Biochem. J. (1990) 265, 109–113 (Printed in Great Britain)



# The mechanism of the reaction between human plasminogen-activator inhibitor 1 and tissue plasminogen activator

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The structural events taking place during the reaction between PAI-1 (plasminogen-activator inhibitor 1) and the plasminogen activators sc-tPA (single-chain tissue plasminogen activator) and tc-tPA (two-chain tissue plasminogen activator) were studied. Complexes were formed by mixing sc-tPA or tc-tPA with PAI-1 in slight excess (on an activity basis). The complexes were purified from excess PAI-1 by affinity chromatography on fibrin–Sepharose. Examination of the purified complexes by SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) and *N*-terminal amino acid sequence analysis demonstrated that a stoichiometric 1:1 complex is formed between PAI-1 and both forms of tPA. Data obtained from both complexes revealed the amino acid sequences of the parent molecules and, in addition, a new sequence: Met-Ala-Pro-Glu-Glu-. This sequence is found in the *C*-terminal portion of the intact PAI-1 molecule and thus locates the reactive centre of PAI-1 to $Arg^{346}$–$Met^{347}$. The proteolytic activity of sc-tPA is demonstrated by its capacity to cleave the 'bait' peptide bond in PAI-1. The complexes were inactive and dissociated slowly at physiological pH and ionic strength, but rapidly in aq. $NH_3$ (0.1 mol/l). Amidolytic tPA activity was generated on dissociation of the complexes, corresponding to 0.4 mol of tPA/mol of complex. SDS/PAGE of the dissociated complexes indicated a small decrease in the molecular mass of PAI-1, in agreement with proteolytic cleavage of the 'bait' peptide bond during complex-formation.

## INTRODUCTION

Fibrin clots in the blood vessels are effectively degraded and removed by the fibrinolytic enzyme system. The system is regulated at many different levels, of which inactivation of tissue plasminogen activator (tPA) by plasminogen-activator inhibitor 1 (PAI-1) is an important part [1]. PAI-1 exists in plasma [2,3], and elevated levels seems to be connected with thrombotic disease [4,5]. It is a glycoprotein with an $M_r$ of about 50000, consisting of 379 amino acids, as revealed from its cDNA sequences [6,7]. This inhibitor is structurally related to the other **serine-p**roteinase **in**hibitors (serpins) in plasma such as $\alpha_1$-antitrypsin, antithrombin III, C1-inhibitor and $\alpha_2$-antiplasmin [8,9]. The inhibition mechanism is quite well known for the serpins, and involves a proteolytic attack by the enzyme on a specific 'bait' peptide bond in the *C*-terminal portion of the inhibitor. The intermediate acyl-enzyme complex formed is hydrolysed only very slowly, and the enzyme is thus trapped in an inactive state [8].

The only data published so far about the structure of the PAI-1–tPA complexes are from gel-filtration experiments on SDS/polyacrylamide-gel electrophoresis (SDS/PAGE), which have demonstrated an $M_r$ of about 110000 for the complex, suggesting a stoichiometric 1:1 complex [4,10].

A major problem in studies of PAI-1 is its poor stability under physiological conditions [11,12]. Therefore the purified PAI-1 preparations obtained so far are at most 10% active [13]. PAI-1 is produced in an active form [12,14], but it is rapidly inactivated [12,15]. The inactive PAI-1 can be re-activated by treatment with denaturating agents such as guanidinium chloride [16]. Similar, if not identical, kinetic properties of re-activated and native PAI-1 have been reported [17,18].

In the present work we have investigated the complexes between re-activated PAI-1 and different tPA forms by structural methods. In this way the 'bait' peptide bond in PAI-1 has been unequivocally identified.

## MATERIALS AND METHODS

### PAI-1

Latent PAI-1 was purified to homogeneity from the human fibrosarcoma cell line HT 1080-conditioned medium by heparin–Sepharose chromatography, gel filtration on Sephadex G-150 and chromatography on carboxymethyl cellulose as described in [11]. The inactive PAI-1 was re-activated by treatment with guanidinium chloride (4 mol/l; pH 5.5) [14]. The specific activity obtained was about 400000 units/mg of PAI-1. Thus about 50% of the PAI-1 was activated and about 50% remained inactive.

### Fibrin–Sepharose

Fibrinogen was coupled to CNBr-activated Sepharose CL 4B (Pharmacia AB, Uppsala, Sweden) to about 10 mg of protein/ml of gel. In order to convert fibrinogen into fibrin the gel was suspended in about 10 vol. of sodium phosphate (0.05 mol/l) buffer, pH 7.3, containing

Abbreviations used: sc-tPA, single-chain tissue plasminogen activator; tc-tPA, two-chain tissue plasminogen activator; PAI-1, plasminogen-activator inhibitor 1; serpin, **serine p**roteinase **in**hibitor; PAGE, polyacrylamide-gel electrophoresis; PTH, phenylthiohydantoin; S2288, D-Ile-Pro-Arg *p*-nitroanilide.

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NaCl (0.1 mol/l), thrombin (Topostasine, Roche, Basel, Switzerland) [10 NIH (National Institutes of Health) units/ml] and aprotinin (Trasylol; 10 kallikrein-inhibitory units/ml; Bayer AG, Leverkusen, Germany). The suspension was stirred for 4 h at room temperature and then washed on a Büchner funnel with the same buffer, but without thrombin.

### Reagents

Pure sc-tPA and tc-tPA were generously given by Biopool AB, Umeå, Sweden (courtesy of Dr. Mats Rånby). The specific activities of sc-tPA and tc-tPA were checked [19] against the W.H.O. international standards for these proteins. The specific activity was 650000 i.u./mg for both proteins.

Sephacryl S-300 and CNBr-activated Sepharose CL 4B were from Pharmacia. CM-52 cellulose was from Whatman. Acrylamide and SDS were obtained from Bio-Rad, Richmond, CA, USA. Chromogenic peptide substrate for determination of the amidolytic activity of tPA (D-Ile-Pro-Arg *p*-nitroanilide, S-2288) was from Kabi Diagnostica, Mölndal, Sweden. Other chemicals were of analytical grade and mostly purchased from Merck, Darmstadt, Germany.

### Determination of PAI-1 activity and antigen

PAI-1 activity was determined by a coupled spectrophotometric assay essentially as described in [19], utilizing a kit (Spectrolyse-fibrin) from Biopool. The samples were diluted with phosphate buffer (0.02 mol/l), pH 7.3, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l), and then purified sc-tPA was added to a final concentration of 25 i.u./ml. The mixture was incubated for exactly 10 min at 25 °C, after which the reaction was stopped by acidification. Residual tPA activity was measured by addition of a reagent containing plasminogen, solubilized fibrin and the chromogenic substrate D-Val-Phe-Lys *p*-nitroanilide in Tris/HCl buffer (0.05 mol/l), pH 8.8, containing Tween 80 (0.1 g/l). After incubation for 75 min at 37 °C, the absorbance at 405 nm was read; 1 arbitrary unit of PAI-1 was defined as the amount that inhibits 1 i.u. of sc-tPA [19].

PAI-1 antigen was quantified by a double-antibody radioimmunoassay utilizing polyclonal rabbit antibodies towards human PAI-1 as previously described [20]. Purified latent PAI-I from HT 1080 cells was used as standard and, after labelling with $^{125}I$, as a tracer.

### Determination of tPA activity and tPA antigen

tPA antigen was determined by an e.l.i.s.a. method, utilizing a kit (Tintelize-tPA) from Biopool. tPA activity was determined spectrophotometrically by utilizing the chromogenic substrate S-2288 (0.6 mmol/l) in Tris/HCl (0.05 mol/l) buffer, pH 7.8, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l).

### SDS/PAGE

SDS/PAGE was performed by an established procedure [21], using a flat-bed electrophoresis apparatus (Pharmacia). The gels were stained with silver [22].

### Amino acid sequence analysis

*N*-Terminal amino acid sequences were determined as described by Edman & Henschen [23] using a pulsed liquid-phase sequencer (model 477A; Applied Biosystems, Foster City, CA, U.S.A.) equipped with an on-line PTH 120 analyser. A standard program was used and the reagents were provided by the manufacturer.

### Formation of complexes between tPA and PAI-1

sc-tPA or tc-tPA (60 $\mu$g/ml) was mixed with re-activated PAI-1 in about 10% excess (on an activity basis) at neutral pH and incubated for 20 min at room temperature. The mixture was applied to a fibrin–Sepharose column (2 $cm^2$ cross-sectional area × 5 cm long) equilibrated with sodium phosphate (0.05 mol/l) buffer, pH 7.3, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l). Elution was performed with KBr (2 mol/l) in this buffer. The fractions containing protein were subsequently dialysed against the equilibration buffer and then concentrated by ultrafiltration. The purified complex was desalted by gel filtration on a Sephacryl S-300 column (2 $cm^2$ × 30 cm), equilibrated, developed with 10% (v/v) acetic acid, and then freeze-dried.

### Dissociation of the tPA–PAI-1 complexes

Freeze-dried complex was dissolved in three different solutions to a final concentration of 240 $\mu$g/ml. The solutions used were aq. $NH_3$ (0.1 mol/l) or sodium phosphate (0.5 mol/l) buffer, pH 7.3, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l) or Tris/HCl (0.05 mol/l) buffer, pH 7.8, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l). Samples (10 $\mu$l) were taken for assaying tPA activity as described above. Samples for SDS/PAGE were incubated overnight in aq. $NH_3$ (0.1 mol/l), freeze-dried and then dissolved in the SDS sample buffer.

## RESULTS

### Purification of complexes of PAI-1 with sc-tPA and tc-tPA

Complexes were formed by mixing sc-tPA or tc-tPA with PAI-1 in excess (10–20% on an activity basis). In order to purify the complexes from excess active PAI-1 and the inactive PAI-1, affinity chromatography on fibrin–Sepharose was used (Table 1). The purified complexes had no detectable PAI-1 or tPA activities. As Table 1 shows, the molar ratios of PAI-1 antigen to tPA antigen in both complexes were close to 1, indicating a 1:1 stoichiometry.

The complexes were examined by SDS/PAGE followed by silver staining. As Fig. 1 shows, the complex of sc-tPA with PAI-1 has an $M_r$ of about 110000, both before and after reduction. In contrast, the complex between tc-tPA and PAI-1 has an $M_r$ of 110000 before reduction. After reduction three major bands are seen, one at $M_r$ 80000 and a doublet at about 30000, corresponding to a complex between the B-chain of tPA and PAI-1 and the free A-chain of tPA respectively (Fig. 1). The A-chain is known to occur in two variants having different $M_r$ values, attributable to differences in glycosylation [24]. In addition to the complexes, faint bands corresponding to the parent molecules are also visible on the gels; this is most likely due to small amounts of the complexes dissociating during the sample preparation. Indeed, this phenomenon was much more pronounced if the samples were boiled in the SDS buffer before PAGE instead of being incubated at 80 °C.

**Table 1. Purification of the complexes between sc-tPA or tc-tPA and PAI-1 by affinity chromatography on fibrin–Sepharose**

PAI-1 antigen and tPA antigen were determined in the different steps.

| Complex... | sc-tPA–PAI-1 | | | tc-tPA–PAI-1 | | |
|---|---|---|---|---|---|---|
| Step | PAI-1 antigen (μg) | tPA-antigen (μg) | tPA/PAI-1 quotient (mol/mol) | PAI-1 antigen (μg) | tPA-antigen (μg) | tPA/PAI-1 quotient (mol/mol) |
| Starting material | 1017 | 498 | 0.36 | 1076 | 513 | 0.37 |
| Break-through, fibrin–Sepharose | 564 | <1 | – | 277 | <1 | – |
| Eluate, fibrin–Sepharose | 189 | 264 | 1.03 | 289 | 365 | 0.97 |

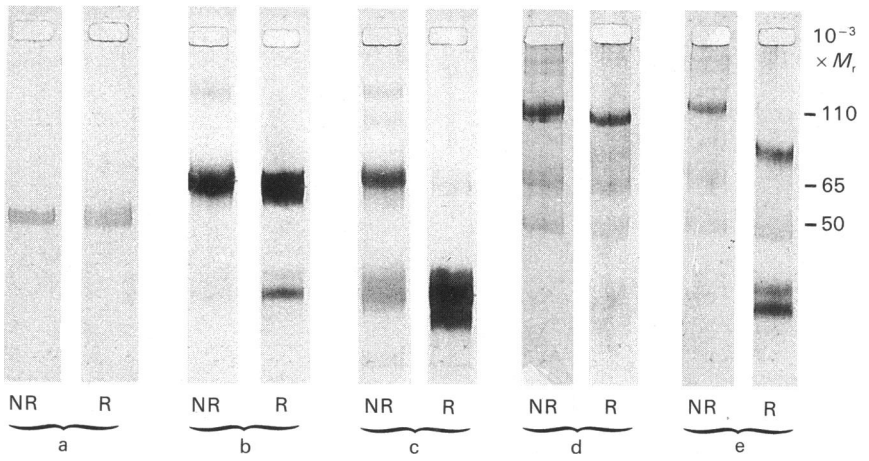


**Fig. 1. SDS/PAGE of the complexes between sc-tPA (or tc-tPA) and PAI-1**

The gels [10% (w/v) polyacrylamide] were subjected to electrophoresis with non-reduced (NR) and reduced (R) samples and subsequently stained with silver. The samples were as follows: a, PAI-1; b, sc-tPA; c, tc-tPA; d, sc-tPA–PAI-1 complex; e, tc-tPA–PAI-1 complex.

**Table 2. *N*-Terminal amino acid sequences of the complexes sc-tPA–PAI-1 and tc-tPA–PAI-1**

About 290 pmol of sc-tPA–PAI-1 complex and 340 pmol of tc-tPA–PAI-1 complex were analysed. All amino acid phenylthiohydantoin derivatives reported in the first five steps were clearly identified (44–95 pmol of each residue for sc-tPA–PAI-1 complex and 88–130 pmol of each residue for tc-tPA–PAI-1 complex respectively. In addition, some trace amounts (tr) were seen.

| | | Amino acids | |
|---|---|---|---|
| Step | Complex... | sc-tPA–PAI-1 | tc-tPA–PAI-1 |
| 1 | | Ser Met Val His(tr) Gly(tr) | Ser Ile Met Val |
| 2 | | Tyr Ala His Lys(tr) | Tyr Lys Ala His |
| 3 | | Gln Pro His Val(tr) | Gln Gly Pro His Val(tr) |
| 4 | | Val Glu Pro Leu(tr) | Val Gly Glu Pro |
| 5 | | Ile Glu Pro Tyr(tr) | Ile Leu Glu Pro |

## *N*-Terminal amino acid sequence analysis of tPA PAI-1 complexes

In order to establish whether PAI-1 was proteolytically cleaved by sc-tPA or tc-tPA during complex-formation, *N*-terminal amino acid sequence analysis was performed on the complexes. The results are summarized in Table 2.

In the complex between sc-tPA and PAI-1, three residues in about equal amounts were identified for the first five steps. In the complex between tc-tPA and PAI-1, four clearly identified residues in about equal amounts were found. The data are consistent with the known sequences in PAI-1, sc-tPA and tc-tPA respectively. However, in addition, in both complexes a new sequence was found,

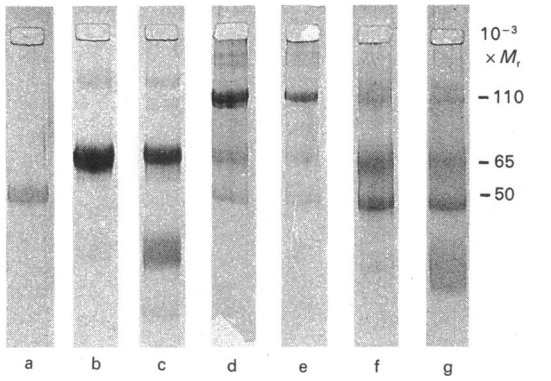


**Fig. 2. SDS/PAGE of the complexes between sc-tPA (or tc-tPA) and PAI-1 and after dissociation in aq. $NH_3$ (0.1 mol/l)**

The gels [10% (w/v) polyacrylamide] were run using non-reduced samples only and subsequently stained with silver. The samples were as follows: a, PAI-1; b, sc-tPA; c, tc-tPA; d, intact sc-tPA–PAI-1 complex; e, intact tc-tPA–PAI-1 complex; f, sc-tPA–PAI-1 complex after treatment with $NH_3$; g, tc-tPA–PAI-1 complex after treatment with $NH_3$.

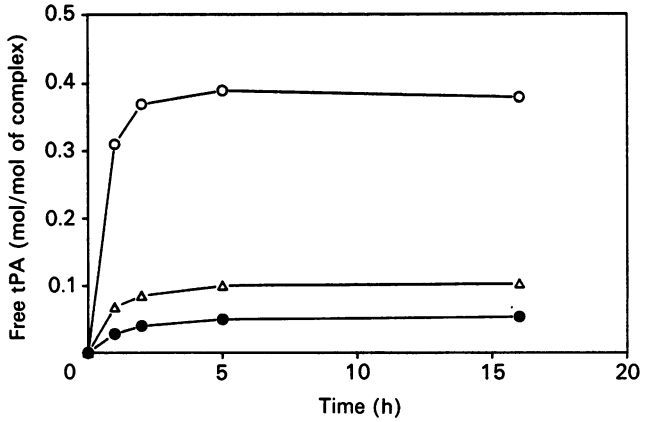


**Fig. 3. Generation of tPA activity by treatment of the tc-tPA–PAI-1 complex with $NH_3$**

The enzyme–inhibitor complex (240 mg/l) was dissolved in phosphate (0.05 mol/l) buffer, pH 7.3, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l) (●), Tris/HCl (0.05 mol/l) buffer, pH 7.8, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l) (△) or $NH_3$ (0.1 mol/l) (○) and incubated at 25 °C. After different periods of time, 10 μl samples were taken for assay of tPA activity using the chromogenic substrate S-2288 [0.6 mmol/l in Tris/HCl (0.05 mmol/l) buffer, pH 7.8, containing NaCl (0.1 mol/l) and Tween 80 (0.1 g/l)].

namely Met-Ala-Pro-Glu-Glu-. This sequence originates from the *C*-terminal portion of the intact PAI-1 molecule and corresponds to amino acid residues 347–351 [6].

### Dissociation of complexes between PAI-1 and sc-tPA or tc-tPA

The stability of the complexes was studied. In a neutral phosphate buffer the complexes were hydrolysed slowly, but treatment of the complexes with the nucleophilic agent $NH_3$ (0.1 mol/l) caused a complete dissociation, as demonstrated by SDS/PAGE (Fig. 2). The $M_r$ values for sc-tPA and tc-tPA released from the complexes were unchanged. By contrast the $M_r$ of PAI-1 was slightly decreased to about 47000, in agreement with the release of a small peptide.

The appearance of tPA activity on incubating the complexes in different solutions was also studied. Treatment of the complex between tc-tPA and PAI-1 with $NH_3$ generated up to 0.4 mol of tPA/mol (Fig. 3). However, in Tris/HCl buffer, pH 7.8, and in a sodium phosphate buffer, pH 7.3, less amidolytic activity was generated, corresponding to 0.10 and 0.05 mol of tPA/mol respectively. Similar results were obtained with the sc-tPA–PAI-1 complex (results not shown), but the experiments were technically more difficult, owing to the poor sensitivity of the substrate towards sc-tPA.

## DISCUSSION

Our group has previously investigated the kinetics of the reaction between PAI-1 and plasminogen activators [17]. The reaction rates were very high and dependent on a 'second-site'-mediated interaction in addition to the interaction between the enzyme active sites and the inhibitor 'reactive site'.

In the present work we have studied the structural events taking place during the reaction between PAI-1 and the plasminogen activators sc-tPA and tc-tPA. It has been very difficult to obtain functionally active PAI-1 in a pure form, because the molecule is labile [11,12,15,18]. Therefore the present investigation was carried out with guanidinium chloride-re-activated PAI-1, containing about equal amounts of active and inactive material. The complexes obtained between PAI-1 and tPA forms were effectively purified from excess PAI-1 by using affinity chromatography on fibrin–Sepharose. The subsequent structural analysis of the complexes by SDS/PAGE and *N*-terminal amino acid sequence determination demonstrated that a stoichiometric 1:1 complex is formed between PAI-1 and both forms of tPA. In agreement with the function of other serpins [8,25–27], the 'bait' peptide bond in PAI-1 is cleaved in its complexes with both tc-tPA and sc-tPA. The data thus gives important information on two different aspects of these proteins: first, tPA is definitely proteolytically active in its single-chain form, since it has the capacity to cleave the 'bait' peptide bond in PAI-1. Secondly, the 'bait' peptide bond in PAI-1 has been unequivocally identified as $Arg^{346}$–$Met^{347}$, situated 33 residues from the *C*-terminus. The same Arg–Met peptide bond was suggested previously from sequence similarities between PAI-1 and the other serpins [6]. Also, this peptide bond was found to be cleaved by enzymic amounts of plasminogen activators using highly denatured PAI-1 [28].

In agreement with results for other serpins [26,27,29,30], the complexes of PAI-1 with sc-tPA and tc-tPA are also sensitive to nucleophilic agents. Complete dissociation is obtained in aq. $NH_3$ at 0.1 mol/l, and about 0.4 mol of functionally active tPA is recovered per mol of complex. By SDS/PAGE the PAI-1 moiety obtained from the complex in this way had a slightly lower $M_r$ when compared with that of the 'virgin' inhibitor. This accords well with the observation that the *C*-terminal 33 residues have been cleaved off. In many of the serpins this peptide remains tightly bound to the

complex. Indeed, in spite of the fact that the 'bait' peptide bond is cleaved in the complexes, separation of the peptide from the rest of the complex has only been achieved in the presence of SDS [27,31]. Mainly because of limited availability of the PAI-1–tPA complexes, we have not yet been able to isolate the *C*-terminal PAI-1 peptide from these complexes. Nevertheless, all the available data suggest that the inhibitor mechanism for PAI-1 is quite similar to that of the other inhibitors in the serpin family.

Financial support was obtained from the Swedish Medical Research Council (Project no. 05193), the Magnus Bergvalls Foundation, the Karolinska Institute, Biopool AB and the Swedish Cancer Society (2515-B89-02XB). Excellent technical assistance by Ms. Tove Lindén, Ms. Anita Lundblad and Ms. Iréne Sjöström is gratefully acknowledged.

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Received 22 May 1989/14 August 1989; accepted 22 August 1989