Purification and Characterization of a Plasminogen Activator Inhibitor from the Histiocytic Lymphoma Cell Line U-937*

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We report the production, purification, characterization, and partial amino acid sequence of a plasminogen activator (PA-I). The starting material is culture fluid from phorbol myristate 13-acetate-treated U-937 cells and the isolation steps consist of preparative isoelectric focusing followed by affinity chromatography on Cibacron Blue-Sepharose. PA-I migrates as a closely spaced doublet of 47-kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and forms covalent complexes with urokinase and two-chain tissue-type plasminogen activator, displaying second order rate constants of $10^{-1}$ M$^{-1}$ s$^{-1}$, respectively. Upon treatment with 1 M NH$_4$OH, the covalent complexes were hydrolyzed, yielding a 35-kDa inhibitor fragment. A partial amino acid sequence of PA-I showed that it belongs to the antithrombin III family of inhibitors. PA-I is immunologically related to a PA-inhibitor from human placenta. mRNA from phorbol myristate 13-acetate-treated U-937 cells directed, in a rabbit reticulocyte derived cell-free system, the biosynthesis of only one 47-kDa protein that could be immunoprecipitated with anti-PA-I IgG, indicating that the two molecular forms of PA-I are the products of post-translational processing.

Plasminogen activators (PA') convert the zymogen plasmin into the protease plasmin (EC 3.4.21.7) and can thus generate systemic or localized proteolysis. They participate in several important physiological reactions such as fibrinolysis (1), inflammation (2) or malignant invasion, and metastasis (3, 4). For a coordinate action of PA's a precise regulation of their activity in space and time is required. In principle, regulation is possible on the level of transcription, mRNA processing, translation, and secretion of PAs but also through the production of specific inhibitors. As it turns out, some early reports on the hormonal regulation of PA biosynthesis actually dealt with hormonal regulation of PA-inhibitor secretion (5, 6).

PA-inhibitors are found in many tissues and have been described in the conditioned medium of endothelial cells (7, 8), fibroblasts (9, 10), hepatoma cells (11), leukocytes (12-15), in human plasma (16-19) and in extracts of human platelets (20, 21), human placenta (22), or human carcinomas (23). The relationship between these PA-inhibitors is still unclear. At present at least three different PA-inhibitors have been purified to homogeneity: protease-nexin (24), a PA-inhibitor from bovine endothelial cells (25), and one from human placenta (26).

Recently Vassalli et al. (14) reported that human monocytes, macrophages, and the human monocye-like histiocytic lymphoma cell line U-937 secrete a PA-inhibitor, which is different from protease-nexin (14). The following report describes the production and the purification of this inhibitor, as well as a partial primary sequence and a kinetic analysis of the reaction of PA-I with several serine proteases to determine the specificity of PA-I.

EXPERIMENTAL PROCEDURES

Materials—Uttrogal and the Amphiolines, pH 3.5-10, 4-6, and 5-7, were obtained from LKB, Bromma, Sweden; albumin (bovine fraction V) from Miles, Elkhart, IN; Na$^{+}$(180,000 units/mg) was kindly provided by Dr. Alzawa, Mochida Pharmaceuticals, Tokyo, Japan. The molar concentration of the u-urokinase (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000) from Pharmacia, Uppsala, Sweden; L-pyro-glutamyl-Gly-Arg-p-nitroanilide (Pyr-Gly-Arg-pNA) from Bachem, Bubendorf, Switzerland; H-D-Phe-Pip-Arg-pNA (S2238), H-Ile-Pro-Arg-pNA (S-22881, and H-o-Pro-Phe-Arg-pNA (S2302) from Kabi Diagnostics, Stockholm, Sweden; p-nitrophenyl p' guanidinobenzoate (NPGB) from Sigma; thrombin (Topostasin) and heparin (Liquemin) from Hoffmann-La Roche. The World Health Organization International Reference Preparation of human t-PA (NIBSC 83/517) was provided by Dr. P. J. Gaffney from the National Institute for Biological Standards and Control, London, United Kingdom. The buffer used for all experiments unless stated otherwise was 50 mM Tris-HCl, 0.1% Tween 80, 0.05% NaN$_3$, pH 7.4.

Protein determinations were done according to the method of Bradford (27).

Plasminogen Activators—Pure high $M_r$ urokinase (HM$_r$-u-PA, specific activity 100,000 international units/mg) was a gift of Dr. Sauser, Serono Laboratories, Cosins, Switzerland, and pure low $M_r$-u-PA (180,000 units/mg) was kindly provided by Dr. Aizawa, Mochida Pharmaceuticals, Tokyo, Japan. The molar concentration of the u-
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PA solutions used in the kinetic experiments was determined by active site titration using NPGB (28). HM-u-PA was radiolabeled using IODO-GEN (29) as previously described (17). Human t-PA was purified from Bowes melanoma cells as previously described (30); it was more than 90% pure. It was converted to two-chain (tc) t-PA by treatment for 90 min at 37 °C of 4 μg/ml of t-PA with 40 ng/ml of plasminogen activator inhibitor (PAI) t-PA. A specific monoclonal antibodies was a gift of Dr. M. Rånby (Biopool, Umeå, Sweden). Attempts to measure the molar concentration of t-PA solutions by active site titration were unsuccessful; therefore molar concentrations of t-PA were estimated from activity comparisons with the International Reference Preparation of t-PA (1 IU is equivalent to 2 ng (0.03 pmol) of t-PA).

Other Enzymes—Glandular kallikrein from porcine pancreas (Pudatin, 1180 IU/mg) was obtained from Bayer Leverkusen, Federal Republic of Germany. Plasma kallikrein (its specific activity on the chromogenic substrate Pro-Phe-Arg-pNA was 35 μmol of substrate converted at 25°C/min/mg of kallikrein) was purified as described elsewhere (31). The molar concentration of the plasma and glandular glandular kallikreins preparations were estimated from their activity on the chromogenic substrates Pro-Phe-Arg-pNA and Val-Leu-Arg-pNA. Glu-plasminogen was prepared by affinity chromatography on lysine-Sepharose. Trypsin was characterized as described before (33). It was converted into plasmin by incubation with urokinase immobilized to CNBr-activated Sepharose. The molar concentration of the plasmin preparation was determined by active site titration using NPGB (27). Thrombin was further purified by affinity chromatography on heparin-Sepharose (34) and had a specific activity of 500 units/mg. The molar concentration was estimated using the value of 2000 units/mg reported for highly purified thrombin (34).

PA-Inhibitor from Human Placenta—A PA-inhibitor was partially purified from human placenta essentially as described by Kawano et al. (22) up to the ion exchange step where CM-Affi-Gel Blue (Bio-Rad) was used instead of CM-Sepharose. The preparation had a specific activity of about 50 units/mg.

Radioiodination of PA-Inhibitor—Purified PA-I was labeled with 125I using IODO-GEN (29) to a specific radioactivity of 20 μCi/μg (2 molecules of 125I/molecule of PA-I). Radioiodinated inhibitor was separated from iodide by gel filtration on a 12-ml column of Sephadex G-25.

Cell Culture—U-937 cells were grown in suspension culture at 37 °C in a 5% CO2, 95% air mixture in RPMI 1640 (Biochrom, West Berlin, Germany) containing 5% fetal bovine serum (GIBCO, Paisley, United Kingdom) in 150-cm2 tissue culture flasks (Corning Glass Works, Corning, NY). When the culture reached a density of about 2 × 106 cells/ml, the cells were centrifuged (10 min, 200 g, 20 °C), washed twice with phosphate-buffered saline, and suspended in RPMI 1640 containing 30 ng/ml of phosphol 12-myristate 13-acetate (PMA) (P-L Biochemicals). Fifty ml of U-937 cell suspension (cell density: 2 × 106/ml) was added to each 150-cm2 tissue culture flask and incubated at the conditions described above. Upon stimulation by PMA the U-937 cells attached quantitatively to the culture flask.

Antibodies—Antisera against PA-I were raised in rabbits after 3-4 monthly intradermal injections of 100 μg of pure antigen, mixed with 0.1% SDS and Freund’s adjuvant. The antisera inhibited PA-I activity and double immunodiffusion of antiserum against various PA-I-Sepharose, prepared by coupling 1 mg of pure PA-I to 1 ml of CNBr-activated Sepharose. Immunopurified anti-PA-I IgG was prepared by passage of 5 mg of total IgG over a 1-ml column of PA-I-Sepharose, prepared by coupling 1 mg of pure PA-I to 1 ml of CNBr-activated Sepharose.

Amino Acid Sequence Analysis—PA-I (300 μg/ml), in 6 μg guanidine HCl, 0.15 M NaCl, pH 7.35 (modification buffer), was reduced with 140 μM 2-mercaptoethanol at 100 °C for 1 min and alkylated with 160 μM Na iodoacetate for 30 min at 23 °C in the dark, under N2. After dialysis against 5 M guanidine HCl, 0.15 M NaCl, pH 7.35 (active site titration buffer), the inhibitor was radioiodinated were visualized by SDS-PAGE (30) and autoradiography as described before (17).

Visualization of Complexes of PA-Inhibitor with u-PA and t-PA—Enzyme-inhibitor complexes, in which either the enzyme or the inhibitor was radioiodinated were visualized by SDS-PAGE (30) and autoradiography as described before (17).

PA + PA-I → k1 Cm + k2 Cc

in which Cm and Cc are, respectively, the reversible and irreversible enzyme-inhibitor complexes. The kinetic parameters k1, k2, and k3 were estimated by nonlinear least square regression analysis, using rate equations, derived from the general inhibition mechanism: the first describing the formation of a reversible complex from free PA and PA-I.

Using these equations simulated inhibition curves were constructed by calculating the changes in PA, PA-I, and reversible complex concentration for each small incremental incubation period. Sets of values for k1, k2, and k3 were taken and the sums of squares of differences between the experimentally determined and the calculated percentages of free enzyme determined. By iterating this procedure for a great number of values for k1, k2, and k3, contour maps were constructed (38) and those values of the kinetic constants determined that led to the least sum of squares. We also calculated the least sum of squares for inhibition mechanisms in which an irreversible complex is formed without the formation of a reversible intermediate complex (i.e. k1 = 0) or in which only a reversible complex is formed (i.e. k2 = 0).

Inhibition of Glandular Kallikrein, Plasma Kallikrein, Plasma, and Thrombin by PA-Inhibitor—Several serine proteases (concentration: 10 nM) were incubated up to 2 h at 25 °C with 476 nM of PA-I. At various time intervals remaining enzyme activity on chromogenic substrates was estimated from the initial rate of t-PA increase. The following enzymes and chromogenic substrates were used: glandular kallikrein (activity measured using 0.2 mM Pro-Phe-Arg-pNA), plasma kallikrein (0.4 mM Pro-Phe-Arg-pNA), plasmin (0.7 mM Val-Leu-Lys-pNA), and thrombin (0.7 mM Val-Leu-Lys-pNA), the latter in the presence or absence of 25 units/ml heparin.

Antibodies—Antisera against PA-I were raised in rabbits after 3-4 monthly intradermal injections of 100 μg of pure antigen, mixed with 0.1% SDS and Freund’s adjuvant. The antisera inhibited PA-I activity and double immunodiffusion of antiserum against various amounts of PA-I indicated an antibody titer of 0.15 μg/ml. The antiserum precipitated only one 47-kDa protein from an in vitro translation mixture of stimulated U-937 mRNA (Fig. 2). Total IgG was precipitated from antiserum using 0.9 M (0.4 mM Pro-Phe-Arg-pNA), plasma kallikrein (0.4 mM Pro-Phe-Arg-pNA), plasmin (0.7 mM Val-Leu-Lys-pNA), and thrombin (0.7 mM Val-Leu-Lys-pNA), the latter in the presence or absence of 25 units/ml heparin.

Amino Acid Sequence Analysis—PA-I (300 μg/ml), in 6 μg guanidine HCl, 0.15 M NaCl, pH 7.35 (modification buffer), was reduced with 140 μM 2-mercaptoethanol at 100 °C for 1 min and alkylated with 160 μM Na iodoacetate for 30 min at 23 °C in the dark, under N2. After dialysis against 5 M guanidine HCl, 0.15 M NaCl, pH 7.35 (active site titration buffer), the inhibitor was radioiodinated were visualized by SDS-PAGE (30) and autoradiography as described before (17).

Determination of the Kinetic Parameters of the Inhibition of u-PA or t-PA by PA-Inhibitor—The inhibitor, titrated as described above, was incubated with HM-u-PA, LM-u-PA, or t-PA at the concentrations and incubation times indicated under "Results." At the end of the incubation period chromogenic substrate was added (0.6 mM Leu-Pro-Arg-pNA for t-PA) and remaining enzyme activity calculated from the initial rate of absorbancy change at 405 nm in a Cary 219 spectrophotometer. For the calculation of the kinetic parameters the inhibition reaction was assumed to follow the general mechanism of inhibition of serine proteases by their inhibitors (36,37):

\[ d[PA-I]/dt = d[PA]/dt = -k_1[PA][PA-I] + k_2[PA] \]

\[ d[PA]/dt = d[PA-I]/dt = -k_1[PA][PA-I] + k_2[PA][PA] \]

The second describing the capability of the reversible complex to dissociate or to transform itself into an irreversible complex.

\[ d[PA]/dt = d[PA-I]/dt = -k_1[PA][PA-I] + (k_1 + k_2)[PA] \]

Using these equations simulated inhibition curves were constructed by calculating the changes in PA, PA-I, and reversible complex concentration for each small incremental incubation period. Sets of values for k1, k2, and k3 were taken and the sums of squares of differences between the experimentally determined and the calculated percentages of free enzyme determined. By iterating this procedure for a great number of values for k1, k2, and k3, contour maps were constructed (38) and those values of the kinetic constants determined that led to the least sum of squares. We also calculated the least sum of squares for inhibition mechanisms in which an irreversible complex is formed without the formation of a reversible intermediate complex (i.e. k1 = 0) or in which only a reversible complex is formed (i.e. k2 = 0).

\[ d[PA]/dt = d[PA-I]/dt = -k_1[PA][PA-I] + (k_1 + k_2)[PA] \]
 injected onto a reverse phase high pressure liquid chromatography column ($C_{18}$, Spectra Physics, 4.6 x 250 mm) equilibrated with 0.1% trifluoroacetic acid at a flow rate of 1.4 ml/min at 40 °C. Bound components were eluted with a 95-5 min gradient of 0-75% acetonitrile in 0.1% trifluoroacetic acid, 200 half-min fractions were collected and stored at −20 °C.

Individual tryptic peptides of carboxymethylcysvinyl-PA-I (500-750 pmol) were subjected to protein microsequence analysis by sequential automated Edman degradation in an Applied Biosystems 470A gas-phase sequencer (40), in the presence of 3'-Omethane. Prior to loading 50 μg of SDS was added to samples prepared by high pressure liquid chromatography as described, the volume reduced to about 100 μl in a SpeedVac (Savant), and the samples boiled for 2 min. Identification of the phenylthiohydantoin-derivative from each cycle was performed using two independent reverse phase high pressure liquid chromatography separation methods on 5-μm cyanophe column (46 x 250 mm, IBM Instruments, Wallingford, CT) (41, 42). The column effluent was monitored spectrophotometrically at both 283 and 335 nm.

**Immunoblotting (43)—** After SDS-PAGE the proteins were transferred to nitrocellulose by overnight electrophoresis at 30 V in 0.02 M Tris, 0.15 M glycine, pH 8.8, containing 17% methanol. To facilitate transfer of the proteins, a filter paper soaked in 1% SDS was placed on the cathodic side of the gel. The nitrocellulose was washed briefly with water and incubated for 2 x 15 min in 40 ml of BLOTTO (44), overnight in 40 ml of BLOTTO containing 10 μg/ml of immunopurified rabbit anti-PA-I IgG and 4 h at room temperature with BLOTTO containing goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad; used at a dilution of 1/200). Peroxidase staining was done with the staining reagent of Bio-Rad according to the instructions of the manufacturer.

**In Vitro Translation—** mRNA from PMA-stimulated U-937 cells was prepared by a combination of SBS/proteinase K digestion, phenol extraction, and hybridization to oligo(dT)-cellulose as will be described in detail elsewhere and translated in the reticulocyte lysate system of Amersham (Amersham, United Kingdom), using [35S]methionine as a label. A one-hundred-μl translation mixture was added to 10 μl of anti-PA inhibitor IgG (0.2 mg/ml), incubated overnight at 4 °C, and mixed with 100 μl of a 25% suspension of cellulose coated with donkey anti-rabbit-IgG antibodies (SeccoCell, Welcome, Beckamonds, United Kingdom). After 30 min at 37 °C the cellulose was pelleted by centrifugation and washed three times with 1.5 ml of 40 mM Tris, 120 mM NaCl, 5 mM EDTA, 1% Triton X-100, and subsequently boiled in 50 μl of SDS-PAGE sample buffer, containing 10 mM dithiothreitol. As a control, rabbit preimmune IgG was used in the first incubation. The samples were subjected to SDS-PAGE in a 10% gel and, after treatment with a scintillation mixture (EN'HANCE, New England Nuclear), to autoradiography.

**RESULTS**

**Purification of PA-Inhibitor—** The level of PA-I in the conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 units/ml). Six liters of conditioned medium of U-937 cells grown in the presence of 30 ng/ml PMA was 150–400 units/ml (in the absence of PMA, PA-I levels never exceeded 20 ..
Purification of a Plasminogen Activator Inhibitor

**Summary table of the purification of u-PA-inhibitor from the conditioned medium of PMA-stimulated U-937 cells**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
<th>Recovery</th>
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</thead>
<tbody>
<tr>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Conditioned medium*</td>
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<td>350</td>
<td>640,000</td>
<td>1,800</td>
<td>1</td>
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<tr>
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<td>5.4</td>
<td>184,000*</td>
<td>34,000</td>
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<td>2.0</td>
<td>126,000</td>
<td>63,000</td>
<td>35</td>
</tr>
</tbody>
</table>

*The values given are those after concentration by ultrafiltration and ammonium sulfate precipitation.

Approximately 95,000 units were associated with the pl 4.4 peak (not included), and an additional 90,000 units contained in fractions on both sides of the main peak of pl 5.0 were not included in the pool.

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**Complex Formation of PA-I with u-PA and t-PA**—To study complex formation of PA-I, it was radiiodinated and incubated (1 h, 37°C) with HM-u-PA or LM-u-PA (final concentrations 1 μg/ml), respectively, with 10 μg/ml of two-chain t-PA. After incubation with HM-, t-PA activity losses were less than 10%. We may therefore assume that possible trace amounts of t-PA did not influence the inhibition curves. With 320 and 700 nM of PA-I, activity was progressively inhibited and linear semilogarithmic plots of activity versus incubation time were obtained (Fig. 6). From activity half-lives (41 and 18 min, respectively) the second order rate constant of the reaction was calculated to be 0.9 × 10^8 M^{-1} s^{-1}, i.e. at least 2 orders of magnitude lower than that of t-PA. However, the curves did not allow the calculation of k_1 and k_2 values.

**Inhibition of Other Serine Proteases by PA-I**—The activity of 10 nM of glandular or plasma kallikrein or of thrombin (in the presence or absence of 25 units/ml heparin) was not significantly inhibited within 2 h by 476 nM of PA-I. Plasmin was slowly inhibited by 476 nM of PA-I, but activity losses were only 40% in 2 h, indicating a second order rate constant in the order of 10^9 M^{-1} s^{-1}.
Percentage of added u-PA activity. The data points give the mean deduced from the initial rate of A405 change and expressed in percentage.

43,000, k-., 0.6 mM Pyr-Gly-Arg-pNA was added and remaining u-PA activity HM,-u-PA were subjected to SDS-PAGE in a 10% gel, followed by autoradiography. The position of the single chain and double chain t-PA with radiolabeled PA-I.

Kinetics of the inhibition of HM,-u-PA by PA-I. HM,-u-PA (9.7 nM final concentration) was incubated with PA-I (final concentration of active inhibitor: 22.5 nM). At different times, 0.6 mM Pyr-Gly-Arg-pNA was added and remaining u-PA activity deduced from the initial rate of A405 change and expressed in percentage of added u-PA activity. The data points give the mean ± S.D. (n = 4). The drawn curves were calculated using those values of k1, k-1, and k2 that led to minimal least squares of differences.

Discussion

PA-I purified from the conditioned medium of U-937 cells has a PI of 5.0 and migrates in SDS-PAGE as a closely spaced doublet of 47 kDa; each species reacting equally well with u-PA. Since the in vitro translation product of U-937 mRNA consisted of a single band after immunoprecipitation with anti-PA-I antibodies, the doublet is probably the result of limited proteolysis or another post-translational modification. The high degree of homology of a trypic fragment of PA-I with several other inhibitors of serine protease and with ovalbumin indicates that it belongs to the serine protease inhibitor family of proteins (45-48). In region 329-353 over 80% of the positions identical or structurally similar amino acids were found. This high degree of conservation suggests that the functional activity of the serine protease inhibitors requires a special structure at this region, which does not allow much deviation from the optimal amino acid sequence.

In support for this notion rontgen diffraction analysis of α1-protease inhibitor suggested that this region, just before the active site, constitutes a strained loop, which serves to expose the active site amino acid (49, 50).

A very striking feature of the sequence comparisons was the homology of the PA-I fragment with the corresponding region of barley protein Z was as great as that with the antithrombin III and contrapsin but greater than with antichymotrypsin and α1-protease inhibitor. This observation suggests that the structural constraints of this region are such that it has been conserved without much alteration for over a billion years since the time the animal kingdom and the plant kingdom separated. An alternative, although at present highly speculative explanation, might be that the ancestor gene for protein Z was acquired by plants much later by horizontal gene transfer after the divergence of some of the members of the serine protease inhibitor family.

PA-I forms complexes with u-PA and t-PA at high rates. The molecular mass of these complexes is 15 kDa smaller than would be expected from the sum of the molecular weights of the starting products. This is the result of a cleavage, which converted PA-I into a 35-kDa form. In this respect, PA-I reacts according to the classical mechanism of serine protease inhibitor reactions, part of which involves cleavage of the inhibitor (37).

Our study demonstrates that PA-I is a fast and specific inhibitor of u-PA and to a lesser degree of tc t-PA. Nonlinear least squares analysis of the inhibition curves of u-PA and tc t-PA suggests a standard inhibition mechanism (37) in which formation of a reversible complex is followed by conversion into an irreversible complex. The second order rate constant of the reaction of u-PA by PA-I was determined using PA-I that had been titrated with an active site titrated u-PA solution. The value of 0.9 × 10^8 m^-1 s^-1 is typical for specific inhibitors of serine proteases (36) and is about 5 times higher than that reported for protease-nexin (51), but 1 order of magnitude smaller than the one of a PA-inhibitor in human plasma (52). Previously, Christensen et al. (53) studied the reaction of LM,-u-PA with a partially purified PA-inhibitor from human placenta. The second order rate constant of the reaction (3 × 10^6 m^-1 s^-1) is quite close to our value. The dissociation constant of the reversible complex of u-PA with...
The PA-I we calculated (4 nM) is considerably higher than the value of about 0.01 nM estimated by Christensen et al. (53). These authors have, however, incubated enzyme and inhibitor for 1 h. Under these conditions the conversion of reversible into irreversible complex leads to a continuous shift in equilibrium, which results in a considerable decrease of free enzyme concentration. This would explain why the dissociation constant calculated by these authors is lower than that found by us.

LMu and HMu-PA are inhibited by PA-I at a similar rate. The kringle in the light chain of HMu-PA (54) thus does not seem to contribute to the inhibition reaction in contrast to the kringleS of plasmin, which accelerate the inhibition of plasmin by α2-antiplasmin (55).

The second order rate constant of the reaction of PA-I with tc t-PA was four times lower than with u-PA. Furthermore, the dissociation constant of the reversible complex of t-PA with PA-I was 20 times higher. U-937 derived PA-I is therefore a better inhibitor of u-PA than of tc t-PA.

PA-I is a relatively poor inhibitor of sc t-PA. Its rate of inhibition of sc t-PA was 2 orders of magnitude lower than that of tc t-PA. A similar although less striking difference in reactivity of sc t-PA and tc t-PA has been observed previously for the inhibition of t-PA by several other protease inhibitors (51, 56-58).

PA-I is different from the PA-inhibitor purified from endothelial cells (EC-PA-I) in the following respects: PA-I is a fully active inhibitor, whereas, EC-PA-I is a latent inhibitor which is activated by SDS (59); PA-I has a Mr of 47,000 and EC-PA-I of 52,000; EC-PA-I binds to ConA-Sepharose, whereas PA-I does not; the PA-inhibitor found in human plasma and platelet extracts bound to immobilized antibodies to EC-PA-I (60), whereas PA-I did not.

In contrast to protease-nexin, PA-I does not react with thrombin, not even in the presence of heparin. This, in combination with other results such as the lack of affinity of PA-I to heparin-Sepharose, the difference in pl (24) and the difference in inhibition kinetics clearly demonstrates that PA-I and protease-nexin are different proteins. PA-I is an inefficient inhibitor of plasmin. The inhibition rate constant is 5 orders of magnitude lower than that of α2-antiplasmin (55). It is therefore unlikely that PA-I contributes to the inhibition of plasmin. Glandular and plasma kallikrein were not inhibited by PA-I.

The immunological relationship of PA-I to a PA-inhibitor partially purified from human placenta is shown by (a) the line of complete identity upon double immunodiffusion, and (b) the identical Mr of the placental PA-inhibitor and the smaller form of the PA-I doublet at 47 kDa. Since the in vitro translation showed only one band of PA-I it is possible that the smaller of the two forms is generated by proteolytic degradation of PA-I, which in conditioned media was only partial and in placenta extracts complete. The similar kinetics of the inhibition of u-PA by PA-I and a partially purified PA-inhibitor from human placenta (53) provides further evidence for the identity of these two PA-inhibitors. At present it is not yet established if PA-inhibitor is identical to the one purified by Åstedt et al. (26), but even if future research should provide hard evidence as to their identity, conditioned medium of U-937 cells appears to be superior to human placenta as a source of PA-I, because the latter contains 1000-fold more contaminating proteins. Purification of PA-I from placenta extracts is difficult. Despite the use of monoclonal anti-placental PA-inhibitor antibodies, a recently published purification procedure consisted of six steps and the final recovery was less than 3% (26). In contrast, our two-step procedure has a good yield and does not require the use of not generally available monoclonal antibodies.

The production and purification procedure for PA-I outlined here will allow its comparison with PA-inhibitors from other sources and aid in the study of their function. Our results, notably the high rates of inhibition of PAs and the lack of significant interaction with other proteases, suggest that the primary role of PA-I is to regulate the activity of u-PA and to a lesser extent tc t-PA.

**Note Added in Proof**—At the 32nd annual meeting of the International Committee on Thrombosis and Haemostasis, the name PA-inhibitor 2 was assigned to the PA-inhibitor purified from U-937 cells and from placenta, whereas the name PA-inhibitor 1 was assigned to the PA-inhibitor from endothelial cells.

**REFERENCES**

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