. The kringle 1+2+3 domain, kringle 4 fragment and mini-plasminogen (kringle 5 and protease domain) were prepared by elastase digestion of plasminogen and purified as described (*4*). Glu-, Lys-, and mini-plasminogen were treated with aprotinin immobilized on Sepharose before being stored at −80°C, and after having been thawed, they were treated with 1 mM pAPMSF for 3 h before experiments. Under the experimental conditions, the inhibitory activity of 1 mM pAPMSF against plasmin was undetectable after 3 h. The concentration of plasminogen was determined at 280 nm, using $A_{1\%,1cm}=16.1$ for Glu- and Lys-plasminogen and 14.0 for mini-plasminogen (*21*). Plasmin contamination in the plasminogen preparations was measured by incubation of 10 mM plasminogen with 1 mM S-2251 in the absence or presence of 1,000 KIU/ml of aprotinin essentially as described by Collen *et al.* (*3*). Only a negligible amount of plasmin or mini-plasmin activity existed in the plasminogen preparations (<0.001% molar ratio) after the aprotinin-Sepharose and pAPMSF treatments.

Plasmin or mini-plasmin was generated by UK-coupled Sepharose in the presence of 25% glycerol as described (*22*). The specific activities of Glu-plasminogen and Lys-plasminogen (amidolytic activity/absorbance at 280 nm) were comparable after activation by UK. Plasmin and mini-plasmin were titrated with NPGB prior to use (*3, 23*).

Effect of EACA on the Activation of Plasminogen with SAK—The effect of increasing EACA concentration on the amidolytic activity of mixtures of SAK and Glu-plasminogen or Lys-plasminogen was monitored as follows: plasminogen (final concentration 0.11 μM) was incubated with SAK (final concentration 0.035 μM) in the presence of 1.2 mM S-2251 and various concentrations of EACA (0-50 mM) at 25°C in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4 containing 0.1% BSA and 0.01% Tween 80 (TBSTA). The absorbance at 405 nm of the mixture was continuously measured following addition of SAK.

Kinetics of Activation of Plasminogen by a Catalytic Amount of (Mini-)Plasmin-SAK Complex—For kinetic analysis of plasminogen activation, plasmin or mini-plasmin (final concentration 1.5 μM) and SAK (final concentration 1.5 μM) were pre-incubated for 10 min at 37°C in TBSTA and then diluted with the same buffer. The complexes (final concentration, 0.5 nM) were incubated with plasminogen (final concentration, 0.5-20 μM) in the absence or presence of EACA at 37°C in TBSTA. Under these experimental conditions, there was no lag phase in the generation of plasmin. Generated plasmin activity was measured at intervals (0-10 min) with 1.2 mM S-2251 after 50-fold dilution of the sample. Initial activation rates were obtained from linear plots of the concentration of generated plasmin *versus* time. The concentration of plasmin or mini-plasmin was determined from a standard curve constructed from NPGB-titrated plasmin or mini-plasmin and S-2251.

Effect of EACA on S-2251 Hydrolysis by Plasmin or Plasmin-SAK Complex—The effect of EACA concentration on the amidolytic activities of plasmin or the plasmin-SAK complex was observed as follows: 0.06 μM plasmin and 0.6 μM SAK were preincubated for 5 min at 37°C. EACA was added at 0-500 mM (final) and the solutions were incubated for 3 min in the presence of 0.5-3 mM S-2251 in TBSTA. The reaction was stopped with 0.3% citric acid and the absorbance at 405 nm was measured.

Gel Filtration of Plasmin(ogen)-SAK Complex—In the presence of three different concentrations of EACA (final concentration 0, 1, or 25 mM), SAK was added to Glu-plas-

minogen, Lys-plasminogen, or plasmin (final concentration 5.5 μM) in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4, containing 0.01% Tween 80 and 10 mM GBM and incubated at 4°C for 2 h. Each mixture (200 μl) was applied to a Superdex 75 prep grade column (0.7×50 cm; 7.5 ml/h) equilibrated with 50 mM phosphate buffer, 0.15 M NaCl, pH 7.3, containing 0.01% Tween 80, 10 mM GBM, and the same concentration of EACA as in the mixture. Fractions of 0.5 ml were collected for determination of SAK-related antigen by ELISA (*18*), and analysis by SDS-PAGE.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Tricine-SDS-PAGE with 10%T and 3%C separating gels (*24*) under reducing conditions was used for the analysis of SAK and the chain composition of plasmin. Samples fractionated by SDS-PAGE were stained with a silver staining kit, or further processed by autoradiography, or Western blotting as described (*25, 26*). The protein standard consisted of phosphorylase *b* (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400).

RESULTS

Effect of EACA on Plasminogen Activation by SAK—Activation of plasminogen in the presence of SAK occurred progressively, with a marked lag phase followed by an exponential growth phase (Fig. 1). Under these experimental conditions, neither the plasminogens nor SAK alone reacted with S-2251 substrate. Addition of EACA to the mixture of 110 nM plasminogen and 33 nM SAK resulted in a concentration-dependent increase in the activation rate of Glu-plasminogen (Fig. 1A). In the presence of 5 mM EACA, the activation rate of Glu-plasminogen reached a plateau and was similar to that of Lys-plasminogen. Above 10 mM EACA, the initial lag phase of Glu-plasminogen activation was prolonged (Fig. 1A). In contrast, while the initial lag phase of Lys-plasminogen activation in the presence of SAK was prolonged in an EACA-concentration dependent manner, the activation rate did not change (Fig. 1B). When the time required to obtain 15% hydrolysis of S-2251 is plotted as a function of EACA concentration, the time for Lys-plasminogen activation was prolonged in a concentration-dependent manner (Fig. 1C). In contrast, the time for Glu-plasminogen activation shortened below 5 mM EACA, and was then prolonged, like that of Lys-plasminogen, above 5 mM EACA. SDS-PAGE of the mixtures under reducing conditions revealed that the appearance of amidolytic activity in the presence of EACA corresponded to plasminogen-plasmin conversion (data not shown).

Kinetic Analysis of the EACA-Effect on the Activation of Plasminogen by Plasmin-SAK Complex—The plasmin generated after we added SAK to purified plasminogen can be explained in terms of complex formation between SAK

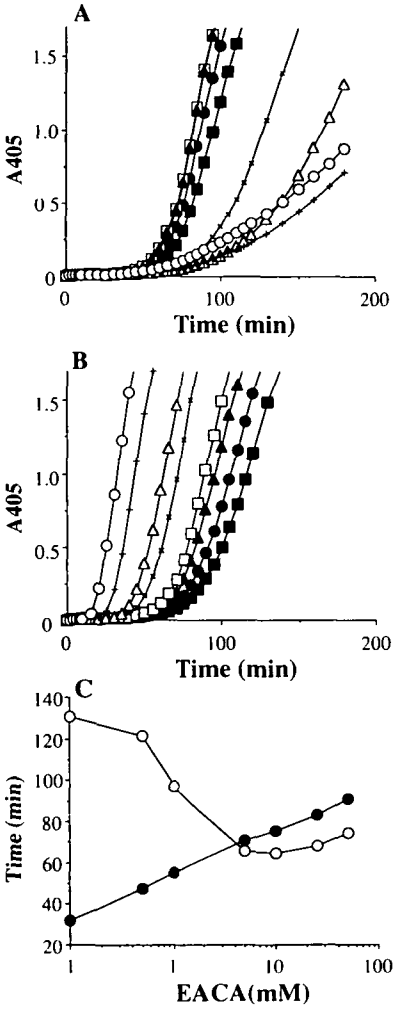

Fig. 1. **Effect of EACA on plasminogen activation by staphylokinase.** Glu- (A) or Lys-plasminogen (B) at 110 nM was incubated at 25°C with 37 nM SAK in the presence of various concentrations of EACA (○, 0; +, 0.1 mM; △, 0.5 mM; ×, 1 mM; □, 5 mM; ▲, 10 mM; ●, 25 mM; ■, 50 mM) and 1.2 mM S-2251. Each point shows the mean of triplicate experiments. The time required for 15% hydrolysis of S-2251 by SAK-Glu-plasminogen (○) or Lys-plasminogen (●) mixture in the presence of various concentrations of EACA is shown in C.

TABLE I. **Kinetic parameters of plasminogen hydrolysis by plasmin- or mini-plasmin-SAK complex.**

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	r
Plasmin-SAK	Glu-plasminogen (−)[a]	23.0	0.929	0.0404	0.986
Plasmin-SAK	Glu-plasminogen (+)	ND	ND		
Plasmin-SAK	Lys-plasminogen (−)	1.59	1.95	1.23	0.999
Plasmin-SAK	Lys-plasminogen (+)	27.5	1.78	0.0647	0.998
Plasmin-SAK	mini-plasminogen (−)	5.65	5.03	0.89	0.993
Mini-plasmin-SAK	Lys-plasminogen (−)	1.08	2.98	2.76	0.995
Mini-plasmin-SAK	mini-plasminogen (−)	4.62	5.70	1.23	0.985

[a](−) in the absence and (+) in the presence of 5 mM EACA. ND, not determined because of rapid conversion of Glu-plasminogen to Lys-plasminogen during assay; r, correlation coefficient determined by linear-regression analysis of Lineweaver-Burk plots constructed with 6-8 substrate concentrations.

and a very small amount of contaminating plasmin in the sample. Thus, kinetic constants for the activation of different molecular forms of plasminogen in the presence or absence of EACA by a preformed catalytic amount of plasmin-SAK complex were determined. Activation of plasminogen or mini-plasminogen by preformed (mini-) plasmin-SAK complex obeyed Michaelis-Menten kinetics, as revealed by linear double-reciprocal plots of the initial activation rate *versus* plasminogen concentration (*27*). Although similar experiments have been reported using 2 nM plasmin-SAK complex in the absence of EACA (*27*), we employed 0.5 nM preformed plasmin-SAK complex and pAPMSF-pretreated plasminogens to prevent the conversion of Glu-plasminogen to Lys-plasminogen during the incubation. Under these conditions, the K_m and catalytic efficiency for the Glu-plasminogen-activation was

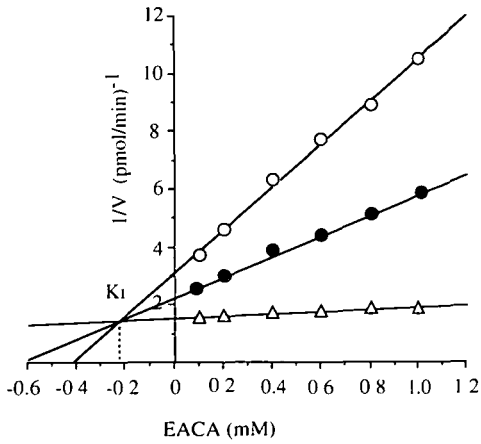

Fig. 2. **Inhibition by EACA of activation of Lys-plasminogen by plasmin-SAK complex.** Different concentrations of Lys-plasminogen (△, 5 μM; ●, 2.5 μM; and ○, 1.25 μM) were activated at 37°C with 0.5 nM plasmin-SAK in the presence of various concentrations of EACA. Generated plasmin was measured at different time intervals with S-2251 substrate as described under "MATERIALS AND METHODS." The data are presented in the form of a Dixon plot.

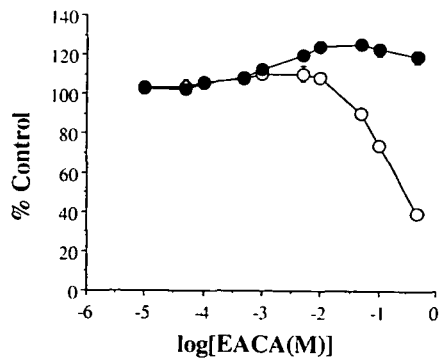

Fig. 3. **Effect of EACA on plasmin-SAK complex-catalyzed reaction.** Plasmin (○) or plasmin-SAK complex (●) at 0.06 μM was incubated with 1 mM S-2251 and the indicated concentrations of EACA at 37°C for 3 min as described under "MATERIALS AND METHODS." The absorbance at 405 nm of each sample in the presence of EACA was compared with that in the absence of EACA (control) and expressed as percent of the control. Data represent the mean ± SD of triplicate experiments.

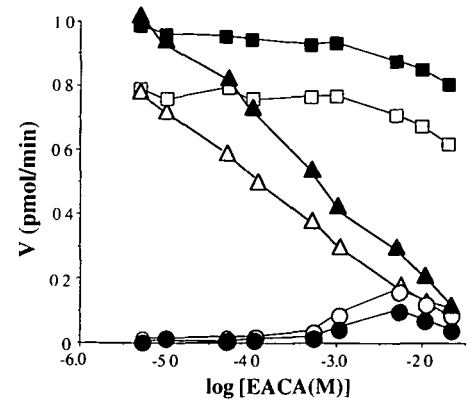

Fig. 4. **Effect of EACA on the activation of different molecular forms of plasminogen by plasmin or mini-plasmin.** Glu- (○, ●), Lys-plasminogen (△, ▲), or mini-plasminogen (□, ■) at 6 μM was activated with 0.5 nM plasmin- (open symbols) or mini-plasmin- (closed symbols) SAK complex for 5 min at 37°C in the presence of various concentrations of EACA. The amidolytic activity was measured with 1.25 mM S-2251 after a 50-fold dilution of each sample. The amount of (mini-)plasmin generated was calculated by reference to a standard curve constructed with purified materials.

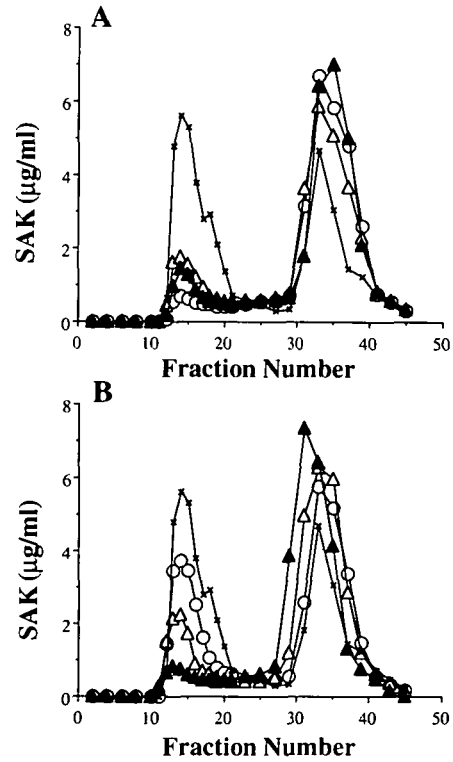

Fig. 5. **Gel-filtration of plasmin- or plasminogen-SAK complexes with EACA.** Plasmin (A, B: ×), Glu-plasminogen (A), or Lys-plasminogen (B) at 5.5 μM was mixed with 11 μM SAK in the presence of 10 mM GBM and EACA at final concentrations of 0 (○), 1 mM (△), or 25 mM (▲) and incubated at 4°C for 2 h. Each mixture (200 μl) was applied to a Superdex 75 column (0.7 × 50 cm) equilibrated with 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.3, containing 10 mM GBM and the same concentration of EACA as in each sample. Since the elution profiles of plasmin in the absence or presence of 1 or 25 mM EACA were almost the same, we present the data (×) in the presence of 25 mM EACA. The column was developed with the same buffer and the concentration of SAK in each fraction was measured as described under "MATERIALS AND METHODS." The data shown are representative of 4 runs.

14-fold higher and 30-fold lower, respectively, than those for the Lys-plasminogen activation (Table I). In the presence of 5 mM EACA, the activation of Glu-plasminogen did not obey Michaelis-Menten kinetics, probably because the conversion of Glu-plasminogen to Lys-plasminogen, which was confirmed by SDS-PAGE (data not shown), was remarkably accelerated. In the case of Lys-plasminogen, the initial rate of activation of Lys-plasminogen was inhibited in the presence of 5 mM EACA, because the K_m increased about 17-fold (Table I).

The mode of inhibition by EACA of the activation of plasminogen by the plasmin-SAK complex was further analyzed using three different concentrations of Lys-plasminogen and various concentrations of EACA. After 10 min at 37°C, the samples were diluted 50-fold and the amount of plasmin formed was measured. The data are shown in Fig. 2 in the form of a Dixon plot. Because the three lines intersect above the abscissa, the mode of inhibition by EACA is competitive. The point of intersection of the three lines indicates that the K_i for EACA is 0.23 mM. We obtained K_i values of >10, 0.95, and 0.02 mM, for arginine, lysine, and tranexamic acid, respectively, using similar inhibition experiments.

Effect of EACA on Plasmin-SAK Complex-Catalyzed Reaction—To determine whether EACA directly inhibited the plasmin-SAK complex activity, the effects of EACA on the amidolytic activity toward S-2251 of plasmin and plasmin-SAK complex were examined. Based on the K_m values of the two enzymes (plasmin, 0.38 mM, and plasmin-SAK complex, 3.74 mM) (*28*), we used substrate concentrations of 0.5, 1, and 3 mM. At any substrate concentration, in the presence of more than 50 mM EACA, plasmin activity was inhibited in a dose-dependent manner. In contrast, even 500 mM EACA did not inhibit the amidolytic activity of the plasmin-SAK complex (Fig. 3). When S-2444 or S-2288 was used as a substrate, EACA also had no inhibitory effect on the plasmin-SAK complex amidolytic activity (data not shown).

Effect of Kringle 1+2+3+4 Domain of Plasminogen on the Activation of Plasminogen by SAK—Interestingly, the K_m values for the activation of mini-plasminogen with the plasmin-SAK complex and the mini-plasmin-SAK complex were about 3.5-fold higher than those for Lys-plasminogen (Table I). These data suggest that the K1+2+3+4 of plasminogen plays a role in enzyme-substrate complex formation between plasminogen and the plasmin-SAK complex. To test whether EACA competes with plasmin-SAK complex for interaction with the K1+2+3+4 of plasminogen, the effect of EACA on the activation of plasminogen or mini-plasminogen by preformed (mini-)

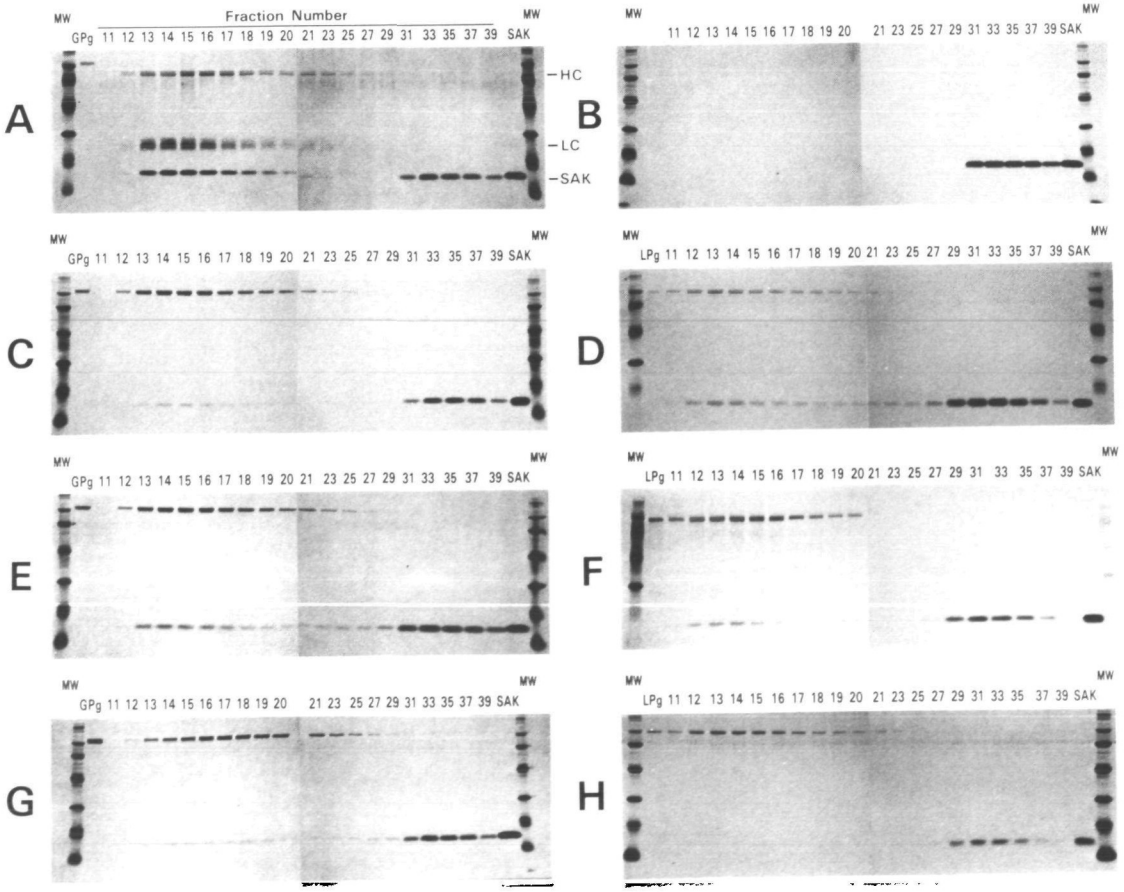

Fig. 6. **SDS-PAGE analysis of gel filtration samples.** Tricine-SDS-PAGE under reducing conditions was performed on fractions from the Superdex 75 column described in Fig. 5. Gels were stained with silver stain. A, plasmin-SAK; B, SAK; C, Glu-plasminogen-SAK without EACA; D, Lys-plasminogen-SAK without EACA; E, Glu-plasminogen-SAK with 1 mM EACA; F, Lys-plasminogen-SAK with 1 mM EACA; G, Glu-plasminogen-SAK with 25 mM EACA; and H, Lys-plasminogen-SAK with 25 mM EACA. GPg, LPg, HC, LC, and MW represent Glu-plasminogen, Lys-plasminogen, heavy chain of plasmin, light chain of plasmin, and protein molecular weight standards, respectively.

TABLE II. **The amount of SAK-binding to plasminogen in the presence of EACA.** Samples were applied to Superdex 75 and eluted as described in Fig. 6. The amount of SAK in each fraction was measured by ELISA, and expressed as percent of SAK recovered from the column. The data are mean ± SD of three experiments.

EACA (mM)	Glu-plasminogen			Lys-plasminogen		
	0	1	25	0	1	25
Recovery (%)	91±1	90±2	92±3	93±4	94±4	96±4
Binding (%)[a]	7.9±2	17±3	12±2	31±2	19±3	10±2

[a]The amount of SAK eluted from fraction 11 to 21.

plasmin-SAK complexes was examined. As shown in Fig. 4, the effect of EACA on the initial rate of (mini-)plasmin generation was independent of the enzyme form in the (mini-)plasmin-SAK complex. In contrast, the rate was dependent on the substrate form since EACA had a minimal effect on the conversion rate from mini-plasminogen to mini-plasmin by the (mini-)plasmin-SAK complex.

Effect of EACA on the Complex Formation between Plasmin(ogen) and SAK—The effect of EACA on complex formation was examined by gel filtration in the presence of serine protease inhibitor (Fig. 5). Samples containing 5.5 μM plasmin(ogen), 11 μM SAK, and 0-25 mM EACA were pre-incubated for 2 h at 4°C in the presence of 10 mM GBM to prevent activation. The mixtures were applied to a Superdex 75 column equilibrated with buffer containing the inhibitor. In the presence of GBM, the plasmin-SAK complex eluted at a volume similar to that of the plasmin-SAK complex without GBM, and EACA at any concentration had no effect on the elution position or the amount of SAK (about 49.5% of total SAK) in the plasmin-SAK complex (data not shown), in accordance with the findings of Silence *et al.* (28). The plasminogen-SAK complexes eluted at a volume similar to that of the plasmin complex. In the presence of GBM, single-chain plasminogen was not converted to a two-chain plasmin derivative as judged by SDS-PAGE under reducing conditions (Fig. 6). SAK bound to Lys-plasminogen was approximately four times higher than that bound to Glu-plasminogen in the absence of EACA under the conditions used (Table II). The amount of SAK-binding to Glu-plasminogen was increased by addition of 1 mM EACA to the mixture, but remained only about half of that bound to Lys-plasminogen in the absence of EACA (Fig. 5 and Table II). With Lys-plasminogen, however, the addition of EACA inhibited the complex formation in a concentration-dependent manner (Fig. 5B and Table II).

DISCUSSION

In this study, we have evaluated the roles of the lysine binding sites in kringle domain of plasminogen in plasminogen activation with the plasmin-SAK complex.

The plasminogen-SAK complex does not show plasminogen activator activity before conversion to the two-chain form, plasmin (*3*). Lijnen *et al.* have asserted that the lysine binding sites of plasminogen do not contribute to plasminogen-binding to SAK nor to its enzyme activity in the complex with SAK in a purified system (*12*). However, as described in the introduction, they actually observed the effect of EACA on the binding of plasmin and SAK and the enzyme activity of the plasmin-SAK complex. We, therefore, using EACA, examined the effect of lysine binding sites on the activation of purified plasminogen by plasmin-SAK complex and on the complex formation between plasminogen and SAK. The remarkable change in the activation rate of Glu-plasminogen by EACA may reflect the conformational change from the Glu-form to the Lys-form, since the effect of EACA reached a plateau at about 5 mM EACA. This concentration is close to the K_d value of the low affinity binding site of EACA to Glu-plasminogen, occupancy of which is sufficient to induce the conformational change (*10*). The prolongation of the lag phase in Lys-plasminogen activation should correspond to a decrease in plasmin generation, because addition of a catalytic amount of plasmin-SAK complex shortened the lag period (*3*).

The effect of EACA on plasminogen activation by plasmin-SAK was confirmed by activating plasminogen with a catalytic amount of plasmin-SAK complex. The difference in K_m between Glu-plasminogen and Lys-plasminogen and the effects of EACA on the initial rate of Lys-plasminogen activation (Table I) appear to be similar to those observed using urokinase (*29, 30*). The interaction of plasminogen with urokinase is dependent upon the conformation of plasminogen as well, and lysine competitively inhibits the plasminogen activation by urokinase with a K_i of 0.1 M (*30*). In addition, Petersen *et al.* (*31*) demonstrated that the inhibitory effect of tranexamic acid on the plasminogen activation with urokinase was due to interaction of the ligand with the active site of urokinase by using a urokinase-specific synthetic substrate. In the case of the plasmin-SAK complex, however, the inhibitory effect of EACA on plasminogen activation should be different from that in the case of urokinase, because the K_i for lysine is 0.95 mM [100 times lower than the previous report (*30*)] and 750 mM EACA did not inhibit plasmin-SAK activity toward S-2251, S2288, or S2444. Using mini-plasminogen as a substrate, the K_m for activation with either plasmin or mini-plasmin was 3.5-fold higher than that obtained when Lys-plasminogen was the substrate (Table I). In addition, EACA no longer affected the activation of mini-plasminogen by a catalytic amount of plasmin or mini-plasmin-SAK complex (Fig. 4). Accordingly, the K1+2+3+4 domain of Lys-plasminogen probably plays a role in binding the plasmin-SAK complex. Interestingly, the K_i value of 0.23 mM for EACA obtained from Dixon plot analysis agrees with the K_d of 0.26 mM for the higher affinity EACA-binding site in Lys-plasminogen, which does not appear in Glu-plasminogen (*4-6, 29*). In the case of Glu-plasminogen, the effects of the EACA-induced conformational change on activation, and competition with plasmin-SAK for this secondary site could be observed simultaneously in the same sample.

In attempting to confirm the binding of plasminogen and SAK quantitatively, we encountered many obstacles. Plasminogen is converted into two-chain plasmin during

assay in the presence of SAK. Since this conversion is probably triggered by the complex between contaminating plasmin and SAK, the amount of plasmin generated should be dependent upon the plasminogen concentration. In addition, the binding constants between plasminogen and SAK varied depending on the SAK-immobilization method, whether directly or through anti-SAK antibodies, as well as on the labeling method of SAK, either with $Na^{125}I$, peroxidase, or biotin (data not shown). The main reason for this is probably the fact that 30% of the amino acids in SAK are charged (*1*). Hence, we determined the plasminogen-SAK binding in the presence of EACA and a serine protease inhibitor using gel-filtration methods, thereby eliminating the need to modify any of the ligands. Although the amount of observed complex was smaller than expected, probably because of the considerably higher dissociation constants, Lys-plasminogen appeared to bind to SAK at least 4-fold more efficiently than Glu-plasminogen (Table II). Furthermore, the Lys-plasminogen binding was inhibited by EACA in a concentration-dependent manner (Fig. 5 and Table I), suggesting that the lysine binding site contributes significantly to Lys-plasminogen binding to SAK. In contrast, addition of EACA to Glu-plasminogen induced biphasic effects on the complex formation. Complex formation increased, probably through a change in conformation from the Glu-form to the Lys-form, and then decreased by the same mechanism as observed for Lys-plasminogen. After plasminogen conversion to plasmin, the affinity of the light chain (containing the active site) of plasmin and SAK becomes so high that EACA can not dissociate the plasmin-SAK complex.

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