Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Plasminogen activator inhibitor 1 (PAI-1) in its active conformation: crystallization and preliminary X-ray diffraction data

Pieter K. A. Willems,[a] Anja Rabijns,[a] Kathleen Aertgeerts,[a] Nele Vleugels,[b] Isabelle Knockaert,[b] Hendrik L. De Bondt,[a] Camiel J. De Ranter[a] and Paul J. Declerck[b]*

[a]Laboratory for Analytical Chemistry and Medicinal Physicochemistry, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium, and [b]Laboratory for Pharmaceutical Biology and Phytopharmacology, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium

Correspondence e-mail: paul.declerck@farm.kuleuven.ac.be

Received 22 July 1998
Accepted 15 October 1998

Because of its intrinsic lability, wild-type plasminogen activator inhibitor 1 (PAI-1) cannot be crystallized in its active conformation. Therefore, a stable variant of PAI-1 was used to retain the active conformation during crystallization. Four different crystallization conditions were evaluated in detail and two major types of crystals were detected. Whereas solutions consisting of either (i) cacodylate and sodium acetate, (ii) lithium sulfate and polyethylene glycol 4K, or (iii) imidazole, sodium chloride and sodium potassium phosphate buffer revealed thin platelet crystals, a solution (iv) containing ammonium acetate, citrate and polyethylene glycol 4K appeared to enhance the formation of clustered brush-like crystals. Crystals grown under condition (iii) were found to be suitable for X-ray data collection and consequent structural investigation. Data collection was 79.8% complete with a maximum resolution of 2.92 Å. Importantly, PAI-1 retained its functional properties under all conditions.

1. Introduction

Plasminogen activator inhibitor 1 (PAI-1) is a glycoprotein with an apparent molecular mass of 50 kDa (Van Mourik *et al.*, 1984). It is considered to be the major physiological inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Sprengers & Kluft, 1987; Alessi *et al.*, 1988). Increased levels of PAI-1 in plasma are often associated with an increased thrombotic risk (Declerck *et al.*, 1994; Kruithof, 1988).

PAI-1 belongs to the serpin (serine-protease inhibitors) superfamily, a group of more than 40 proteins which share a sequence homology of 35% (Pannekoek *et al.*, 1986; Ny *et al.*, 1986; Ginsburg *et al.*, 1986; Andreasen *et al.*, 1986; Huber & Carrell, 1989; Gils & Declerck, 1999).

A special feature of PAI-1 is that it can adopt three different conformations: latent, substrate and active. The active conformation reacts with its target proteinase (Hekman & Loskutoff, 1985) by the formation of a Michaelis complex, followed by the formation of an SDS-stable covalent complex. The substrate conformation (Declerck *et al.*, 1992; Urano *et al.*, 1992; Munch *et al.*, 1993) interacts with the target proteinase, resulting in the cleavage of the bait peptide bond (P1–P1′) of PAI-1 without formation of a covalent complex (Declerck *et al.*, 1992). The latent conformation, formed spontaneously through conformational transitions occurring in the labile active conformation, does not react with the target proteinase (Hekman & Loskutoff, 1985).

Elucidation of the three-dimensional structure of the latent conformation showed that the latency arose from to a partial insertion (P16 to P4) of the reactive-site loop into β-sheet *A*, while the other part (P3 to P10′) remained as an extended loop on the outer surface (Mottonen *et al.*, 1992). The structure of a cleaved substrate variant of PAI-1 shows an insertion of the N-terminal part of the reactive-site loop (P16-P1) into β-sheet *A*, resulting in a separation of the two newly generated ends (P1 and P1′ residues) by 70 Å (Aertgeerts *et al.*, 1995).

In the current report, we describe the crystallization of an active stable PAI-1 variant (Berkenpas *et al.*, 1995) and the collection of preliminary X-ray diffraction data from thin platelet crystals. Density studies were carried out to determine the number of active PAI-1 molecules in the unit cell.

2. Materials and methods

2.1. Expression and purification of the PAI-1 stab-mutant

A stable variant of PAI-1 (PAI-1-stab) containing the mutations N150H, K154T, Q319L and M354I (Berkenpas *et al.*, 1995) was constructed by site-directed mutagenesis using the appropriate oligonucleotides. Expression in *Escherichia coli* and subsequent purification was performed as described in Gils *et al.* (1996). Briefly, *E. coli* MC1061 cells transformed with the appropriate expression plasmid were grown and PAI-1 synthesis was

induced. Subsequently, cells were harvested, lysed and the obtained supernatant was subjected to purification. Purification was performed at 277 K using ion-exchange chromatography including SP–Sepharose and heparin–Sepharose columns.

2.2. Crystallization and data collection

The hanging-drop vapour-diffusion technique at 296 K was used to crystallize PAI-1-stab. The wells of a Linbro multiwell tissue-culture plate (Linbro, catalogue No. 76-033-05) were filled with 1 ml of the different crystallization solutions of the sparse screening method of Jancarik & Kim (1991). The 8 µl protein droplets consisted of 4 µl of protein solution (3.0 mg ml^{-1}) in 50 m*M* *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethane (HEPES) buffer pH 7.5, 0.6 *M* sodium chloride, 2 m*M* glutathione and 4 µl reservoir solution. Microscope cover slips were siliconized with Prosil, prior to the addition of the droplets. Four conditions were selected for further investigation. These were: (i) 0.1 *M* cacodylate and 1.3 *M* sodium acetate, (ii) 0.8 *M* lithium sulfate and 2% polyethylene glycol 4K, (iii) 0.1 *M* imidazole, 10% sodium chloride and 0.2 *M* sodium potassium phosphate buffer and (iv) 0.2 *M* ammonium acetate, 0.1 *M* citrate and 30% polyethylene glycol 4K.

Collection of diffraction data was performed at DESY/EMBL (Hamburg) using an MAR Research image-plate detector and synchrotron radiation of wavelength 0.84 Å (BW7B). The software packages *DENZO* and *SCALEPACK* (Otwinowski, 1993) were used for data reduction and scaling, respectively.

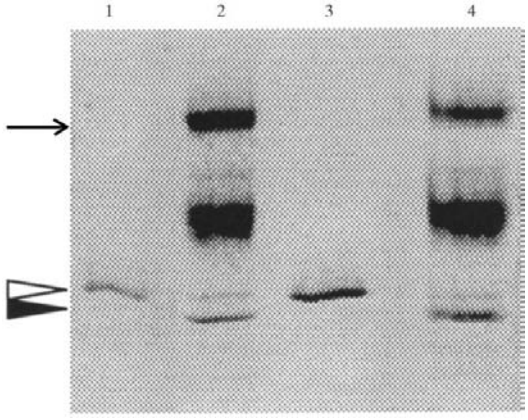

Figure 1
SDS–PAGE of PAI-1-stab. Lanes 1 and 2, PAI-1-stab before crystallization; lanes 3 and 4, PAI-1-stab after crystallization. PAI-1-stab was subjected to SDS–PAGE as such (lanes 1 and 3) or after incubation with a twofold molar excess of t-PA (lanes 2 and 4). PAI-1-stab was analyzed at a concentration of 100–250 µg ml^{-1}. The arrow indicates the migration position of the PAI-1-stab–t-PA complex, originating from the active form of PAI-1-stab. The open and closed arrow head indicate the migration position of intact and cleaved PAI-1-stab, respectively.

Figure 2
A typical PAI-1-stab crystal as grown in 0.1 *M* imidazole buffer, 0.2 *M* sodium/potassium phosphate and 10% NaCl. The crystal dimensions are approximately 0.4 × 0.2 × 0.01 mm.

2.3. Densitometric study

Densitometry was performed using the Ficoll solutions technique (Westbrook, 1985). Briefly, Ficoll solutions of increasing density (ranging between 1.07 and 1.24 g cm^{-3}) were loaded on a 1.5 mm diameter capillary. Crystals were added to the capillary and their relative position was measured after centrifugation.

3. Results and discussion

Thin platelet crystals appeared typically after 4 d in solutions containing (i) 0.1 *M* cacodylate, 1.3 *M* sodium acetate, (ii) 0.8 *M* lithium sulfate, 2% polyethylene glycol 4K and (iii) 0.1 *M* imidazole, 10% sodium chloride, 0.2 *M* sodium potassium phosphate buffer pH 7.5, reaching a maximum size of 0.2 × 0.6 × 0.02 mm after 10 d at 296 K. Growth of brush-like crystals was supported in 0.2 *M* ammonium acetate, 0.1 *M* citrate and 30% polyethylene glycol 4K.

SDS–PAGE analysis (Fig. 1) of fully grown crystals obtained under condition (iii) revealed that the mutant was crystallized in its functionally active conformation, an observation which is in agreement with its highly stable properties (Berkenpas *et al.*, 1995). It is also of interest to note that the currently used conditions contain a high sodium chloride concentration which is known to stabilize the active conformation of PAI-1 (Sancho *et al.*, 1994).

Table 1
Data-collection and reduction statistics.

Values in parentheses indicate data in the highest resolution shell.

	PAI-1-stab crystals†
Wavelength used (Å)	0.84
Resolution limit (Å)	2.92 (2.97–2.92)
Total observations	46813 (1963)
Unique reflections	29848 (1436)
Completeness of all data (%)	79.8 (75.3)
Completeness of data ($I > 2\sigma$) (%)	74.5 (62.5)
Mean I/σ	9.8 (6.4)
R_{sym} value‡ (%)	5.5 (13.0)

† Crystals grown under condition (iii). ‡ R_{sym} (%) = $100 \times \sum_i |\langle I_i \rangle - I_i| / \sum_i I_i$, where $\langle I_i \rangle$ is the average of the I_i over all symmetry equivalents.

The diffraction data obtained from platelet crystals grown under condition (iii) (Fig. 2) demonstrate the presence of a unit cell with parameters a = 65.3, b = 75.1, c = 103.1 Å and α = 85.5, β = 86.0, γ = 64.3°. The crystals belong to the triclinic space group *P*1. According to the method of Westbrook (1985), the density of the crystal is 1.17 g cm^{-3}, which is in agreement with the calculated value of 1.19 g cm^{-3}. Based on the density measurements of the crystal and the volume of the unit cell, it can be estimated that the unit cell contains four PAI-1 molecules.

According to the method of Matthews (1968), the V_m was calculated to be 2.27 Å^3 Da^{-1}, assuming four molecules in the asymmetric unit, with a solvent content of 45.8%, which is in compliance with the generally accepted solvent content of protein crystals.

Data collection was 79.8% complete with a maximum resolution of 2.92 Å. Detailed statistics are given in Table 1.

4. Conclusions

We report the crystallization and preliminary X-ray data up to a resolution of 2.92 Å for the active conformation of plasminogen activator inhibitor-1 (PAI-1). To the best of our knowledge, crystallization of PAI-1 in its active conformation has not previously been reported.

Further refinement and structure investigation will provide better insight into the molecular determinants of the functional

and conformational properties of the unique serpin PAI-1.

HDB is a Postdoctoral Research Fellow of the Fund for Scientific Research (FWO-Vlaanderen, Belgium). AR is a recipient of a fellowship of the 'Vlaams Instituut voor de Bevordering van het Wetenschappelijk Onderzoek in de Industrie (IWT)'. NV is a Research Assistant of the Fund for Scientific Research (FWO-Vlaanderen, Belgium). This work was supported in part by a grant from the Fund for Scientific Research (FWO-Vlaanderen, Belgium, project G.0266.97). We thank the European Union for support of the work at EMBL Hamburg through the HCMP to Large Installations Project, contract No. CHGE-CT93-0040.

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