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Proteolytic Activation of Single-chain Tissue-type Plasminogen Activator by Protease/α_2-Macroglobulin Complex Isolated from Human Ovarian Follicular Fluid

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ABSTRACT—α_2-Macroglobulin is a high-molecular-weight glycoprotein that inhibits a variety of endoproteases. Proteolytic enzymes associated with this inhibitor are thought to be unable to act on protein substrates. This paper reports that the α_2-macroglobulin fraction isolated from the follicular fluid of human ovaries is capable of proteolytically activating human single-chain tissue-type plasminogen activator. We demonstrated that a bound protease unlike plasma kallikrein was involved in the activation. This activity was maximally detected at pH values in the ranges 6–9, and was strongly inhibited by diisopropylfluorophosphate and aprotinin, indicating that the enzyme responsible for the activation is a serine protease. In summary, this paper describes for the first time that a protease complexed with α_2-macroglobulin exhibits detectable proteolytic activity toward the protein substrate single-chain tissue-type plasminogen activator.

INTRODUCTION

α_2-Macroglobulin is a glycoprotein that exists in plasma as a tetramer composed of identical subunits, with a total molecular weight of 725,000 (Barrett and Starkey, 1973; Sottrup-Jensen, 1989). It functions as an inhibitor of all four major classes of endoproteases. This inhibiting mechanism is unique in that limited proteolysis of α_2-macroglobulin at a “bait region” located near the middle of each subunit results in a conformational change in the inhibitor that physically entraps the protease. Because of this unique mechanism, proteases with molecular weights smaller than about 80,000 can be trapped by the inhibitor. Plasma kallikrein and plasmin are the largest proteases that the inhibitor is reported to bind (Barrett, 1981).

α_2-Macroglobulin is also known to be present in the follicular fluid of mammalian ovaries (Andersen *et al*, 1976; Curry *et al.*, 1990; Gaddy-Kurten and Richards, 1991; Zhu and Woessner, 1991; Ohnishi *et al.*, 1997). Recently, we have reported that the plasma kallikrein activity in human ovarian follicular fluid is largely associated with α_2-macroglobulin (Ohnishi *et al.*, 1997). It has been well established that proteases bound to α_2-macroglobulin maintain their activity in regard to small substrates, but have diminished effect on macromolecular substrates (Barrett and Starkey, 1973; Barrett, 1981; Bieth *et al.*, 1981; Gonias and Pizzo, 1983; Sottrup-Jensen, 1989; Sakurai *et al.*, 1996; Uchino *et al.*, 1993). Therefore, we presumed that little or no activity would be detected for α_2-macroglobulin fraction containing active plasma kallikrein regarding protein substrates. However, we observed the fraction inciting considerable activation of single-chain tissue-type plasminogen activator (sctPA), a protein known to be expressed in ovarian follicles upon ovulation (Beers, 1975; Beers *et al.*, 1975; Ny *et al.*, 1985; Reich *et al.*, 1985; Reinthaller *et al.*, 1990; Leonardsson *et al.*, 1995). In this report, we show that a serine protease complexed with α_2-macroglobulin converts sctPA to two-chain tissue-type plasminogen activator (tPA). The present data also indicate that plasma kallikrein is not the enzyme responsible for this activation.

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MATERIALS AND METHODS

Materials

Ovarian fluids were obtained from follicle aspirates of women participating in an *in vitro* fertilization program (Ohnishi *et al.*, 1997; Kudo *et al.*, 1997). *t*-Butyloxycarbonyl(Boc)-Gln-Gly-Arg-4-methylcoumaryl-7-amide (MCA), Boc-Val-Leu-Lys-MCA, Pro-Phe-Arg-MCA, succinyl(Suc)-Leu-Leu-Val-Tyr-MCA, E-64, and antipain were obtained from the Peptide Institute (Osaka, Japan). Diisopropylfluorophosphate (DFP), phenylmethanesulfonyl fluoride (PMSF), N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK), N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TPCK), benzamidine-HCl, aprotinin, and *p*-chloromercuribenzoate (PCMB) were purchased from Sigma Chemical Co. (St. Louis, USA). Sephacryl S-300 was obtained

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from Pharmacia Biotech (Uppsala, Sweden). Human sctPA , two-chain tPA, and goat anti-human melanoma tPA antibody were obtained from Biopool AB (Umeå, Sweden). Sheep anti-human α_2-macroglobulin IgG (Cosmo Bio, Tokyo, Japan) and rabbit anti-human plasma kallikrein IgG (Binding Site Ltd., England) were purchased from the indicated sources. An ECL Western blot detection kit was obtained from Amersham (Buckinghamshire, England). Polyvinylidene difluoride membrane was obtained from Nihon Millipore Ltd. (Tokyo, Japan). Gradient non-denaturing polyacrylamide gel electrophoresis (PAGE) gels (PAG plate 2/15) were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Other reagents were of the highest grade available.

Enzyme Activity

Enzyme activities toward synthetic peptide MCA substrates were assayed according to Barrett (1980) with a slight modification. Enzyme reactions were carried out at 37°C in a 0.5 ml solution containing the enzyme sample, 0.1 M Tris-HCl buffer (pH 8.0), and 0.1 mM substrate. The reaction was stopped by the addition of 2.5 ml of 30 mM sodium acetate buffer (pH 4.3) containing 100 mM monochloroacetic acid. The release of fluorophore 7-amino-4-methylcoumarin was measured by spectrofluorometry using an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Enzyme activity was expressed as the amount of released 7-amino-4-methylcoumarin under the stated conditions.

sctPA Activation

Human sctPA was incubated at 37°C with the α_2-macroglobulin fraction in 0.1 M Tris-HCl buffer (pH 8.0) under the stated conditions. After incubation, aliquots of the reaction mixture were taken and incubated with aprotinin (0.5 mg/ml) for 10 min at 37°C. The mixtures were then assayed for enzyme activity in a 0.5 ml-system using Boc-Gln-Gly-Arg-MCA (0.1 mM) as described above. This procedure allowed us to determine specifically activated tPA activity (Ohnishi *et al.*, 1995; Ohnishi *et al.*, 1997).

Electrophoresis and Western Blot Analysis

The α_2-macroglobulin sample was electrophoresed on a 2–15% gradient non-denaturing PAGE according to the method described by Laemmli (1970). The sctPA sample incubated with the α_2-macroglobulin fraction was electrophoresed on 12% PAGE gel in the presence of sodium dodecyl sulfate (SDS) under reducing conditions. The separated proteins were blotted onto polyvinylidene difluoride membrane according to the method of Towbin *et al.* (1979). Immunoreactive signals were detected using an ECL Western blot detection kit.

Immunoprecipitation

The α_2-macroglobulin samples were mixed individually with anti-human α_2-macroglobulin IgG and anti-human plasma kallikrein IgG in 0.1 M of Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl for a total volume of 100 µl. The mixtures were incubated at 4°C for 16 hr, and then processed as described previously (Ohnishi *et al.*, 1997). The recovered supernatants were used for determining both plasma kallikrein and sctPA-activating activities.

RESULTS AND DISCUSSION

The protease inhibitor α_2-macroglobulin can be purified from the follicular fluid of human ovaries obtained during *in vitro* fertilization procedures by a series of column chromatographic steps on DE-52, Sephacryl S-300, and heparin-Cellulofine (Ohnishi *et al.*, 1997). In this study, we obtained the α_2-macroglobulin fraction according to the above method. The chemical entity and purity of the fraction were confirmed by electrophoretic and immunological analyses, as described previously (Ohnishi *et al.*, 1997). The sample thus prepared contained largely α_2-macroglobulin in free form. In addition, the sample was shown to contain in part α_2-macroglobulin complexed with plasma kallikrein. In view of the general understanding that proteases entrapped in α_2-macroglobulin no longer act on protein substrates because their free contact becomes sterically impossible (Barrett and Starkey, 1973; Barrett, 1981; Sottrup-Jensen, 1989), we assumed that the α_2-macroglobulin fraction would exhibit no significant activity toward protein substrates. However, in a preliminary experiment, sctPA (Mr = 60,000) upon incubation with the franction was found to be activated substantially. This unexpected result was initially thought to be due to a still-contaminating, unbound protease(s) in the α_2-macroglobulin sample. Therefore, the sample obtained from heparin-Cellulofine column chromatography was fractionated once more through a column of Sephacryl S-300 (Fig. 1). The fractions were monitored by assay with three MCA-substrates: Pro-Phe-Arg-MCA (substrate for kallikrein), Boc-Val-Leu-Lys-MCA (substrate for plasmin or endopeptidases with cleavage specificity for Lys-X peptide bonds), and Suc-Leu-Leu-Val-Tyr-MCA (substrate for proteasome). The enzyme activity toward Pro-Phe-Arg-MCA appeared in a single peak at the position corresponding to a molecular weight of 725,000. No detectable activity was observed in the fractions eluted at the position for lower molecular weight proteins. Little activity was detected with Boc-Val-Leu-Lys-MCA and Suc-Leu-Leu-Val-Tyr-MCA throughout

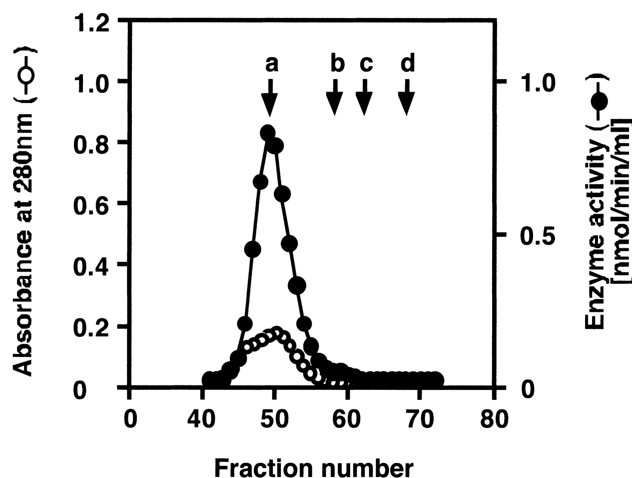

Fig. 1. Sephacryl S-300 column choromatography of α_2-macroglobulin fraction isolated from human ovarian follicular fluid. The α_2-macroglobulin fraction (1.2 mg) obtained from a heparin-Cellulofine column (Ohnishi *et al.*, 1997) was concentrated, applied to a column (1.5 × 54 cm) of Sephacryl S-300, and eluted in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl. Flow rate was 20 ml/h, and fractions of 1 ml were collected. Absorbance at 280 nm and enzyme activities toward three MCA-substrates (Pro-Phe-Arg-MCA, Boc-Val-Leu-Lys-MCA, and Suc-Leu-Leu-Val-Tyr-MCA) of the fractions were determined. Little enzyme activity was detected with the latter two substrates, and only Pro-Phe-Arg-MCA-hydrolyzing activity is shown. Fractions 45–54 were pooled for further study. Elution positions of four molecular weight markers are indicated: a) porcine plasma α_2-macroglobulin (725 kDa); b) rabbit muscle aldolase (160 kDa); c) human plasma kallikrein (90 kDa); d) bovine serum albumin (65 kDa).

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the fractions. These results indicate that the sample obtained from the heparin-Cellulofine column did not contain proteolytic activities of plasmin, Lys-X-hydrolyzing endopeptidases, or proteasome. Therefore, we tentatively presumed that the α_2-macroglobulin fraction had not been seriously contaminated with unbound proteases. However, we cannot exclude completely the possibility that proteases having activities toward appropriate substrates other than the above three MCA substrates are present. The fractions 45–54 were pooled and used in the following experiments.

The α_2-macroglobulin fraction's effect on sctPA was examined. As shown in Fig. 2, sctPA was converted to its two-chain form when incubated with the fraction. The result illustrated in Fig. 3 is direct evidence that the protein possesses an enzyme activity toward sctPA. These observations suggest that sctPA is still to some extent accessible to a protease associated with α_2-macroglobulin. Since the fraction contained bound plasma kallikrein (Ohnishi *et al.*, 1997), its part in sctPA activation was examined. Our previous data suggested that the enzyme activity detected with the substrate Pro-Phe-Arg-MCA was almost exclusively due to the action of bound plasma kallikrein (Ohnishi *et al.*, 1997). Using the active site titrant *p*-nitrophenyl-*p*'-guanidinobenzoate HCl (Chase and Shaw, 1967), the active bound enzyme in the fraction was quantified. Fig. 4 shows the comparison of sctPA activation with plasma kallikrein and the α_2-macroglobulin fraction containing the same number of the active sites. sctPA was activated with the fraction faster than was free plasma

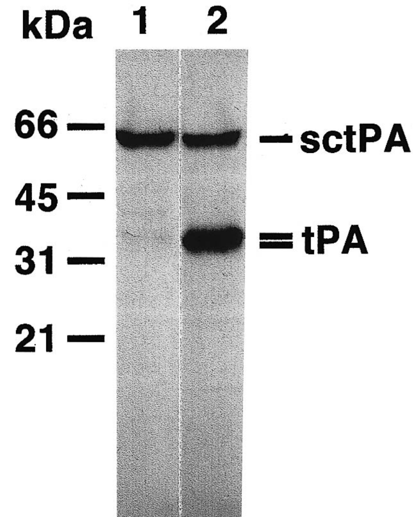

Fig. 2. Proteolytic conversion of sctPA to two-chain tPA by the α_2-macroglobulin fraction. Human sctPA (0.1 µg) was incubated at 37°C with the fraction (0.3 µg) in a total volume of 50 µl in 0.1 M Tris-HCl buffer (pH 8.0) containing bovine serum albumin (0.5 mg/ml). Aliquots (10 µl) of the mixture were taken for reducing SDS-PAGE/Western blot analysis at 0 (lane 1) and 4 hr (lane 2) of incubation. The polyvinylidene difluoride membrane with blotted proteins was first incubated with goat anti-human tPA IgG in a 1:5,000 dilution, and then with donkey anti-goat IgG in a 1:2,000 dilution. The 34 kDa chain of tPA is strongly detected by the antibody, but the 32 kDa chain is slightly visible in this detection system. Positions of molecular weight markers are shown on the left.

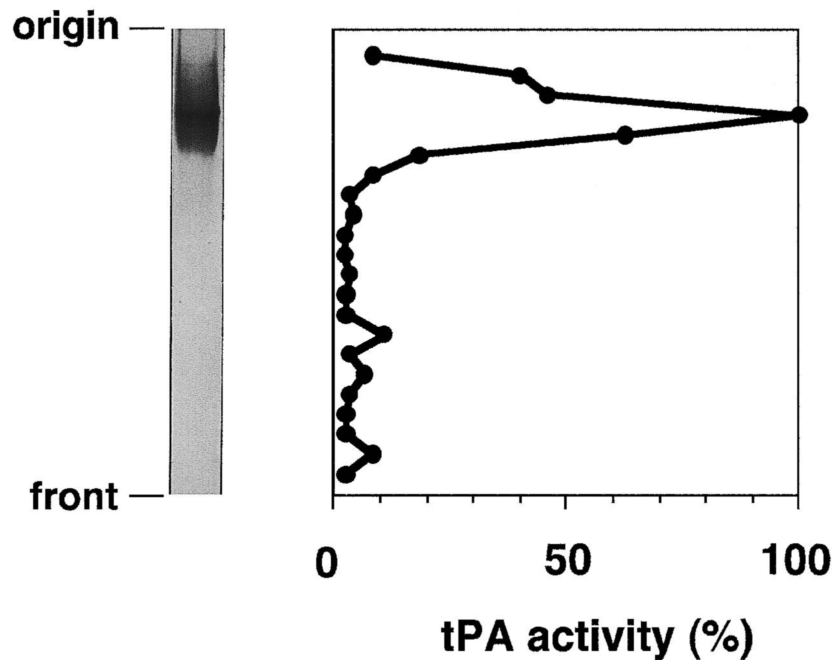

Fig. 3. Association of sctPA-activating activity with the α_2-macroglobulin fraction. The α_2-macroglobulin fraction (20 µg) was separately applied to two well positions of a 2–15% gradient polyacrylamide gel without SDS. After electrophoresis at 4°C, one lane was stained with Coomassie Brilliant Blue R-250, while the other one was sliced into pieces 3 mm wide for overnight extraction in 600 µl of 0.1 M Tris-HCl buffer (pH 8.0). Aliquots (487 ?l) of each extract were incubated with sctPA (0.3 µg) in a total volume of 500 µl at 37°C for 3 hr, then the mixtures were assayed for activated tPA activity using Boc-Gln-Gly-Arg-MCA in the presence of aprotinin. Activities relative to the highest value are shown.

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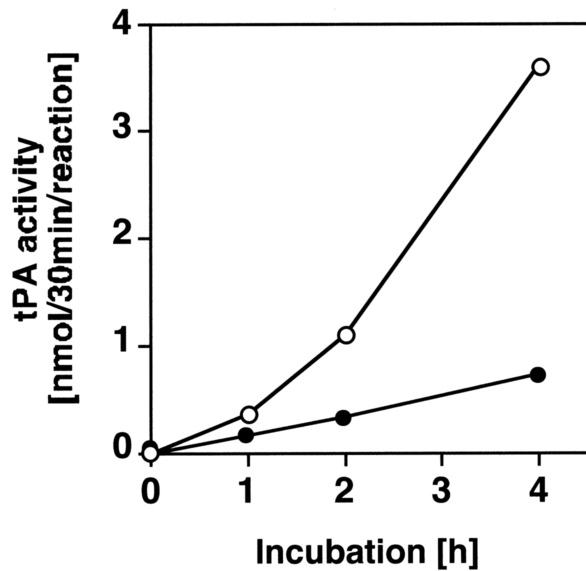

Fig. 4. sctPA activation by the α_2-macroglobulin fraction and plasma kallikrein. Human sctPA (2 µg) was incubated at 37°C with the α_2-macroglobulin fraction (5.8 µg, enzyme activity of 0.18 nmol/min toward Pro-Phe-Arg-MCA) in a total volume of 1 ml in 0.1 M Tris-HCl buffer (pH 8.0) containing bovine serum ablumin (0.5 mg/ml). For comparison, a sctPA activation experiment was conducted under conditions using free human plasma kallikrein with the same number of the active sites. At the indicated times, aliquots of 200 µl of the incubation mixture were taken for determination of the activated tPA activity. Activities were assayed with Boc-Gln-Gly-Arg-MCA at 37°C in the presence of aprotinin, and are expressed as the amount of 7-amino-4-methylcoumarin released per 30 min. ○, sctPA activation by the α_2-macroglobulin fraction; ●, sctPA activation by plasma kallikrein.

kallikrein. It shoud be noted that activation by the fraction apparently accelerated with incubation time. It is not known at present why the activation proceeds non-linearly. We next conducted sctPA activation experiments using the α_2-macroglobulin fraction that had been previously treated with either anti-human α_2-macroglobulin IgG or anti-human plasma kallikrein IgG. As shown in Fig. 5A, the greater part of the fraction's Pro-Phe-Arg-MCA-hydrolyzing activity was immunoprecipitated with both IgGs. The result is consistent with our previous data (Ohnishi *et al.*, 1997). Under these conditions, the sctPA-activating activity was largely immunoprecipitated by the α_2-macroglobulin IgG. However, the plasma kallikrein IgG-treated α_2-macroglobulin fraction was still capable of activating sctPA (Fig. 5B). These results clearly indicate that plasma kallikrein associated with α_2-macroglobulin is not involved in the activation of sctPA.

The bound protease was stable. No significant loss of sctPA-activating activity of the fraction was observed in several months of incubation at pH 8.0 and 4°C. Fig. 6 shows the effect of pH on the sctPA-activating activity of the α_2-macroglobulin fraction. A bell-shaped curve revealed a large plateau at pH values in the ranges of 6–9. The effects of various protease inhibitors were examined and the results are shown in Fig. 7. The conversion of sctPA to tPA by the bound enzyme was strongly inhibited by aprotinin and DFP. Other inhibitors were without effect. These results indicate that a serine protease is involved in the conversion. We believe that autocatalytic activation of sctPA is probably not a mechanism underlying the conversion of sctPA to tPA. If this would be the

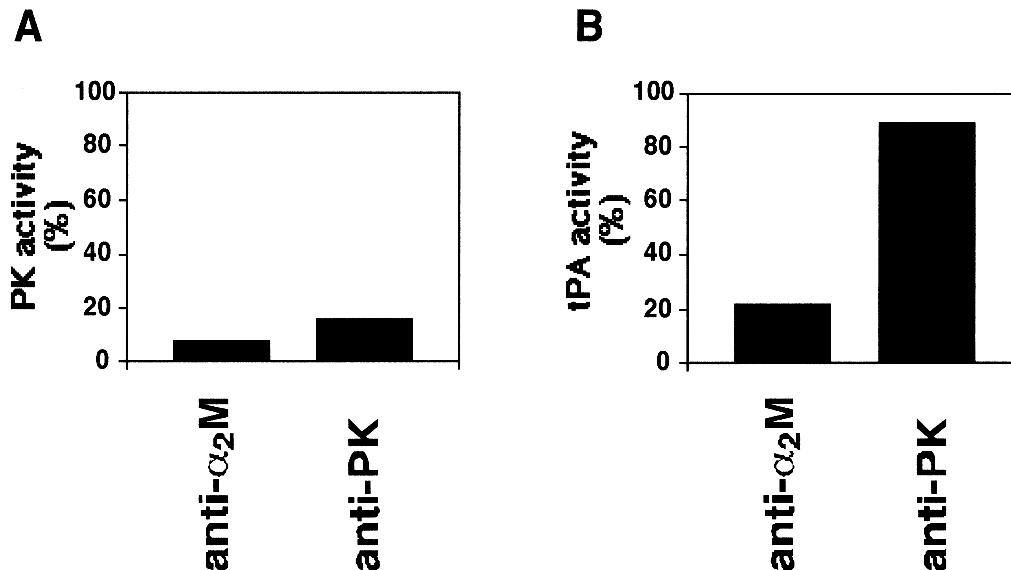

Fig. 5. Immunoprecipitation experiments of the bound sctPA-activating activity. The α_2-macroglobulin fraction (78 µg) was mixed with sheep anti-human α_2-macroglobulin IgG (120 µg) or rabbit anti-human plasma kallikrein IgG (100 µg) in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl in a volume of 100 µl and incubated at 4°C for 16 hr. One hundred microlitres of Staphylosorbe (10% v/v, binding capacity of 5 to 6 mg of human IgG/ml suspension) were added and the mixtures were incubated at 4°C for 3 hr with intermittent mixing every 30 min. After centrifuging at 10,000 × g for 10 min, the supernatants were assayed for enzyme activities. (A) The supernatants were directly assayed with Pro-Phe-Arg-MCA in order to detect plasma kallikrein (PK) activity. (B) The supernatants were incubated with human sctPA (0.2 µg) at 37°C for 3 hr, and then the activated tPA was assayed at 37°C with Boc-Gln-Gly-Arg-MCA in the presence of aprotinin. Enzyme acitivities are expressed as percentages of the controls that were similarly processed without antibodies.

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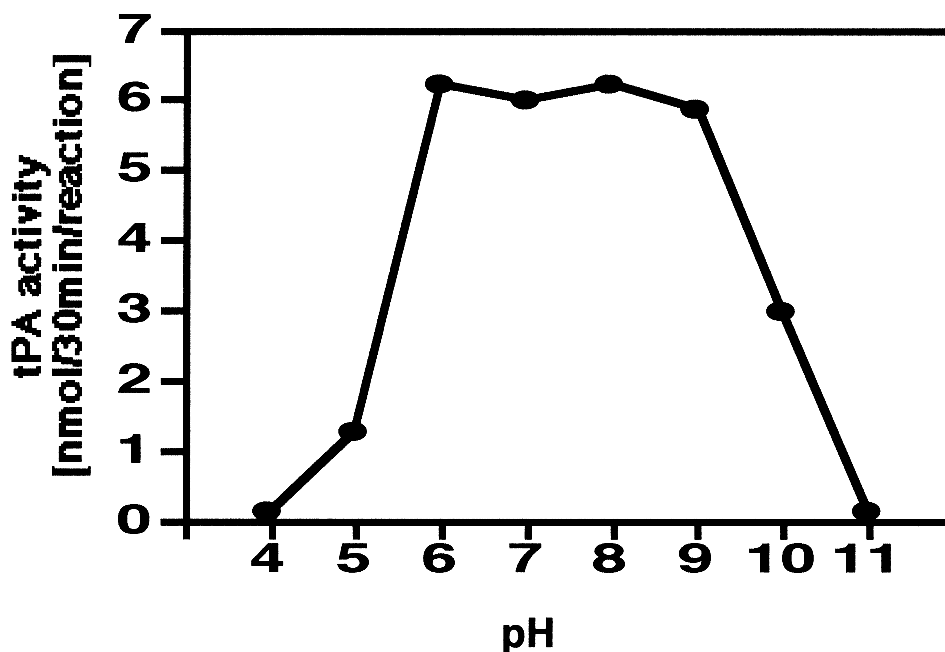

Fig. 6. Effect of pH on sctPA-activating activity. Human sctPA (0.4 μg) and the α_2-macroglobulin fraction were incubated at 37°C in a volume of 100 μl in 0.1 M sodium acetate buffer (pH 4, 5 or 6) or 0.1 M Tris-HCl buffer (pH 7, 8 or 9) or 0.1 M glycine-NaOH buffer (pH 10 or 11). After incubation for 1 hr, activated tPA activity was determined at 37°C in 0.1 M Tris-HCl buffer (pH 8.0) using Boc-Gln-Gly-Arg-MCA in the presence of aprotinin. Activities are expressed as the amount of 7-amino-4-methylcoumarin released per 30 min.

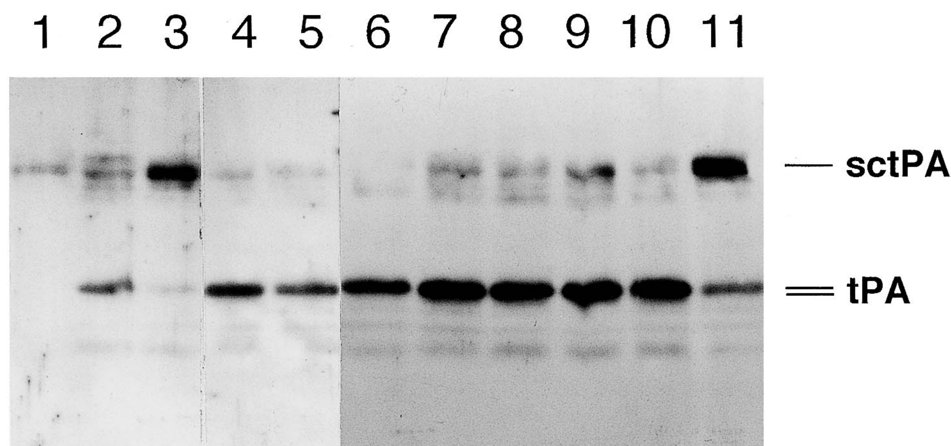

Fig. 7. Effects of various inhibitors on sctPA-activating activity. Human sctPA (0.2 μg) was incubated at 37°C with the α_2-macroglobulin fraction in 16 μl in 0.1 M Tris-HCl buffer (pH 8.0) in the presence of inhibitors. After 3 hr of incubation, the samples were mixed with 4 μl of 5 × SDS sample solvent, boiled, and electrophoresed in SDS under reducing conditions. Blotted sctPA and tPA were visualized as described in MATERIALS AND METHODS. Lane 1, sctPA only; lane 2, without inhibitors; lane 3, aprotinin (0.4 mg/ml); lane 4, antipain (40 μM); lane 5, benzamidine (2 mM); lane 6, PMSF (2 mM); lane 7, TLCK (0.4 mM); lane 8, TPCK (0.4 mM); lane 9, PCMB (0.4 mM); lane 10, E-64 (0.2 mM); and lane 11, DFP (4 mM). Note that aprotinin and DFP strongly inhibit tPA production.

case, the conversion should have proceeded irrespective of whether or not aprotinin, which has no effect on tPA, was included in the reaction.

We initiated the present study following the unexpected observation that the α_2-macroglobulin fraction obtained from the follicular fluid of human ovaries showed substantial proteolytic activity on the protein substrate sctPA. Because our attempt to dissociate and isolate the responsible enzyme from the protease/α_2-macroglobulin complex was unsuccessful, information regarding the protease is at present very limited. We previously reported (Ohnishi *et al.*, 1997) that free plasma kallikrein and the α_2-macroglobulin fraction isolated from the human ovarian follicular fluid showed essentially the same substrate specificity when tested using various MCA substrates. These observations, together with the present finding that the bound plasma kallikrein of the α_2-macroglobulin fraction is not responsible for the activation of sctPA, suggest that the sctPA-activating enzyme perhaps exhibits diminished

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enzyme activity toward MCA substrates. However, further studies are necessary to know more about the enzyme itself. Furthermore, the mechanism of tPA production by the enzyme/ α_2-macroglobulin complex as well as the biological meaning of its occurrence in human ovarian follicular fluid ramain to be elucidated. Nevertheless, this is the first demonstration that a protease complexed with α_2-macroglobulin exhibits detectable proteolytic activity toward sctPA.

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