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# Plasminogen activator inhibitor 1 (PAI) is bound to vitronectin in plasma

Björn Wiman*, Åsa Almquist, Olöf Sigurdardottir and Tomas Lindahl

*Department of Clinical Chemistry, Karolinska Hospital, S-104 01 Stockholm, Sweden*

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Functionally active plasminogen activator inhibitor 1 (PAI) is bound to a discrete binding protein in plasma [(1988) Thromb. Haemost. 59, 392–395]. The binding protein has now been partially purified using conventional chromatographic techniques. After addition of active PAI its complex with the binding protein was purified by chromatography on insolubilized monoclonal antibodies towards PAI. Dodecylsulphate (polyacrylamide gel electrophoresis revealed two main compounds with molecular masses of 50 and 75 kDa respectively. $NH_2$-terminal amino acid sequence analysis and immunoblotting analysis suggested that the two compounds were PAI (50 kDa) and vitronectin (75 kDa). We conclude that the PAI-binding protein is identical to vitronectin.

Plasminogen activator inhibitor; Vitronectin; Protein-protein interaction; Fibrinolysis

## 1. INTRODUCTION

On gel filtration of functionally active plasminogen activator inhibitor 1 (PAI) in the presence of human plasma [1] or fetal calf serum [2] it behaves as a high molecular mass protein. In contrast, functionally inactive or so called 'latent' PAI filters as a 50 kDa compound [2,3], in agreement with its suggested primary structure [4]. Recently we presented evidence that the deviating gel filtration behaviour of functionally active PAI in the presence of plasma/serum is due to the existence of a discrete PAI-binding protein in plasma/serum [3].

In the present work we have purified the complex between PAI and the binding protein from human plasma and then characterized the compounds by structural and immunochemical methods.

*Correspondence address:* B. Wiman, Department of Clinical Chemistry, Karolinska Hospital, S-104 01 Stockholm, Sweden

## 2. MATERIALS AND METHODS

### 2.1. *Proteins*

PAI, mostly in a functionally inactive form, was purified from conditioned medium from the fibrosarcoma cell-line HT 1080 by heparin-Sepharose chromatography, gel filtration on Sephadex G-150 and chromatography on carboxymethyl cellulose. The procedure is described in detail elsewhere [5]. The latent PAI was reactivated by treatment with 4 mol/l guanidinium chloride at pH 5.5 as described [5]. Specific activities of about 250000–500000 arbitrary units/mg were typically obtained.

Vitronectin was purified according to a published procedure [6]. The monoclonal and polyclonal antibodies towards human PAI have been described earlier [7,8]. The rabbit antiserum towards human vitronectin initially used, was a kind gift from Dr Björn Dahlbäck (Dept of Clinical Chemistry, Malmö General Hospital, Sweden), but in the published experiments another antiserum was used, produced by immunization of a rabbit with purified vitronectin.

### 2.2. *Plasma*

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

### 2.3. *Reagents*

Sephadex, Sepharose derivatives, Sephacryl and DEAE-Sephacel were from Pharmacia (Uppsala, Sweden).

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Acrylamide, SDS, 4-chloro-1-naphthol and nitrocellulose sheets were from BioRad (Richmond, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG was obtained from Dakopatts (Copenhagen, Denmark). Aprotinin (Trasylol® ) was from Bayer AG (Leverkusen, FRG).

2.4. *Functional and immunological determination of PAI*

PAI activity was measured by the method of Chmielewska and Wiman [9] utilizing a commercially available kit (Spectrolyse fibrin, Biopool AB, Umeå, Sweden). One unit of inhibitor was defined as the amount which inhibited one IU of one-chain tPA.

Determination of PAI antigen was performed with a double antibody radioimmuoassay as described previously [8]. Purified latent PAI from HT 1080 cells was utilized as standard and after labelling with $^{125}I$ as tracer.

2.5. *Determination of PAI-binding capacity*

The PAI-binding capacity was routinely determined by mixing reactivated PAI (500 arb units) with the sample to be tested, followed by gel filtration on a Sephadex G-150 column (2 $cm^2$ × 18 cm) equilibrated with 0.05 mol/l sodium acetate buffer, pH 5.5, containing 0.5 mol/l NaCl and 0.1 g Tween 80/1. The fractions were tested for PAI activity and the total amount of PAI activity in the first peak was used as an estimate of the PAI-binding capacity [3].

2.6. *SDS-polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS was performed by an established procedure [10], using a flat bed electrophoresis apparatus (Pharmacia, Uppsala, Sweden). Staining was performed by the silver staining procedure [11]. Alternatively immunoblotting was performed. The proteins were transferred electrophoretically to a nitrocellulose sheet using a BioRad (Richmond, USA) transblot cell. After blocking with Tween 20, the nitrocellulose sheet was treated with monospecific rabbit anti-human PAI IgG, or rabbit anti-human vitronectin IgG preparations and subsequently with horseradish peroxidase-labelled goat anti-rabbit IgG. The development was performed with 4-chloro-1-naphthol and $H_2O_2$ [11].

2.7. *Determination of $NH_2$-terminal amino acid sequences*

This was performed with a pulsed liquid phase sequencer (model 477A, Applied Biosystems, Foster City, CA, USA) equipped with an on-line PTH 120A analyzer. A standard programme was used and the reagents were provided by the manufacturer.

## 3. RESULTS

### 3.1. *Purification of the PAI-binding protein*

A three step procedure was used to partially purify the PAI-binding protein from plasma: gel filtration on Sephacryl S-300 (fig.1), DEAE-cellulose chromatography (fig.2) and Blue Sepharose chromatography (fig.3). With this procedure the PAI-binding protein was purified about 170-fold with a yield of about 25% (table 1).

### 3.2. *Purification and analyses of the complex between PAI and the PAI-binding protein*

The material obtained from the Blue Sepharose column (fig.3) was mixed with an equal (on activity basis) amount of reactivated PAI and subjected to gel filtration on Sephacryl S-300. The main PAI activity peak, eluting as a high molecular mass compound together with the main protein peak, was further purified by affinity chromatography on insolubilized monoclonal antibodies towards human PAI. The column was washed with 0.05 mol/l sodium phosphate buffer, pH 7.3, containing 0.1 mol/l NaCl and 0.1 g/l Tween 80.

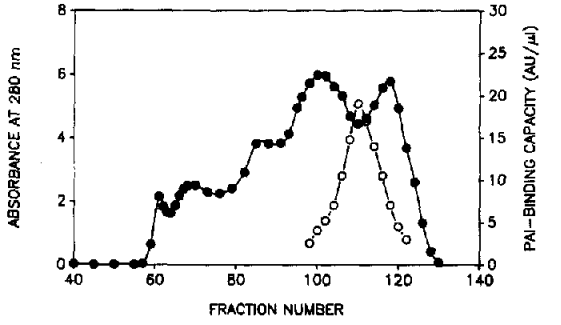


Fig.1. Gel filtration of human plasma (45 ml) on Sephacryl S-300 (20 $cm^2$ × 75 cm) equilibrated with 0.05 mol/l sodium phosphate buffer, pH 7.0, containing 0.05 mol/l NaCl and 10 units aprotinin/ml. The flow rate was 60 ml/h and 10 ml fractions were collected. Absorbance at 280 nm (●—●); PAI-binding capacity (○—○).

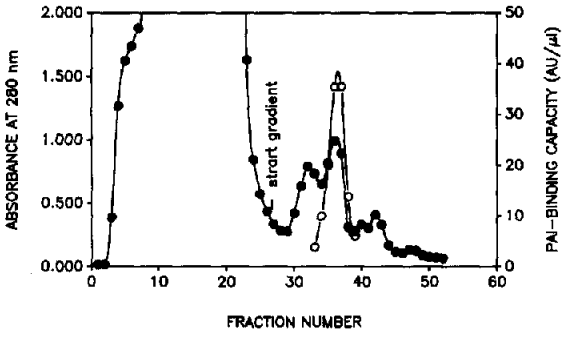


Fig.2. DEAE-cellulose chromatography of the pool of PAI-binding protein from the S-300 chromatogram in fig.1. The column (5 $cm^2$ × 6 cm) was equilibrated with the same phosphate buffer as the S-300 column. Elution was performed with a linear gradient (300 ml total volume) to 0.05 mol/l sodium phosphate buffer, pH 7.0, containing 0.4 mol/l NaCl and 10 units aprotinin/ml. The flow rate was 25 ml/h and 10 ml fractions were collected. Absorbance at 280 nm (●—●); PAI-binding capacity (○—○).

Table 1

Partial purification of PAI-binding protein, purification factors and yields in the different steps

| Step | Volume (ml) | Absorbance at 280 | PAI-binding capacity (U/ml) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| Plasma | 45 | 62 | 55000 | 1 | 100 |
| S-300 | 150 | 4.9 | 12000 | 2.8 | 73 |
| DEAE-cellulose | 40 | 0.70 | 24000 | 39 | 39 |
| Blue Sepharose | 10 | 0.41 | 62000 | 170 | 25 |

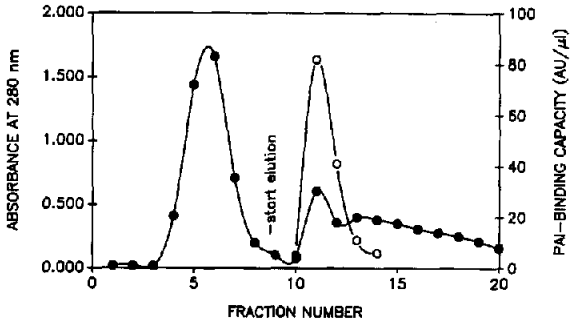


Fig.3. The PAI-binding protein peak from the DEAE-cellulose chromatography was dialyzed against 0.05 mol/l Tris buffer, pH 7.4, containing 0.15 mol/l NaCl, 0.01 mol/l EDTA and 10 units aprotinin/ml and subsequently applied to a Blue Sepharose column (2 $cm^2$ × 10 cm) equilibrated with the same buffer. Elution was performed with this buffer containing 1.15 mol/l NaCl. The flow rate was 20 ml/h and 5 ml fractions were collected. Absorbance at 280 nm (●—●); PAI-binding capacity (○—○).

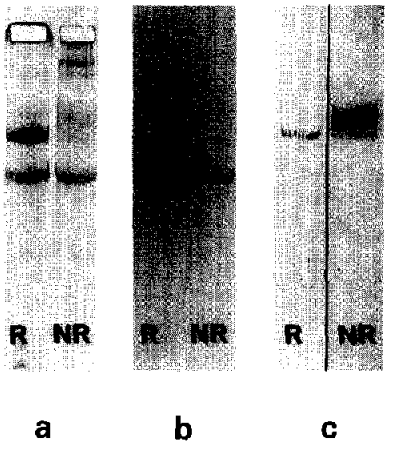


Fig.4. SDS-polyacrylamide gel electrophoresis of the purified complex between PAI and its binding protein from human plasma. The gels (reduced samples, R, and non-reduced samples, NR) were stained with silver (a) or used for immunoblotting with monospecific antisera towards human PAI (b) or human vitronectin (c).

Subsequently, elution was performed with 3 mol/l KSCN in this buffer. The eluted material was dialyzed against the phosphate/NaCl buffer, pH 7.3, and then subjected to different analytical procedures. SDS-polyacrylamide gel electrophoresis (fig.4) displayed two major bands (after reduction) with molecular masses of 50 and 75 kDa, respectively. Immunoblotting using monospecific polyclonal antisera against PAI or vitronectin demonstrated that the 50 kDa band was PAI and that the 75 kDa band was vitronectin (fig.4). Due to unknown reasons the vitronectin band is not clearly displayed on the silver stained gels prior to reduction. However, a clearly visible band slightly above 75 kDa is still obtained in the immunoblot utilizing anti-vitronectin.

$NH_2$-terminal amino acid sequence analysis on microgram quantities of the PAI/PAI-binding protein complex revealed two parallel sequences: Val/Asp-His/Gln-His/Glu-Pro/?-Pro/?-?/Lys-Tyr/?-Val/?-. This is in agreement with the known amino acid sequence of PAI: Val-His-His-Pro-Pro-Ser-Tyr-Val- [4] and that of vitronectin: Asp-Gln-Glu-Ser-Cys-Lys-Gly-Arg- [12].

## 4. DISCUSSION

We have previously demonstrated that functionally active PAI in plasma [1] or from the fibrosarcoma cell-line HT 1080 in the presence of fetal calf serum [2] behaves as a high molecular mass compound. Recently we provided gel filtration data indicating that PAI in plasma is bound to what looks like a carrier protein [3]. In the present work the binding protein has been partially purified from human plasma using conventional chromatographic procedures. After addition of functionally active PAI to this material, the com-

plex between PAI and the binding protein was purified first by gel filtration and subsequently by affinity chromatography on insolubilized monoclonal antibodies towards human PAI. The complex was analyzed by SDS-polyacrylamide gel electrophoresis, $NH_2$-terminal amino acid sequence analysis and immunoblotting. Thus, the complex contained two main compounds: a 50 kDa compound identical to PAI and a 75 kDa compound identical to vitronectin.

Recently we also produced data suggesting that the high molecular mass PAI form obtained from HT 1080 cells in the presence of fetal calf serum constitutes a complex between PAI and bovine vitronectin [5]. Our data that vitronectin is identical to the PAI-binding protein is in agreement with recent results from Collen and co-workers [13].

So far, the physiological meaning of this interaction is unclear, although a slight stabilization of the PAI activity has been observed [14]. Several groups have demonstrated that PAI is adsorbed to the extracellular matrix [15,16]. It remains to be elucidated if vitronectin also is involved in that interaction.

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