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Evidence for a Discrete Binding Protein of Plasminogen Activator Inhibitor in Plasma

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Key words

Plasminogen activator inhibitor (PAI) – Protein-protein interaction – PAI-binding protein

Summary

Gel-filtration experiments of mixtures of functionally active and inactive forms of plasminogen activator inhibitor (PAI) with human plasma or bovine serum albumin have provided evidence for the existence of a discrete binding protein of PAI in plasma. Most likely it is a glycoprotein with a molecular weight of approximately 150,000. The data suggest that it forms a very stable complex with functionally active forms of PAI, but not with the inactive or "latent" PAI. However, the PAI activity seems not to be significantly altered by the interaction with the binding protein. Assuming that a stoichiometric complex is formed, titration experiments suggest that a pool of normal human plasma contains about 40–50 mg of PAI-binding protein liter.

Introduction

A specific inhibitor of plasminogen activators (PAI) has been demonstrated in human plasma (1–4) and in conditioned media from a variety of cultured cells (5–8). PAI is a member of the serpin superfamily of proteins and consists of 379 amino acid residues as estimated from its cDNA sequence (9, 10). All evidence obtained so far suggests that PAI acts in a similar fashion as the other plasma serine proteinase inhibitors, by formation of a stable stoichiometric 1:1 inactive complex with plasminogen activators (2, 11, 12).

The molecular weight of PAI as estimated by sodium dodecylsulphate polyacrylamide gel electrophoresis (2, 13) or from its primary structure (9, 10) is about 50,000. Nevertheless, by gel-filtration of plasma or serum in the absence of denaturating agents the PAI activity gel-filters as a high molecular weight compound, with an estimated M_r of about 200,000 (11). The reason for this behaviour has not been well understood. In this investigation we present data to suggest that the gel-filtration behaviour of PAI in plasma is due to the presence of a specific high molecular weight PAI-binding protein.

Materials and Methods

Plasminogen Activator Inhibitor (PAI)

PAI was purified from conditioned medium from the human fibrosarcoma cell-line HT 1080 essentially as described earlier (13). The material displayed a homogeneous band with a molecular weight of about 50,000 on dodecylsulphate polyacrylamide gel electrophoresis, both prior to and after reduction. The material was functionally inactive, but could be activated by treatment with denaturating agents such as dodecylsulphate or guanidinium chloride (14, 15). This was performed in the following way: solid guanidinium chloride (final concentration 4 mol/l) was added to a solution of PAI (5–20 mg/l) in 0.05 mol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl. The mixture was incubated at ambient temperature for about 5 minutes and subsequently dialyzed against 0.05 mol/l sodium acetate buffer, pH 5.5, containing 0.1 mol/l NaCl at +5 °C. The PAI activity and PAI antigen concentrations were determined on the samples and a specific activity of 500,000–800,000 U/mg was typically obtained. This is close to the theoretical value for a fully active inhibitor (16). At pH 5.5 the PAI activity was very stable, but at neutral pH a slow decline in PAI activity was always observed.

Plasma

Freshly frozen citrated human plasma was a kind gift from the Blood bank of Karolinska Hospital, Stockholm, Sweden (courtesy of Dr Olof Åkerblom).

Reagents

Sephacryl S-300, concanavalin A-Sepharose and heparin-Sepharose were from Pharmacia AB (Uppsala, Sweden). The chromogenic plasmin substrate D-But-CHT-Lys-pNA (Spectrozym PAR) was a gift from Biopool AB (courtesy of Dr Mats Rånby). Bovine serum albumin, alpha-methyl-mannoside and guanidinium chloride were from Sigma (St Louis, Mo, USA). All other chemicals were of analytical grade and mostly purchased from Merck (Darmstadt, FRG).

Determination of PAI Activity

PAI activity was determined using a slightly modified version of our previously described method (17). The plasmin substrate D-But-CHT-Lys-pNA was used. The PAI activity was expressed in arbitrary units/ml, where one U corresponds to inhibition of 1 IU of one-chain t-PA (17).

Determination of PAI Antigen

PAI antigen was determined with a homogeneous double antibody radioimmunoassay essentially as described (16). The concentration was expressed in µg/l using purified "inactive" PAI from HT 1080 cells as a standard.

Determination of PAI-Binding Protein

In order to quantify the PAI-binding protein, a sample containing this compound was mixed with an excess of reactivated PAI (600 U) in a final volume of 0.5 ml (in 0.05 mol/l sodium phosphate buffer, pH 7.3, containing 0.1 ml/l NaCl). The mixture was incubated for 5 minutes at ambient temperature, and subsequently gel-filtered at +5 °C on a small Sephacryl S-300 column (2 cm^2 × 25 cm) equilibrated and developed with 0.05 mol/l sodium acetate buffer, pH 5.5, containing 0.1 mol/l NaCl. The chromatogram was monitored by A_{280} and PAI activity measurements. Two peaks of PAI activity were obtained of which the activity found in the high molecular weight fraction was taken as an estimate of the PAI-binding capacity.

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Results

Demonstration of the Formation of a Complex Between Functionally Active PAI and a High Molecular Weight Compound in Plasma

Purified reactivated PAI (450 U or 900 U in 0.05 mol/l sodium phosphate buffer, pH 7.3, containing 0.1 mol/l NaCl) was mixed with 200 µl or 500 µl normal human plasma or 200 µl bovine serum albumin solution (10 g/l in the same phosphate buffer) and subsequently subjected to gel-filtration on a Sephacryl S-300 column, equilibrated and developed in 0.05 mol/l sodium acetate buffer, pH 5.5, containing 0.1 mol/l NaCl (Fig. 1a, 1b and 1c). The chromatograms were monitored by A_{280} and PAI-activity. In the presence of bovine serum albumin only (Fig. 1a), the PAI-activity gel-filtered as a 50,000 M_r protein. However, in the presence of "excess" plasma (Fig. 1b) all the PAI-activity eluted with an apparent molecular weight of about 200,000. If the ratio of PAI-activity to plasma was increased so that the PAI-binding capacity was exceeded, two peaks of PAI-activity were obtained: one high M_r form and one 50,000 M_r form (Fig. 1c).

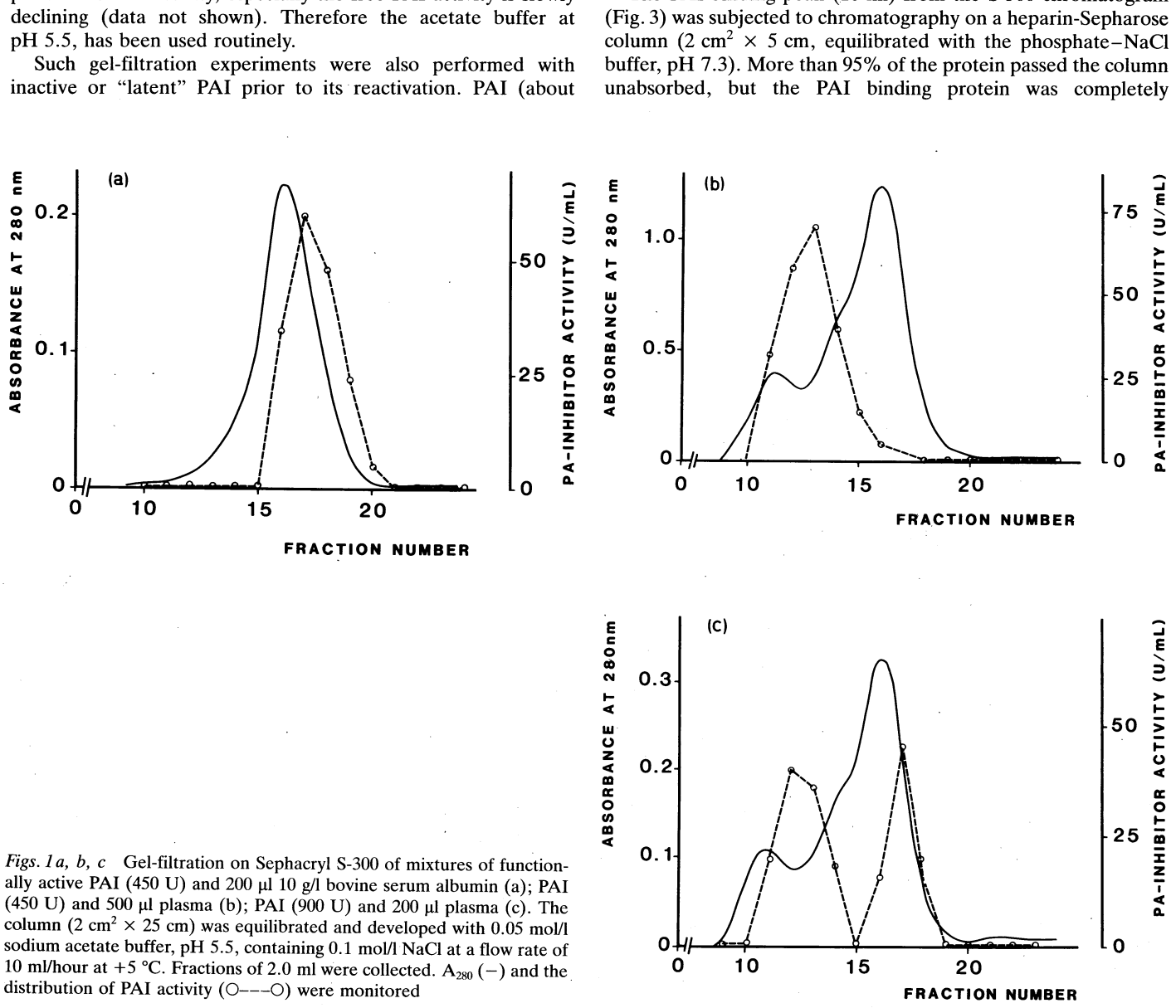
Similar results have been obtained by performing the gel-filtration step at pH 4, pH 5.5 and pH 7.0. However, above pH 6.0 the PAI-activity, especially the free PAI-activity is slowly declining (data not shown). Therefore the acetate buffer at pH 5.5, has been used routinely.

Such gel-filtration experiments were also performed with inactive or "latent" PAI prior to its reactivation. PAI (about 4.0 µg in phosphate–NaCl buffer) was mixed with either 1 ml/ bovine serum albumine solution (10 g/l) or 1 ml normal human plasma and subsequently gel-filtered on Sephacryl S-300 as described above. In these experiments no peaks of PAI activity could be detected, and only one peak of PAI-antigen was found, eluting at 50,000 M_r, both in the presence of bovine serum albumin and in the presence of plasma (Fig. 2a and 2b, respectively).

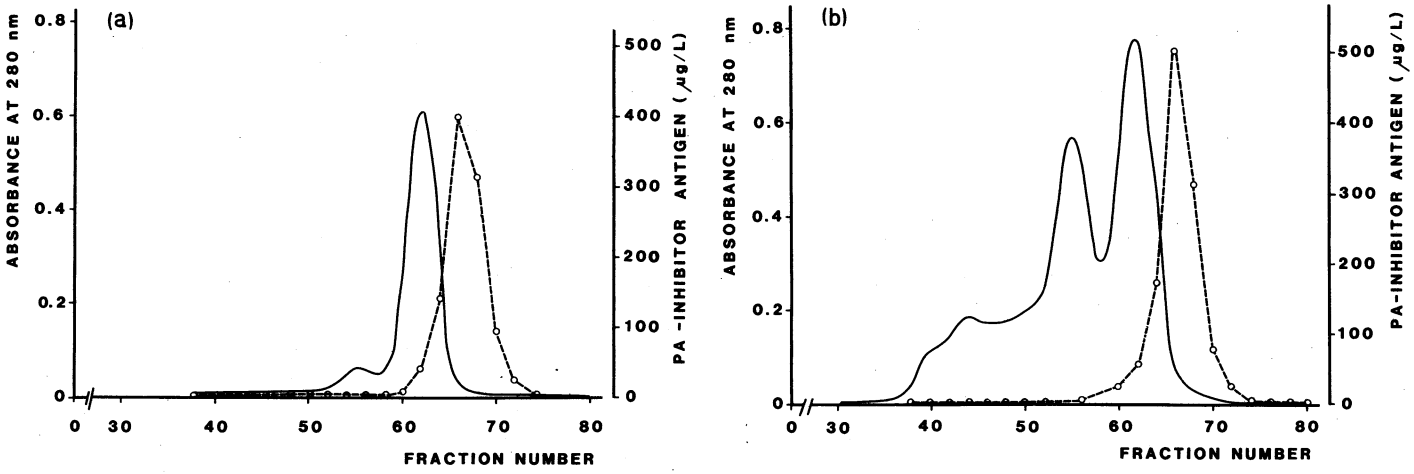
Chromatographic Behaviour of the PAI-Binding Protein in Human Plasma

Pooled normal human plasma was subjected to gel-filtration on Sephacryl S-300 (20 cm^2 × 70 cm, equilibrated and developed in the phosphate–NaCl buffer, pH 7.3) in a preparative way and the chromatogram was monitored by A_{280}. In addition, the capacity of the different fractions to transform 50,000 M_r reactivated PAI to a high M_r compound was assayed as described in methods. This capacity was found in a single peak, almost coinciding with the IgG peak (Fig. 3). Thus, these data suggest that a PAI-binding protein with a molecular weight of approximately 150,000 exists in plasma.

The PAI-binding peak (10 ml) from the S-300 chromatogram (Fig. 3) was subjected to chromatography on a heparin-Sepharose column (2 cm^2 × 5 cm, equilibrated with the phosphate–NaCl buffer, pH 7.3). More than 95% of the protein passed the column unabsorbed, but the PAI binding protein was completely



Figs. 1a, b, c Gel-filtration on Sephacryl S-300 of mixtures of functionally active PAI (450 U) and 200 µl 10 g/l bovine serum albumin (a); PAI (450 U) and 500 µl plasma (b); PAI (900 U) and 200 µl plasma (c). The column (2 cm^2 × 25 cm) was equilibrated and developed with 0.05 mol/l sodium acetate buffer, pH 5.5, containing 0.1 mol/l NaCl at a flow rate of 10 ml/hour at +5 °C. Fractions of 2.0 ml were collected. A_{280} (–) and the distribution of PAI activity (O---O) were monitored



Figs. 2a, b Gel-filtration on Sephacryl S-300 of mixtures of "latent" PAI (about 4 µg) and 1 ml 10 g/l bovine serum albumin (a); PAI (about 4 µg) and 1 ml plasma (b). The same conditions as in Fig. 1 were used but the column size was 5 cm^2 × 42 cm. The flow rate was 25 ml/hour and fractions of 2.5 ml were collected. A_{280} (−) and PAI antigen (O---O) were determined

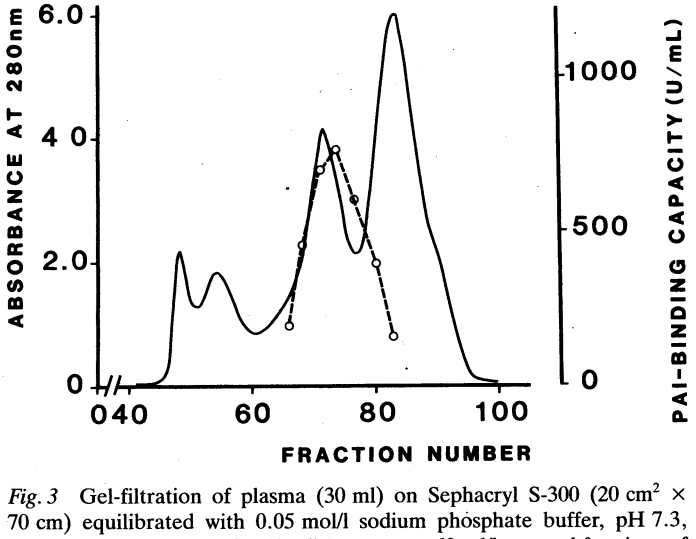


Fig. 3 Gel-filtration of plasma (30 ml) on Sephacryl S-300 (20 cm^2 × 70 cm) equilibrated with 0.05 mol/l sodium phosphate buffer, pH 7.3, containing 0.1 mol/l NaCl. The flow rate was 60 ml/hour and fractions of 10 ml were collected. A_{280} (−) and the PAI-binding capacity (O---O) were determined

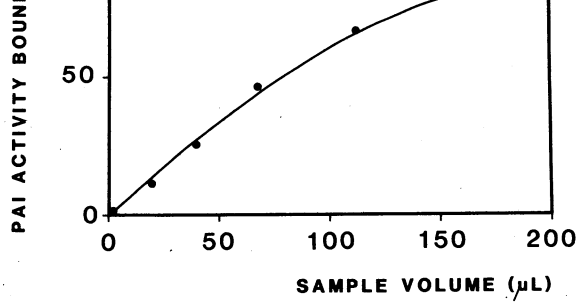


Fig. 4 Titration of the PAI-binding capacity in the pool from the S-300 chromatogram in Fig. 3. Functionally active PAI (600 U) was mixed with different volumes (0–160 µl) of the PAI-binding protein pool and the amount of PAI bound to PAI-binding protein, was estimated as described in methods. The PAI activity bound (% of total) was then plotted against the volume added of the PAI-binding protein pool

adsorbed. Unfortunately, no PAI-binding activity could be eluted with 1.5 mol/l NaCl or 5 mol/l guanidinium chloride when added to the buffer.

The peak (10 ml) containing the PAI-binding capacity from the S-300 column (Fig. 3) was also subjected to chromatography on a small column of concanavalin A-Sepharose (2 cm^2 × 5 cm) equilibrated with the phosphate-NaCl buffer, pH 7.3. The PAI-binding protein was completely adsorbed on the column and about 30% was recovered upon elution with 0.05 mol/l alpha-methylmannoside.

Estimation of the PAI-Binding Capacity

The PAI-binding protein peak from the S-300 chromatogram (Fig. 3) was pooled, concentrated by ultrafiltration to the original plasma volume, and used in a titration experiment: 0–160 µl of the pool were mixed with reactivated PAI (600 units) and gel-filtered on a small S-300 column as described in methods. If the PAI activity found in the high molecular weight fraction was plotted against the volume of the pooled PAI-binding protein added, an almost straight line was obtained (Fig. 4). From these results a binding capacity of 4000 U/ml was calculated. Similar experiments with pooled normal human plasma resulted in a binding capacity of about 15000 U/ml. Assuming a specific activity for PAI of about 800,000 U/ml (16), molecular weights of 50,000 and 150,000 for PAI and the PAI-binding protein, respectively, and that a stoichiometric complex is formed, the PAI binding capacity found in plasma equals about 40–50 mg/l of such a protein.

Stability of the Complex Between PAI and the PAI-Binding Protein

Attempts were performed to dissociate the complex between PAI and the PAI-binding protein. For this purpose preformed complex (600 U PAI and 200 µl of the pooled PAI-binding fraction from the chromatogram in Figure 3) was gel-filtered on small Sephacryl S-300 columns (2 cm^2 × 22 cm) equilibrated with 0.05 mol/l sodium phosphate buffer, pH 7.3 to which 1 mol/l NaCl or 3 mol/l guanidinium chloride had been added. The PAI activity was fully recovered in these chromatograms and no dissociation of the complex was observed neither in 1 mol/l NaCl nor in 3 mol/l guanidinium chloride.

Discussion

On gel-filtration of plasma, rich in PAI activity, this activity elutes as a component with an apparent molecular weight of about 200,000 (11). Functionally active PAI from the fibrosarcoma cell-line HT 1080 produced in the presence of fetal bovine calf-serum also gel-filters as a high molecular weight component (13). In contrast, the inactive or "latent" form of PAI gel-filters as a 50,000 M_r protein (13), which is in agreement with its molecular weight as calculated from the primary structure (9, 10). If the functionally active PAI is gel-filtered in the presence of sodium dodecylsulphate, however, the PAI antigen elutes in a similar fashion as the "latent" PAI form, at an apparent molecular weight of about 50,000 (13).

When we first demonstrated the deviating gel-filtration behaviour of PAI in plasma, we suggested a few different possibilities to explain this phenomenon (11). One of the suggestions was that PAI might form a complex with another plasma protein of higher molecular weight. In the present work we have provided experimental evidence that plasma indeed contains such a PAI-binding molecule, which seems to have a molecular weight of about 150,000. Furthermore, in agreement with our previous results the present data suggest that most likely a stoichiometric complex is formed with functionally active PAI, but not with the "latent" form of the inhibitor. The nature of the interaction is at present not known, but it seems to be strong, since the complex is stable at 1 mol/l or 3 mol/l guanidinium chloride. Furthermore, the activity of PAI is not significantly altered, suggesting that the sites involved in its reaction with plasminogen activators are not primarily affected by the interaction with the PAI-binding molecule.

In the present work we have used reactivated PAI to estimate the PAI-binding capacity. Recently we have also obtained similar results with partially purified spontaneously active 50,000 M_r PAI obtained from HT 1080 cell conditioned medium in the absence of foetal calf serum. Therefore in this respect the properties of reactivated PAI are similar if not identical, to native PAI.

From the binding experiments in the present work we estimated the PAI-binding capacity of pooled normal plasma to about 15000 U/ml. Assuming the formation of a stoichiometric complex, a specific activity of about 800,000 U/mg for reactivated PAI and a molecular weight of about 150,000 for the PAI-binding protein, its concentration in pooled normal human plasma was calculated as about 40–50 mg/l. Thus PAI-binding capacity in plasma exceeds that of PAI by several orders of magnitude. The reason for this is not clear at present. Indeed, the responsible molecule may have other physiological functions than just binding PAI.

The PAI-binding molecule seems to have a strong affinity for heparin. Affinity chromatography of plasma or partly purified fractions of the binding protein on insolubilized heparin, completely removed this compound from the solutions. However, so far we have not been able to elute it from heparin-sepharose, at least not in a functionally active form. However, the PAI-binding activity which we have demonstrated is most likely a glycoprotein since it is adsorbed on concanavalin A-Sepharose and specifically eluted by alpha-methylmannoside. Work with its purification is in progress.

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