Enhancement of fibrin binding and activation of plasminogen by staplabin through induction of a conformational change in plasminogen

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Abstract Staplabin (0.3–0.6 mM), a fungal triprenyl phenol, enhanced 3–6-fold the plasminogen activator-catalyzed activation of Glu-plasminogen and Lys-plasminogen as well as their binding to fibrin. Staplabin was not stimulatory to the amidolytic activity of plasmin and plasminogen activators. Even in the presence of ε-aminocaproic acid (EACA) and fibrinogen fragments, allosteric effectors for Glu-plasminogen, staplabin increased the activation of both forms of plasminogen. In size-exclusion chromatography of Glu-plasminogen and Lys-plasminogen, the molecular elution time, which varies as the conformation of a protein changes, was shortened by staplabin. These results suggest that staplabin causes plasminogens to be more susceptible to activation and fibrin binding by inducing a conformational change that is, at least in part, different from that induced by EACA and fibrinogen fragments.

Key words: Plasminogen activation; Plasminogen binding; Conformational change

1. Introduction

The plasminogen/plasmin system is involved in a variety of physiological and pathological processes requiring localized proteolysis, such as fibrinolysis, inflammation, tissue remodeling, ovulation, tumor metastasis and tissue invasion of pathogens [1–5]. Native plasminogen (Plg) with NH_2-terminal Glu^1 (Glu-Plg) is a single-chain glycoprotein with multiple functional domains, which consist of an NH_2-terminal peptide (NTP), five homologous kringle domains and a trypsin-like serine protease domain [6,7]. Plasmic hydrolysis of Glu-Plg yields a truncated form of Plg called Lys-Plg, which predominantly has Lys^{78} as an NH_2-terminal residue [8,9]. Glu-Plg exhibits a tight spiral structure bringing the NTP into close contact with the protease domain [10]. The tight conformation of Glu-Plg is postulated to be the result of an intramolecular interaction between a lysine residue in the NTP and the aminohexyl site of kringle 5 (K5) in Glu-Plg [11,12]. Lys-Plg, which lacks NTP, adopts a more relaxed and extended conformation than does Glu-Plg [12,13].

The binding of Plg to fibrin and cell surfaces localizes fibrinolytic activity on these surfaces [14]. These Plg bindings are mediated via lysine binding sites (or aminohexyl sites) located within the kringle domains [15,16]. The tight conformation of Glu-Plg attenuates the interaction of the kringle with fibrin and cellular receptors, resulting in a low affinity binding of Glu-Plg to these substrates [17–19]. Lysine analogs, such as ε-aminocaproic acid (EACA), inhibit Plg binding by competing with fibrin or cell surface receptors for the lysine binding sites. On the other hand, binding of lysine analogs to the low affinity lysine binding sites in K5 and K4 (first in K5, then K4) of Glu-Plg induces a large-scale conformational change in Glu-Plg [12,20,21], facilitating its activation to plasmin by Plg activators. A similar conformational change occurs upon binding of Glu-Plg to fibrin [17,22].

We have recently found a novel fungal triprenyl phenol metabolite (designated staplabin) as an activator of the binding of Glu-Plg to fibrin and monocytoid U937 cells [23]. This study was undertaken to explore the mechanism of the staplabin enhancement of Plg binding. As a result, evidence suggests that staplabin induced a conformational change in both Glu-Plg and Lys-Plg to allow them to bind fibrin and to be activated by Plg activators with greater efficiency. These effects of staplabin are quite different from the effects of lysine analogs, which also relax Plg conformation and accelerate Plg activation but inhibit fibrin binding of Plg.

2. Materials and methods

2.1. Materials

Glu-Plg and Lys-Plg were from Enzyme Research Laboratories (South Bend, IN, USA). Two-chain urokinase-type Plg activator (u-PA) was purchased from JCR Pharmaceuticals (Kobe, Japan) and two-chain tissue-type Plg activator (t-PA) from American Diagnostica (Greenwich, CT, USA). Aprotinin was from CosmoBio (Tokyo, Japan). EACA, fibrinogen, thrombin, plasmin and S-2251 (H-D-Val-Leu-Lys-*p*-NA) were from Sigma (St. Louis, MO, USA). Flavigen t-PA (CH_3SO_2-D-HHT-Gly-Arg-*p*-NA) was obtained from Biopool (Umeå, Sweden) and glutaryl-Gly-Arg-7-amino-4-methylcoumarin (Glt-Gly-Arg-AMC) from Cambridge Research Biochemicals (Cheshire, UK). $Na^{125}I$ was purchased from Amersham. Staplabin ($C_{28}H_{39}NO_6$; MW 485) was isolated from cultures of *Stachybotrys microspora* IFO 30018 as described previously [23]. Briefly, 1 l of the culture supernatant was extracted with 1-butanol. The resulting organic extracts were subjected to successive chromatographic fractionation using silica gel, Sephadex LH-20, and silica C_{18} columns, giving 24 mg of purified staplabin. The CNBr fragment of fibrinogen (CNBr-Fbg) was prepared as described [24,25]. Radioiodination of Glu-Plg, Lys-Plg and ovalbumin was performed using the chloramine-T method [26]. The compositions of buffers were: buffer A, 20 mM sodium phosphate and 150 mM NaCl, pH 7.4; buffer B, Hanks' balanced salt solution containing 1 mg/ml bovine serum albumin and 50 mM HEPES, pH 7.4; buffer C, 50 mM Tris-HCl, 100 mM NaCl and 0.01% Tween 80, pH 7.4; buffer D, 3.6% (w/v) SDS, 3.6% (w/v) 2-mercaptoethanol, 0.08% (w/v) bromophenol blue.

2.2. Determination of ^{125}I-Plg binding to fibrin

The assay was performed as described previously [23,27]. Briefly, a 100-μl solution of human fibrinogen (0.2 mg/ml) in buffer A was

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Abbreviations: Plg, plasminogen; EACA, ε-aminocaproic acid; NTP, NH_2-terminal peptide; u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; *p*NA, *p*-nitroaniline; CNBr-Fbg, CNBr fragment of fibrinogen; PAGE, polyacrylamide gel electrophoresis

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dispensed into each well of a 96-well plastic tissue culture plate. After drying the well by incubating at 37°C for 3–5 days, each well received 75 μl of human thrombin (0.68 IU/ml in buffer A) and the plates were incubated at 37°C for 3 h. Fibrin clots formed in the wells were washed 3 times with 100 μl of buffer A, then incubated at 37°C for 60 min with 50 μl of buffer B containing 50 nM ^{125}I-Plg in the presence or absence of 0.2 M EACA. After washing the wells with buffer A (twice with 200 μl, then once with 100 μl), bound ^{125}I-Plg was dissolved in 50 μl of 0.2 M NaOH and 2% (w/v) SDS at 37°C for 30 min. A portion (40 μl) of the lysate was removed and counted for radioactivity using a γ-counter.

2.3. Determination of Plg activation by t-PA and u-PA

Plg activation was assayed either by measuring the initial velocity of plasmin generation using a chromogenic substrate for plasmin or by determining the rate of conversion of ^{125}I-Plg to ^{125}I-plasmin using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In the former assay, 0.1 μM Plg and 0.1 mM S-2251 were incubated in buffer C with either t-PA (1 U/ml) or u-PA (1 U/ml) at 37°C for up to 120 min. From the slope of the plots of A_{405} nm versus t^2 [28], the initial velocity of plasmin generation was calculated. In the latter assay, 0.1 μM ^{125}I-Plg was incubated with t-PA (300 U/ml for incubation with ^{125}I-Glu-Plg and 100 U/ml for ^{125}I-Lys-Plg) and aprotinin (1000 kallikrein inhibitory units/ml) in 30 μl of buffer C. Aprotinin was used to inhibit plasmin activity that forms Lys-Plg during incubation of Glu-Plg [29]. After incubation at 37°C for 120 min, the mixture received 9 mg of solid urea and 10 μl of buffer D, and then was heated at 90°C for 2 min. A portion (10 μl) of the mixture was subjected to SDS-PAGE [30] on a 10% gel. After fixing and drying, the gel was exposed to X-ray film at −80°C for 16 h. Radioactive bands corresponding to Plg and the light and heavy chains of plasmin were excised from the gel and counted for radioactivity in a γ-counter.

2.4. Determination of amidolytic activities of plasmin, t-PA and u-PA

The amidolytic activities of plasmin, t-PA and u-PA were determined in 100 μl of buffer C using S-2251, Flavigen t-PA or Glt-Gly-Arg-AMC, respectively, as a substrate. The release of *p*NA (for S-2251 and Flavigen t-PA) or 7-amino-4-methylcoumarin (for Glt-Gly-Arg-AMC) was measured as an absorbance at 405 nm using a model 450 microplate reader or as fluorescence intensity at an excitation wavelength of 380 nm and an emission at 480 nm using a model 650 fluorescence spectrometer (Hitachi), respectively. The enzyme and substrate concentrations were: 10 nM and 0.1 mM for plasmin; 5 U/ml and 0.1 mM for both t-PA and u-PA.

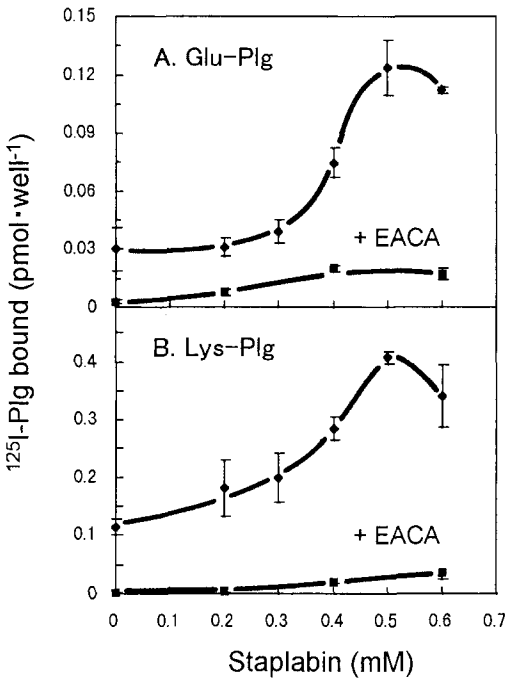

Fig. 1. Enhancement of fibrin binding of ^{125}I-Plg by staplabin. The binding of ^{125}I-Glu-Plg (A) and ^{125}I-Lys-Plg (B) to fibrin was determined in the presence of the indicated concentrations of staplabin. Where indicated, EACA (0.2 M) was included in the reaction mixture. The specific activities of Plg used were 1.98×10^4 cpm/pmol for ^{125}I-Glu-Plg and 2.48×10^4 cpm/pmol for ^{125}I-Lys-Plg. Each value represents the mean ± S.D. from triplicate determinations.

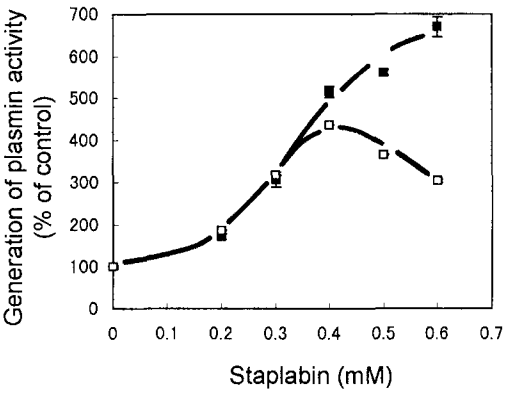

Fig. 2. Enhancement by staplabin of t-PA-catalyzed generation of plasmin activity from Plg. The activation of Glu-Plg (■) and Lys-Plg (□) was determined by measuring the generation of plasmin activity in the presence of the indicated concentrations of staplabin. Each value represents the mean ± S.D. from triplicate determinations. The control values for Glu-Plg and Lys-Plg were 0.10 ± 0.004 and 0.74 ± 0.003 nmol *p*NA/well/h^2, respectively.

2.5. Size-exclusion HPLC

Ten microliters of 0.1 μM ^{125}I-Plg or ^{125}I-ovalbumin (a control for non-allosteric protein) in buffer C were applied to a TSKgel G3000SW column (0.75×60 cm; TOSO Co., Japan) equilibrated with buffer C or buffer C containing 0.4 or 0.6 mM staplabin. The column was developed with the same buffer at a rate of 1 ml/min at 22°C. The eluate fraction (0.33 ml) was counted for radioactivity in a γ-counter.

3. Results

Lys-Plg, which has an extended, relaxed molecular structure, is known to bind to fibrin more efficiently than Glu-Plg, which adopts a closed, tight conformation [17,18]. In the present experiments, the level of fibrin binding of ^{125}I-Lys-Plg is 4 times higher than that of ^{125}I-Glu-Plg (Fig. 1). As described previously, staplabin enhanced ^{125}I-Glu-Plg binding to fibrin 2.5–4-fold at concentrations ranging from 0.4 to 0.6 mM (Fig. 1A). The binding of ^{125}I-Lys-Plg was similarly increased by staplabin at the same concentrations (Fig. 1B). In both cases, the binding was inhibited 74–93% by EACA, suggesting that the binding in the presence of staplabin involved lysine binding sites of Plg.

Since a Plg molecule with a relaxed conformation is efficiently activated by t-PA and u-PA, we tested whether staplabin enhances Plg activation by these Plg activators. When measured as an increase in plasmin amidolytic activity, the rate of t-PA-catalyzed activation of Lys-Plg was seven times as high as that of Glu-Plg. The activation of both Glu-Plg and Lys-Plg by t-PA was significantly enhanced by staplabin (3–6.8-fold at 0.3–0.6 mM; Fig. 2). Similar results were obtained when u-PA was used as a Plg activator (data not shown). In control incubations in the absence of Plg activators, staplabin showed no effect on the hydrolysis of S-2251 by Plg. Staplabin was stimulatory to neither plasmin, t-PA nor u-PA (data not

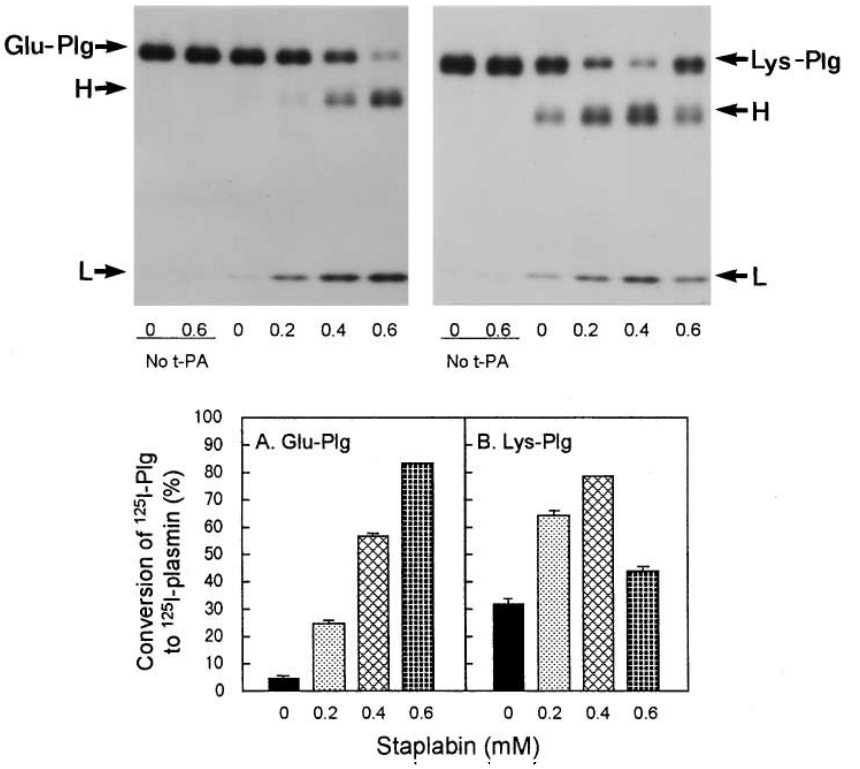

Fig. 3. Enhancement by staplabin of t-PA-catalyzed conversion of ^{125}I-Plg to ^{125}I-plasmin. The t-PA-catalyzed conversion of ^{125}I-Glu-Plg (A) and ^{125}I-Lys-Plg (B) to ^{125}I-plasmin was determined in the presence of the indicated concentrations of staplabin. The specific activities of Plg used were 1.01×10^4 cpm/pmol for ^{125}I-Glu-Plg and 1.26×10^4 cpm/pmol for ^{125}I-Lys-Plg. Top: Representative autoradiograms (H, heavy chain; L, light chain). Bottom: The rate of conversion, which was calculated by dividing the radioactivity of plasmin (heavy chain plus light chain) by the radioactivity of Plg plus plasmin. Each value represents the mean ± S.D. from triplicate determinations.

shown). To test whether these increases in the generation of plasmin activity accompanied the formation of a two-chain form, ^{125}I-Plg incubated with Plg activators in the presence of aprotinin was analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 3, the rate of t-PA-catalyzed conversion of ^{125}I-Glu-Plg to ^{125}I-Glu-plasmin was ~5% in control incubations, while the rate was significantly increased by staplabin (to 57–84% at 0.4–0.6 mM). Similarly, a significant increase in the conversion of ^{125}I-Lys-Plg to ^{125}I-plasmin (from 32 to 79%) was observed in the presence of 0.4 mM staplabin.

Table 1
Reversibility of the effect of staplabin on Glu-Plg activation by t-PA

Staplabin (mM)		Generation of plasmin activity (nmol *p*NA/well/h^2)
At preincubation	At activation assay	
0	0	0.077 ± 0.004
0	0.02	0.081 ± 0.001
0	0.60	0.171 ± 0.003
0.6	0.02	0.080 ± 0.003
0.6	0.60	0.171 ± 0.004

Glu-Plg (3 μM) was preincubated at 37°C for 20 min in the presence of the indicated concentrations of staplabin. Subsequently, a portion of the mixture was removed and diluted 30-fold (so that the concentration of Glu-Plg became 0.1 μM) and assayed for Plg activation by t-PA (1 unit/ml) at 37°C in the presence of the indicated concentrations of staplabin.

Next, the reversibility of the staplabin effect was examined. Glu-Plg was preincubated at 37°C for 20 min with or without staplabin (0.6 mM). Subsequently, the mixture was diluted to assay Plg activation by t-PA at staplabin concentrations of 0.03 and 0.6 mM. Regardless of the preincubation with staplabin, Glu-Plg activation was significantly enhanced only when a sufficient concentration of staplabin was present during the activation incubation, demonstrating that the effect of staplabin was reversible (Table 1).

CNBr-Fbg and EACA induce a large-scale conformational change in the Glu-Plg molecule. These effectors enhanced u-PA-catalyzed activation of Glu-Plg severalfold. When staplabin was present, the effects of CNBr-Fbg and EACA on u-PA-catalyzed Glu-Plg activation were less prominent (Fig. 4). However, the level of activation was still higher in the presence of both staplabin and CNBr-Fbg (or EACA) than in the presence of CNBr-Fbg (or EACA) alone. On the other hand, CNBr-Fbg and EACA caused little change in Lys-Plg activation. Staplabin was also active in enhancing Lys-Plg activation in the presence of these effectors (Fig. 4).

To test whether staplabin induces a conformational change in the Plg molecule, ^{125}I-Glu-Plg and ^{125}I-Lys-Plg were analyzed by size-exclusion HPLC both in the absence and in the presence of staplabin (Fig. 5). By adding staplabin to the elution buffer, the molecular elution time for ^{125}I-Glu-Plg was changed from 17.0 to 16.3 min. Similarly, the elution time for ^{125}I-Lys-Plg was changed from 16.7 to 16.3 min by staplabin. Since the changes in the elution time were too small to account for molecular aggregation of Plgs, these results should represent a structural alteration from a smaller to a

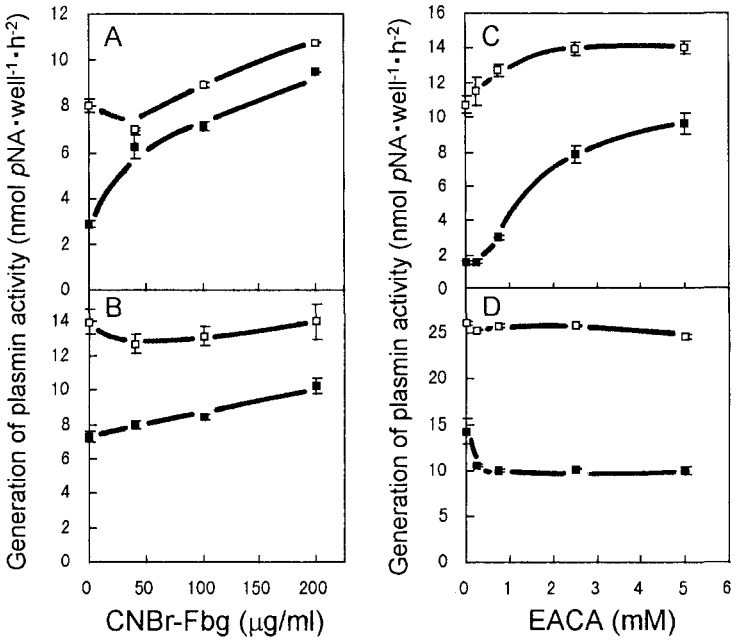

Fig. 4. Effects of CNBr-Fbg and EACA on Plg activation by u-PA in the absence and presence of staplabin. The u-PA-catalyzed generation of plasmin activity from Glu-Plg (A, C) and Lys-Plg (B, D) was assayed in the presence of the indicated concentrations of CNBr-Fbg (A, B) or EACA (C, D). Where indicated (□), the reaction mixture contained 0.6 mM (for Glu-Plg) or 0.4 mM (for Lys-Plg) staplabin. Each value represents the mean ± S.D. from triplicate determinations.

larger molecular shape. No changes in elution time were observed for ^{125}I-ovalbumin, which was used as a non-allosteric protein control [12] (data not shown).

4. Discussion

The present results demonstrate that staplabin enhances both fibrin binding and t-PA- and u-PA-catalyzed activation of both Glu-Plg and Lys-Plg. Changes in conformation of the Plg molecule, from a tight, spiral form to a relaxed, extended one, accompany both an increase in the activation and fibrin binding of Plg [17,18,31,32]. The fact that the molecular elution time for Glu-Plg and Lys-Plg in size-exclusion HPLC is shortened by the presence of staplabin suggests that staplabin causes Plg molecules to adopt more relaxed conformations [12]. In the presence of two allosteric effectors for Glu-Plg, EACA and CNBr-Fbg, staplabin is still active in enhancing Glu-Plg activation. In addition, the activation of Lys-Plg, which has an extended, relaxed structure due to the absence of the intramolecular interaction between a lysine residue in NTP and K5 [11,12], is not enhanced by EACA and CNBr-Fbg but it is by staplabin. Thus, the staplabin-induced conformational change in Plg may not be similar to that induced by dissociation of the intramolecular interaction. Supporting this hypothesis is the observation that u-PA-catalyzed activation of mini-Plg, which lacks NTP and K1–K4, is also elevated by staplabin (data not shown). This finding further suggests that a disruption of the interaction between K4 and the tetranectin-like sequence in K3, which is postulated to participate in maintaining the β-conformation of Lys-Plg [12], is not a mechanism for the action of staplabin. The fibrin binding of Plgs is inhibited by an excess of EACA or several other ligands of lysine binding sites in the kringle. In contrast to these ligands, staplabin increases Plg-fibrin binding, and the staplabin-enhanced binding is inhibited by EACA. These observations suggest that lysine binding sites in the kringle do not play a role in the conformational change induced by staplabin. Taken together, the present results suggest that staplabin causes Plgs to be more susceptible to activation and fibrin binding by inducing a conformational change that is, at least

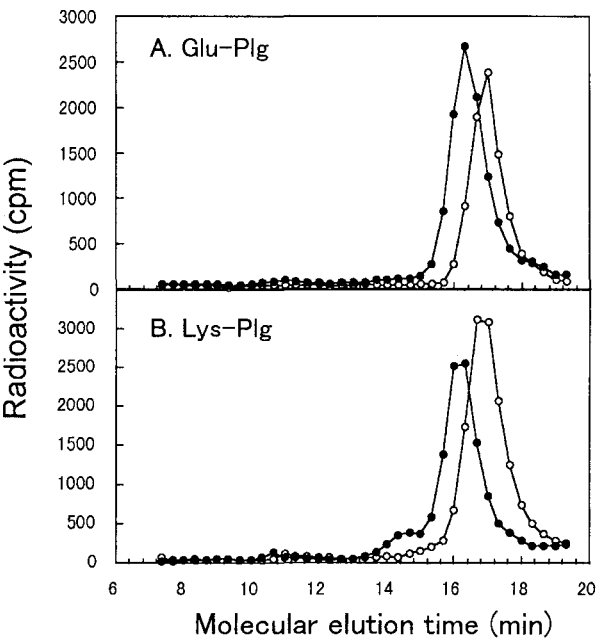

Fig. 5. Size-exclusion HPLC elution profile of ^{125}I-Plg in the absence and presence of staplabin. ^{125}I-Glu-Plg (A) or ^{125}I-Lys-Plg (B) (2.5 pmol each) was analyzed by size-exclusion HPLC in the absence (○) or presence (●) of staplabin (0.6 mM for Glu-Plg and 0.4 mM for Lys-Plg). The specific activities of Plg used were 6.95×10^3 cpm/pmol for ^{125}I-Glu-Plg and 8.92×10^3 cpm/pmol for ^{125}I-Lys-Plg. Each value represents the average of duplicate determinations.

in part, different from that induced by EACA and CNBr-Fbg. At higher concentrations of staplabin, the stimulatory effect on Plg binding to fibrin as well as on Plg activation declines. While the detailed mechanism is currently unknown, it may be related to the extent of the conformational change of Plg.

It would be interesting to ask whether staplabin-induced changes in Plg conformation result in an increase in binding of Plg to streptokinase [33]. In preliminary experiments, staplabin was found to enhance streptokinase-mediated activation of Glu-Plg. Although this effect appears to be due to an acceleration of complex formation between streptokinase and Glu-Plg, details are to be examined in future studies.

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