Purification and Characterization of a Plasminogen Activator Inhibitor 1 Binding Protein from Human Plasma

IDENTIFICATION AS A MULTIMERIC FORM OF S PROTEIN (VITRONECTIN)*

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A binding protein for plasminogen activator inhibitor 1 (PAI-1-BP) was isolated from human plasma by a four-step procedure. 1) The 7 S globulin fraction of plasma was isolated by gel filtration on Sephacryl S-300. 2) Human endothelial cell-type plasminogen activator inhibitor (PAI-1), pretreated with 12 m urea, was added to this fraction (22 μg of PAI-1/ml of plasma), and a PAI-1 antigen peak with apparent mass 450 kDa (representing 65% of PAI-1 antigen and 85% of PAI activity) was isolated by gel filtration of this mixture. 3) The PAI-1-PAI-1-BP complex was further purified by immunoadsorption on an immobilized murine monoclonal antibody directed against PAI-1 (MA-7D4) and by elution with 4 M KSCN. 4) The complex was then dissociated by addition of excess human tissue-type plasminogen activator (t-PA), and t-PA and PAI-1 antigen (t-PA-PAI-1 complexes and free t-PA and PAI-1) were removed by immunoadsorption on monoclonal antibodies directed against t-PA (MA-62E8) and against PAI-1 (MA-7D4 and MA-12A4). Sodium dodecyl sulfate-gel electrophoresis of the purified material under nonreducing conditions revealed two bands with apparent mass ≈ 150 kDa and two bands with mass 74 and 68 kDa. Reduced sodium dodecyl sulfate-gel electrophoresis displayed two main bands with apparent masses of 73 and 64 kDa. The PAI-1-BP reacts with urea-treated, but not with inactive PAI-1. t-PA dissociates the complex between PAI-1 and PAI-1-BP. PAI-1 in complex with PAI-1-BP is 2–3-fold more stable at 37°C than purified PAI-1, suggesting that PAI-1-BP may stabilize PAI-1 in blood. The concentration of PAI-1-BP in plasma determined by titration with PAI-1 is approximately 130 mg/liter. The isolated PAI-1-BP was shown to be identical to S protein (vitronectin) both by cross-reactivity with monospecific rabbit antiserum and by NH2-terminal amino acid sequence analysis. The gel filtration behavior, mobility on sodium dodecyl sulfate-gel electrophoresis, and concentration in plasma suggest that PAI-1-BP is a multimer (presumably a dimer) of S protein accounting for approximately 35% of the S protein in plasma.

Plasminogen activators are serine proteases which convert plasminogen, the proenzyme of the fibrinolytic system in blood, to plasmin which in turn digests fibrin (1). The activity of the plasminogen activators is controlled by plasminogen activator inhibitors (PAI). Three different types of PAI have been identified (2): the endothelial cell-type PAI (PAI-1), the placenta-type PAI (PAI-2) and the urinary-type PAI (PAI-3). PAI-1 appears to be the main physiological inhibitor of t-PA in normal plasma (3) and is increased in many clinical conditions including coronary artery disease, venous thrombosis, obesity, and the postoperative state (4–8). PAI-1 may occur in different molecular forms as revealed by gel filtration (9–11). The occurrence of a specific PAI-1 binding protein in plasma has recently been demonstrated (11). In the present study, we report the isolation and characterization of a PAI-1 binding protein (PAI-1-BP) from human plasma. This protein is identified as a multimer (dimer) of S protein (vitronectin) (13), an adhesive protein which also plays a role in the complement and coagulation systems (14–16).

** EXPERIMENTAL PROCEDURES

Materials

The following proteins were purified as previously described: plasminogen with NH2-terminal glutamic acid (Glu-plasminogen) (17), fibrinogen (17), α2-antiplasmin (18), and two-chain t-PA (19). CNBr-digested fibrinogen was prepared as described by Verheijen et al. (20). The chromogenic substrate d-Val-Leu-Lys-p-NA (S-2251) was obtained from KabiVitrum, o-phenylene-diamine from Fluka, and the protein assay kit, using bovine serum albumin as standard, from BioRad. Murine monoclonal antibodies MA-7D4, MA-7F5, MA-12A4, and MA-15H12 raised against PAI-1 and MA-62E8 raised against t-PA were prepared according to Galfrè and Milstein (21) and are characterized elsewhere (22, 23). Zinc-chelate Sepharose, Sephacryl S-300, TBS, Tris-buffered saline; PBS, phosphate-buffered saline; MA-1-1, PAI-1 binding protein; MA-7D4, MA-7F5, MA-12A4, and MA-15H12 raised against PAI-1 and MA-62E8, murine monoclonal antibody raised against t-PA; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.

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1. The abbreviations used are: PAI, plasminogen activator inhibitor; PAI-1, endothelial cell-type PAI; PAI-2, placenta-type PAI; PAI-3, urinary PAI or activated Protein C inhibitor; t-PA, human tissue-type plasminogen activator; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; MA-1-1, PAI-1 binding protein; MA-7D4, MA-7F5, MA-12A4, and MA-15H12 raised against PAI-1; MA-62E8, murine monoclonal antibody raised against t-PA; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.
S-300, and CNBr-activated Sepharose 4B were purchased from Pharmacia LKB Biotechnology Inc. The International Reference Preparation of t-PA (86/670) was obtained from the National Institute for Biological Standards and Control (London). S protein was purified from human plasma as described previously (24) and was used to prepare monospecific rabbit antisera against S protein. t-PA was purified from conditioned medium of endothelial cells as described previously.2 Some of the experiments reported here, however, were performed with partially purified low molecular weight endothelial type PAI-1. This material was obtained by adsorption of conditioned human umbilical vein endothelial cell culture medium on zinc-chelated Sepharose and elution with 50 mM imidazole. The concentrated eluate was then directly applied to a Sephacryl S-300 column (5 × 85 cm) and the low mass peak of PAI-1 antigen was used.

**Methods**

**Assay of PAI Activity—**PAI activity was determined using the method described by Verheijen et al. (20). Briefly, 50-μl samples, diluted in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4, containing 0.01% Tween 80 (TBS-Tween) were incubated with 50 μl of increasing concentrations of t-PA (0–20 IU/ml) at 37 °C for 10 min in wells of a microtiter plate. Then 100 μl of a solution containing plasminogen (1 μM), CNBr-digested fibrinogen (1 μM), and S-2251 (0.26 mM) were added. One unit of PAI is defined by the amount of PAI neutralizing 1 IU of t-PA.

**Assay of PAI-1 Antigen and t-PA Antigen—**PAI-1 antigen and t-PA antigen were assayed with specific ELISAs based on murine monoclonal antibodies, as described elsewhere (22, 23). PAI-1 antigen was assayed in two ways. One combination, using MA-7D4 (facing) and MA-7F5 conjugated with horseradish peroxidase (tagging antibody) reacts about 10-fold better with free PAI-1 and PAI-1-1 BP complex than with the t-PA-PAI-1 complex. The other combination, using MA-12A4 for tagging, is equally sensitive to all three forms of PAI-1. The assay of t-PA antigen and PAI-1 antigen was performed on a microtiter plate. Then 100 μl of 7S globulin fraction from two gel filtration peaks, or isolated PAI-1 binding protein) was determined by adding urea-treated PAI-1 (250 units corresponding to 1.8 μg) to varying amounts of sample (0–200 μl) in a final volume of 250 μl. This mixture was incubated for 10 min at 37 °C and then applied to the Sephacryl S-300 column (5 × 100 cm). The 7S globulin fraction from two gel filtration peaks was pooled and concentrated to approximately 8 ml by ultrafiltration on an Amicon PM-30 membrane.

Partially purified low mass PAI-1 (containing 180 μg/ml PAI-1 antigen) was reactivated by incubation with 12 μl urea for 30 min at 37 °C, followed by overnight dialysis against PBS at 4 °C, which yielded PAI-1 with a specific activity of 100,000 units/mg PAI-1 antigen. The PAI-1 antigen was then labeled with 125I (0.01% Tween 80 (PBS-Tween), at a flow rate of 150 μl/h at 4 °C. Fractions of 10 ml were collected, and the absorbance at 250 nm was measured. The 7 S globulin fraction was pooled and concentrated to approximately 8 ml by ultrafiltration on an Amicon PM-30 membrane.

**Purification of a PAI-1 Binding Protein from Human Plasma—**Gel filtration of the mixture of the 7 S globulin fraction of plasma with urea-treated PAI-1 on Sephacryl S-300 revealed four peaks reacting in the PAI-1 antigen assay and three peaks of PAI activity (Fig. 3). The main peak with an apparent mass of 450,000, representing 65% of PAI-1 antigen and 85% of PAI activity, was pooled and concentrated 4-fold by ultrafiltration. This material was further purified by adsorption on an insolubilized anti-PAI-1 monoclonal antibody (MA-TD4) column and elution with 4 M KSCN. Gel filtration of this material (not shown) revealed that it consisted mainly of a 450-kDa component containing both PAI activity and PAI-1 antigen and of some inactive low mass PAI-1. This indicated that the presence of 4 M KSCN, and subsequent dialysis did not cause irreversible dissociation of the complex.

Addition of excess t-PA, however, resulted in a complete dissociation of the PAI-1-1 BP complex and a shift of PAI-1 antigen on gel filtration from an elution position with apparent mass 450 kDa to a position with apparent mass 120 kDa (not shown). t-PA and PAI-1 antigen were then removed from this mixture by immunoadsorption on immobilized monoclonal antibodies against t-PA (MA-62E8) and against...
PAI-1 Binding Protein

**FIG. 1.** Gel filtration on a 5 x 100-cm column of Sephacryl S-300 of the mixture of the 7 S globulin fraction (16 ml) of human plasma with urea-reactivated partially purified PAI-1 (1.4 mg). Fractions of 10 ml were collected. The conditions for gel filtration are described under "Methods." ○, absorbance at 280 nm; ▲, PAI-1 activity in units/ml; △, PAI-1-related antigen in μg/ml. The horizontal line indicates the fractions pooled for further purification.

**TABLE I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Material</th>
<th>Volume</th>
<th>PAI-1 binding capacity</th>
<th>Protein</th>
<th>PAI-1 binding capacity/mg protein</th>
<th>Purification factor</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>Plasma</td>
<td>64</td>
<td>4200 (± 1400)*</td>
<td>64</td>
<td>4000 (± 1700)*</td>
<td>1.6</td>
<td>1</td>
</tr>
<tr>
<td>Step 1</td>
<td>7 S globulin fraction</td>
<td>16</td>
<td>690 ± 150</td>
<td>1100 ± 150</td>
<td>2000 (± 400)*</td>
<td>5.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Step 2</td>
<td>Sephacryl S-300</td>
<td>16</td>
<td>690 ± 150</td>
<td>1100 ± 150</td>
<td>2000 (± 400)*</td>
<td>5.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Step 3</td>
<td>Immunoadsorption on MA-7D4 peak I</td>
<td>6</td>
<td>550 ± 25*</td>
<td>0.8 ± 0.2</td>
<td>690 ± 100</td>
<td>430 ± 62</td>
<td>13</td>
</tr>
<tr>
<td>Step 4</td>
<td>Isolated PAI-1-BP</td>
<td>6</td>
<td>210 ± 40*</td>
<td>0.49 ± 0.12</td>
<td>420 ± 94</td>
<td>270 ± 59</td>
<td>5</td>
</tr>
</tbody>
</table>

*The PAI-1 binding capacity is expressed as the amount of PAI-1 (μg) determined by ELISA which can be shifted toward an elution position with mass 450 kDa.

Calculated based on the amount of PAI-1 present in the complex.

Low yield due to incomplete saturation of PAI-1-BP.

PAI-1 (MA-7D4, MA-12A4). The purification procedure and recoveries are summarized in Table I.

**Characterization of the Purified PAI-1-BP**—SDS-gel electrophoresis under nondenaturing conditions (Fig. 2A) revealed several protein bands. The purified PAI-1-BP (lane 1) displayed three bands with mass ≥ 150 kDa, two bands with mass 74 and 68 kDa, and a faint band with mass 50 kDa. The 50-kDa band represented some residual PAI-1 which comigrated with the main PAI-1 band observed in the PAI-1-BP complex (lane 2). Purified S protein (24) (lane 3) contained the two main bands with mass 74 and 68 kDa and a faint large mass component. The 74- and 68-kDa bands and two of the large mass bands contained S protein as revealed by immunoblotting (Fig. 3A).

SDS-gel electrophoresis under reducing conditions (Fig. 2B) revealed two main bands of 73 and 64 kDa in the purified PAI-1-BP (lane 1), in the PAI-1-BP complex (lane 2), and in the purified S protein (lane 3), which were recognized by antibodies against S protein on immunoblotting (Fig. 3B). In addition, the purified PAI-1-BP and PAI-1-BP complex contained a triplet band at 38 kDa which did not cross-react with S protein antibodies and which were not present in the purified S protein preparation.

In order to exclude the possibility that the S protein present in purified PAI-1-BP was a copurified contaminant, the PAI-1-BP was recycled. Therefore an excess of urea-treated highly purified PAI-1 (24,000 units or 180 μg) was added to the purified PAI-1-BP (138 μg), the mixture was incubated at 37 °C for 10 min, the PAI-1-BP was then isolated by immunoabsorption, the complex was dissociated by addition of t-PA, and subsequently PAI-1-BP was purified from this mixture as described above. During this procedure the large mass minor contaminant which was not reactive on immunoblotting was removed, as well as a small amount monomeric S protein (Fig. 4). The recycling procedure had a recovery of 65% of protein and 75% of PAI-1 binding capacity. This final
PAI-1 Binding Protein

PAI-1-BP had a binding capacity of 0.5 mg of PAI-1/mg of PAI-1-BP.

The purified PAI-1-BP was analyzed by double immunodiffusion (Fig. 5). Antiserum against S protein formed precipitin lines with PAI-1-BP, PAI-1·PAI-1-BP complex, and S protein (Fig. 5). The complete fusion is indicative of immunochenical identity. NH₂-terminal amino acid sequence analysis of purified PAI-1-BP gave results shown in Table II. The known NH₂-terminal amino acid sequence of S protein (30) was unequivocally identified. The amino acid composition of the purified PAI-1-BP is shown in Table III and compared with the known amino acid composition of S protein (14, 15, 24).

Cross-immunoelectrophoresis (Fig. 6) using a mixture of murine monoclonal antibodies against PAI-1 in the second dimension showed that PAI-1 has a low electrophoretic mobility (panel A), while PAI-1·PAI-1-BP has a much higher electrophoretic mobility (panel B). Cross-immunoelectrophoresis of the purified PAI-1-BP with monospecific antiserum against S protein (panel C) shows that PAI-1-BP has a high electrophoretic mobility.

Influence of PAI-1-BP on PAI-1 Stability and Inhibition Kinetics—The stability of PAI-1 was studied at 0 and at 37 °C. Both free PAI-1 and PAI-1·PAI-1-BP complex (purified by immunoabsorption on MA-7D4) were stable at 0 °C for the 7-h observation period. At 37 °C, PAI-1 is spontaneously inactivated. The residual activity versus time yielded a straight line on a semilogarithmic plot (Fig. 7). The complex with the PAI-1-BP appeared to be 2.5-fold more stable than the purified PAI-1 (t½ 180 and 75 min, respectively). Adding a 10-fold molar excess of PAI-1-BP to the PAI-1·PAI-1-BP complex yielded similar values as the PAI-1·PAI-1-BP complex (t½ ~ 180 min).

Second-order rate constants of the inhibition of t-PA by PAI-1 and by the PAI-1·PAI-1-BP complex (purified by immunoabsorption on MA-7D4), determined under pseudo first-order conditions are represented in Table IV. The con-
Fig. 4. SDS-gel electrophoresis of PAI-1-BP before and after recycling on the immunoadsorption column. Lane 1, urea-treated highly purified PAI-1 used for the recycling procedure; lane 2, PAI-1-BP; lane 3, PAI-1-BP + urea-treated highly purified PAI-1; lane 4, unadsorbed fraction during immunoadsorption of the PAI-1-PAI-1-BP complex on anti-PAI-1 antibody (MA-7D4); lane 5, KSCN eluate of the immunoadsorption column; lane 6, final product after addition of t-PA to the PAI-1.PAI-1-BP complex and immunoadsorption on anti-t-PA and anti-PAI-1 antibodies (MA-62E8, MA-7D4, and MA-12A4); lane 7, purified S protein; lane 8, protein calibration mixture as in Fig. 2.

Fig. 5. Double immunodiffusion against a monospecific rabbit antiserum raised against S protein (a, 15 μl) and against a mixture of monoclonal antibodies (100 μg/ml) directed toward PAI-1 (b, 15 μl). 1, PAI-1-BP (0.6 μg); 2, S protein (0.6 μg); 3, PAI-1-PAI-1-BP complex (0.6 μg); 4, PAI-1 (0.6 μg).

<table>
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<tr>
<th>NH₂-terminal amino acid sequence of PAI-1-BP and S protein</th>
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<td>Amino acid</td>
<td>1</td>
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<tr>
<td>Expected*</td>
<td>Asp</td>
</tr>
<tr>
<td>Found*</td>
<td>Asp</td>
</tr>
<tr>
<td>Yield*</td>
<td>166</td>
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</table>

* Expected amino acid as published by Suzuki et al. (30).

* Yields obtained at each cycle are expressed in picomoles and were obtained from 163 pmol of starting material. ND, not determined.

**Table II**

**Amino acid composition of PAI-1-BP and S protein (14, 15, 24)**

The number of residues was normalized to a total of 684 (as described previously) (15).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>PAI-1-BP</th>
<th>S protein (14)</th>
<th>S protein (15)</th>
<th>S protein (24)</th>
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<tr>
<td>Asx</td>
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<td>Arg</td>
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</tr>
</tbody>
</table>

**Table III**

**Interaction of PAI-1 with PAI-1-BP and with S Protein**

Complex formation of PAI-1 with purified PAI-1-BP or human S protein was investigated by gel filtration on Sephacryl S-300 after preincubation for 10 min at 37°C (Fig. 8). Purified PAI-1 eluted as a single peak with apparent mass 30 kDa both before (panel A) and after (panel B) reactivation with urea. PAI-1-BP did not form large mass complexes with nonreactivated PAI-1 (panel C) but reacted with urea-reactivated PAI-1 to form primarily the 450-kDa peak but also some 65-kDa material (panel D). When purified S protein, essentially consisting of the 74- and 68-kDa components on SDS-gel electrophoresis (Fig. 2, lane 3) was mixed with inactive PAI-1 (panel E), no complex formation occurred. Purified S protein and urea-reactivated PAI-1 (panel F) formed a minor peak eluting at 450 kDa and a main peak containing both PAI activity and PAI-1 antigen at 65 kDa. Because of the apparent discrepancy between the apparent mass on gel filtration of the complex between S protein and PAI-1 (mass 65 kDa) and the expected mass (130 kDa), the experiment was repeated using a Sephadex G-100 (superfine) column. Again, a main peak was obtained with apparent mass of 68 kDa (results not shown). This component was also formed in small amounts when reactivated PAI-1 was added to the 7 S globulin fraction of plasma (Fig. 1) or to the purified PAI-1-BP (Fig. 8, panel D).

The interaction between PAI-1 and purified S protein was...
PAI-1 Binding Protein

FIG. 6. Cross-immunoelectrophoresis. Samples were applied at the origin (○) and electrophoresis carried out until the tracking dye (bromphenol blue) had migrated to the position indicated by the vertical bars. A mixture of monoclonal antibodies (100 µg/ml gel) against PAI-1 (panels A and B) or monospecific rabbit antiserum (0.5% final concentration) raised against purified S protein (panel C) was added to the gel for electrophoresis in the second dimension. A, reactivated PAI-1 (2 µg); B, PAI-1-PAI-1-BP complex (2 µg); C, PAI-1-BP (2 µg).

confirmed by cross-immunoelectrophoresis with anti-PAI-1 antibodies in the second dimension (Fig. 9). Both inactive (panel B) and urea-treated (panel A) PAI-1 have a slow electrophoretic mobility. Addition of S protein results in the formation of a component with higher electrophoretic mobility with urea-treated PAI-1 (panel C) but not with inactive PAI-1 (panel D).

DISCUSSION

The occurrence of a PAI-1 binding protein in human plasma was recently demonstrated (11). In the present study, we have isolated and characterized this binding protein, PAI-1-BP, with a yield of 5% and a purification factor of 270. SDS-gel electrophoresis showed that the final material consisted of two bands with masses 74 and 68 kDa and two with mass ≈ 150 kDa. After reduction, two main bands with apparent mass of 73 and 64 kDa were displayed and, in addition, a faint triplet band with less mass (≈ 38 kDa). Recycling of this material through complex formation with PAI-1, dissociation of the complex with t-PA, and recovery of the PAI-1-BP did not result in further purification, suggesting that all components displayed on SDS gel were involved in PAI-1 binding.

The protein was initially characterized by its reactivity with monospecific antisera against human plasma proteins at Behringwerke (Marburg/Lahn), which only identified S protein (vitronectin). Further characterization of the PAI-1-BP was then carried out using purified S protein and monospecific antisera produced at the Clinical Research Unit for Blood Coagulation and Thrombosis of the Max-Planck-Gesellschaft (Giessen) (24).

SDS-gel electrophoresis and immunoblotting of PAI-1-BP and S protein revealed that the PAI-1-BP consisted mainly of oligomers (dimers) of S protein which were virtually absent in the purified S protein preparation.

Double immunodiffusion, amino acid composition, and NH₂-terminal amino acid analysis confirmed the similarity between the isolated PAI-1-BP and S protein. The observed differences on SDS-gel electrophoresis suggest that PAI-1-BP consists of a dimeric form of S protein. Our purification procedure, which selected the 450-kDa form of the complex thus resulted in a relatively specific isolation of multimeric S protein.

Analysis of the interaction of purified PAI-1-BP or S protein with PAI-1 showed that: 1) both react with urea-treated PAI-1 but not with inactive PAI-1; 2) both react with PAI-1 to form a complex with increased mobility on agarose gel electrophoresis; 3) both give rise to the occurrence of PAI-1-containing complexes of mass 450 and 65 kDa as sized by gel filtration. While the purified PAI-1-BP mainly reacted to form the 450-kDa moiety, purified S protein mainly produced the 65-kDa form. Addition of urea-treated PAI-1 to plasma,

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**TABLE IV**

*Second-order rate constants (k) of the inhibition of t-PA by PAI-1*

The data represent mean ± S.D. of two experiments with a single concentration of t-PA and three different concentrations of PAI-1 per experiment.

<table>
<thead>
<tr>
<th></th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea-reactivated PAI-1</td>
<td>$4.6 \pm 1.5 \times 10^7$</td>
</tr>
<tr>
<td>PAI-1-PAI-1-BP complex</td>
<td>$3.0 \pm 1.3 \times 10^7$</td>
</tr>
</tbody>
</table>
but preferentially to the multimeric form.

and our finding that PAI-1 binds to both monomeric and oligomeric S protein,

vated PAI-1 a stoichiometry of three molecules of PAI-1 reacting

collected.

The stoichiometry of PAI-1 and PAI-1-BP in the complex

standards; Vmo, void volume.

to the isolated 7 S globulin fraction of plasma, or to isolated PAI-1-BP mainly yielded the 450-kDa form, although some of the 65-kDa form could also be identified. This suggests that PAI-1 binds to both monomeric and oligomeric S protein, but preferentially to the multimeric form.

The stoichiometry of PAI-1 and PAI-1-BP in the complex cannot as yet be deduced unequivocally. Indeed, with a mass for PAI-1 of 50 kDa and of 150 kDa for the PAI-1-BP (dimer) and our finding that 2 mg of PAI-1-BP react with 1 mg of PAI-1, a stoichiometry of three molecules of PAI-1 reacting

with two molecules of PAI-1-BP (i.e. two dimers of S protein) is obtained.

The observation of a shift of urea-treated PAI-1 from 30 kDa to only 65 kDa in the presence of the monomeric form of S protein, which itself has a mass of 75 kDa cannot readily be explained. It is, however, in agreement with the observation that in conditioned cell culture media, PAI-1 elutes with an apparent mass of 30 kDa during gel filtration, while in denaturant-treated conditioned medium it elutes with an apparent mass of 50 kDa (31). This phenomenon might be explained by the presence of S protein in the conditioned medium, resulting in the interaction of reactivated PAI-1 with the S protein.

Titration experiments of plasma with urea-treated PAI-1, performed to determine the maximal binding capacity of plasma, have yielded variable results according to the analytical method used. Initially the binding capacity was titrated by measuring the shift in the elution pattern of PAI activity on gel filtration. Thereby the binding capacity was calculated to be approximately 15 µg of active PAI-1/ml of plasma, which is similar to results reported by others (11). Consequently, PAI-1 was added at a concentration of 22 µg/ml plasma for isolation of the PAI-1-PAI-1-BP complex. With the use of a PAI-1 ELISA to measure PAI-1 antigen in gel filtration fractions, we found that plasma can bind approximately 70 µg of PAI-1/ml, indicating that the binding capacity would be about five times higher than initially anticipated. Based on the stoichiometry of the interaction and the results of the titration experiments the concentration of PAI-1-BP in plasma would be approximately 130 mg/liter. This represents about 35% of the total S protein concentration and is compatible with the hypothesis that PAI-1 binds preferentially with oligomeric forms of S protein.

Our findings suggest a dual regulatory role of the PAI-1-BP in the fibrinolytic system. First, the stabilizing effect of PAI-1-BP on the activity of PAI-1 in circulating blood may play a role in the physiological inhibition of circulating t-PA. However, our results also suggest a potential regulatory role of this interaction in the vessel wall. Indeed, S protein (vitronectin) is an adhesive protein which not only promotes the attachment and spreading of cultured human endothelial cells (32), but which is a major constituent of the extracellular matrix. PAI-1 is also a component of the extracellular matrix of human lung fibroblasts (33), human fibrosarcoma cells (34), and human endothelial cells (35). Our results would suggest that PAI-1 in the extracellular matrix occurs in complex with S protein. The finding that matrix-bound PAI-1

FIG. 8. Analytical gel filtration on Sephacryl S-300 (1.5 x 25 cm) in PBS-Tween at 10 ml/h; fractions of 1 ml were collected. A, nonreactivated PAI-1 (2 µg); B, urea-reactivated PAI-1 (3.7 µg); C, nonreactivated PAI-1 (2.1 µg) + PAI-1-BP (3.5 µg); D, urea-reactivated PAI-1 (8.9 µg) + PAI-1-BP (28 µg); E, nonreactivated PAI-1 (5 µg) + S protein (16 µg); F, urea-reactivated PAI-1 (5 µg) + S protein (16 µg). Samples were incubated for 10 min at 37 °C before application. ■, PAI activity in units/ml; ▲, PAI-1-related antigen in µg/ml. The vertical bars indicate the elution position of the M, standards; Vmo, void volume.

FIG. 9. Cross-immunoelectrophoresis (see legend to Fig. 5). In all cases, a mixture of monoclonal antibodies against PAI-1 was added to the gel for electrophoresis in the second dimension. A, reactivated PAI-1 (6 µg); B, nonreactivated PAI-1 (6 µg); C, reactivated PAI-1 (6 µg) + S protein (25 µg); D, nonreactivated PAI-1 (6 µg) + S protein (25 µg).
mainly occurs in active form (12, 35) whereas in conditioned
cell culture medium it is essentially inactive (22, 35) and
might be explained by the stabilizing effect of S protein on PAI-1.
Alternatively, inactivated PAI-1 could be released from the
matrix, leading to specific accumulation of active PAI-1 in
the extracellular matrix. Furthermore, the dissociation of the
PAI-1 PAI-1-BP complex by t-PA suggests an additional
mechanism for the specific accumulation of active PAI-1 in
the extracellular matrix.

The physiological role of the interaction between PAI-1
and PAI-1-BP for the regulation of humoral and cellular
fibrinolysis suggested by the present findings remains to be
elucidated.

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