High-resolution structure of the stable plasminogen activator inhibitor type-1 variant 14-1B in its proteinase-cleaved form: A new tool for detailed interaction studies and modeling

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(RECEIVED May 29, 2008; FINAL REVISION July 10, 2008; ACCEPTED July 11, 2008)

Abstract

Wild-type plasminogen activator inhibitor type-1 (PAI-1) rapidly converts to the inactive latent state under conditions of physiological pH and temperature. For in vivo studies of active PAI-1 in cell culture and in vivo model systems, the 14-1B PAI-1 mutant (N150H-K154T-Q319L-M354I), with its stabilized active conformation, has thus become the PAI-1 of choice. As a consequence of the increased stability, the only two forms likely to be encountered are the active or the cleaved form, the latter either free or complexed with target proteinase. We hereby report the first structure of the stable 14-1B PAI-1 variant in its reactive center cleaved form, to a resolution of 2.0 Å. The >99% complete structure represents the highest resolved structure of free cleaved PAI-1. This high-resolution structure should be of great use for drug target development and for modeling protein–protein interactions such as those of PAI-1 with vitronectin.

Keywords: crystal structure; plasminogen activator inhibitor type 1; serpin; structure-based drug targeting; vitronectin

Plasminogen activator inhibitor type-1 (PAI-1) is a 379-residue glycoprotein belonging to the serpin family of serine protease inhibitors (for review, see Gettins 2002). PAI-1 is a key regulator of such physiological processes as wound healing and tissue remodeling through its regulation of the urokinase-type and tissue-type plasminogen activators. This key role implicates PAI-1 in many pathophysiological conditions such as cardiovascular

diseases and cancer metastasis and spread (for review, see Durand et al. 2004; Gils and Declerck 2004).

Unlike most serpins, whose metastable active conformations have long-term stability in the purified state under physiological conditions, PAI-1 spontaneously and rapidly converts from the metastable state to the inactive latent state, with a half-life of 1–2 h (for review, see Wind et al. 2002). This conversion involves insertion of the intact reactive center loop into β -sheet A and loss of strand 1 from β -sheet C. It is thought that this is facilitated by conformational changes around α -helices D and E acting as “flexible joints” that permit the small and large serpin fragments to slide apart and hence allow reactive center loop (RCL) insertion (Mottonen et al. 1992). In vivo PAI-1 binds the extracellular matrix protein vitronectin (VN), which leads to a stabilization of the metastable state and hence may serve to modulate the activity of PAI-1 (for review, see Wind et al. 2002). Binding of VN occurs at two sites. A high-affinity

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Abbreviations: PAI-1, plasminogen activator inhibitor type 1; SERPIN, serine protease inhibitor; RCL, reactive center loop; RccPAI-1, RCL-cleaved PAI-1; VN, vitronectin; SomB, N-terminal somatomedin B domain of VN; SER-CAT, Southeast Regional Collaborative Access Team; sX, β -sheet X; s#X, strand # of β -sheet X; hX, α -helix X.

Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.036707.108>.

binding site only found in the active form of PAI-1, and which involves the somatomedin B domain, is localized in the flexible joint region, whereas a secondary lower affinity binding site, which does not involve the somatomedin B domain, present in both active and relaxed PAI-1, is located on hD and hE (Jensen et al. 2002; Schar et al. 2008a,b).

Because of the difficulty in studying the properties of such a conformationally unstable protein, attempts have been made to create a more stable form of PAI-1. Screening of a library of randomly mutated PAI-1 variants produced one, named 14-1B, that contained the four-point mutations N150H-K154T-Q319L-M354I (Berkenpas et al. 1995) and has an increased half-life of ~100 h (Vleugels et al. 2000; Dupont et al. 2006). The 14-1B variant has accordingly been widely adopted as the preferred form of PAI-1 for studying the effect of active PAI-1 in cell culture and in *in vivo* assays. For example, transgenic mice expressing 14-1B have been used to investigate the role of the plasminogen activation system in normal development, polycystic ovarian syndrome, and bone homeostasis (Eren et al. 2002, 2007; Devin et al. 2007; Nordstrom et al. 2007). 14-1B protein has also been used in cell culture assays studying migration and adhesion to extra cellular matrix proteins (Degryse et al. 2004; Stefansson et al. 2007).

Given the widespread use of 14-1B for functional studies, it is important to have high-resolution structural data on it in both the active and cleaved forms, especially since there is evidence, from drug targeting studies and binding of conformationally sensitive proteins, of structural differences between native PAI-1 and the 14-1B variant (Jensen et al. 2006; Li et al. 2008). Whereas there are several X-ray structures of active 14-1B (Sharp et al. 1999; Nar et al. 2000; Stout et al. 2000), no structure of the cleaved form has been available. We here report the first structure of the 14-1B in the cleaved form, at a resolution of 2.0 Å. The high resolution of the structure and consequent high precision of side chain conformations should make it an invaluable tool for understanding protein interactions and subsequent structure-based mutagenesis and model-based drug targeting on PAI-1.

Results and Discussion

Structure of cleaved 14-1B PAI-1

A high-resolution structure of cleaved 14-1B was solved to 2.0 Å resolution (Fig. 1; Table 1). Because of the near completeness of the data set and the consequent high quality of the electron density maps, it was possible to distinguish between even closely related side chains. For example, in the PAI-1 clone used, position 10 is an Arg rather than the His that occurs elsewhere. This was easily

Figure 1. Example of electron density and ribbon representation of the cleaved 14-1B structure. (A) An example of 2fo-fc electron density ($\sigma = 2$). The C_{α} position of Ala335 is marked (white arrow). (B) Ribbon presentation of cleaved 14-1B structure with important structural elements labeled. Color scheme is a B-factor scale (relative B-factor to color conversion shown at bottom left) from dark blue to red. (C) As in B, but rotated 90° anticlockwise around a vertical axis showing the location and conformation of the four substituted side chains.

seen in the calculated difference maps, which predicted the residue difference from the starting model (data not shown). An example of the quality of the maps is shown in Figure 1A, which is a close-up of β -sheet A (sA) centered around Ala335.

The structure of cleaved 14-1B exhibits the classical cleaved serpin form consisting of nine α -helices (hA–hI) and three β -sheets (sA, sB, and sC). The structure is dominated by the large central sheet A forming the scaffold of the molecule. As expected for the cleaved form, the N-terminal part of the RCL, residues 331–346, is inserted as s5A completing the six-stranded antiparallel sheet A. Apart from the N-terminal extension (Met12–Ser1), which is not part of the actual PAI-1 sequence and is expected to be highly flexible, the only residues without visible electron density were the three residues following the P1 cleavage site at Arg 346 (Met347–Ala348–Pro349). These three residues were seen in the 1995 structure of a cleaved substrate form of PAI-1 (A335P variant) extending away from the molecule and supported by neighboring symmetry related molecules (Aertgeerts et al. 1995), suggesting that this was a crystal packing artifact. We conclude that these three residues,

Table 1. *Crystallographic statistics*^a

Space group	P21
Unit cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	76.60, 104.01, 76.43
$\alpha = \gamma$, β (deg)	90.00, 101.93
Resolution limits (Å)	20.0–2.0
Total measurements	241,677
Unique reflections	75,140
Completeness (%)	98.0 (98.6)
$\langle I/\sigma(I) \rangle$	9.31 (2.51)
R_{merge}	0.127 (0.551)
Average redundancy	3.2

^aHighest resolution bin values in parentheses (2.1–2.0).

which extend from beyond the β -sheet C, must be protruding unstructured into the solvent.

From Figure 1B it is clear that the distribution of Wilson B-factors is characterized by generally uniform low values consistent with the well-defined electron density. Some deviations to higher values are seen in such areas as s1A, sC, hE, and hF, which is in agreement with an expected higher degree of flexibility, as these regions can be argued to be less tightly associated with the body of the serpin scaffold.

Structural impact of the four mutations

As seen in Figure 1C, most of the structural effect of the four substitutions present in the 14-1B variant is minimal in the cleaved form because of their now solvent-exposed positions following the transition from the active to the cleaved conformations. The stabilizing interactions between three of the mutated residues (150, 154, and 319) and the 3_{10} helix induced in the loop between hF and s3A, as proposed in Sharp et al. (1999), have been eliminated by the structural rearrangement after the expansion of sA. Although the fourth mutated residue, methionine replacing isoleucine at position 354, is in a buried hydrophobic environment and still makes contacts in the cleaved structure, this position has less structural importance in the cleaved structure. We conclude therefore that our cleaved 14-1B structure must very closely resemble the true cleaved wild-type structure. In contrast, the currently available structure of cleaved “wild-type” PAI-1 is of a substrate form in which residue 335 in the reactive center loop has been replaced by proline (hence making it a substrate form rather than a proteinase inhibitor). In this structure the insertion of proline 335 into β -sheet A results in alterations in the H-bonding pattern of the sheet, and causes perturbation of the local main chain conformation. Our structure of cleaved 14-1B is thus not only of use for comparison with native 14-1B but as the best structure of a cleaved wild-type PAI-1. This is also of

significance since it demonstrates, at least for PAI-1, that introduction of mutations that enhance the stability of the metastable state has not altered the structure of the most stable, cleaved loop-inserted state.

Why the somatomedin B domain of VN does not bind cleaved 14-1B

Given that our present structure is likely to be the most representative of cleaved PAI-1 species, whether for 14-1B or for wild type, it can now be used to provide insight into why the somatomedin B (SomB) domain of VN shows greatly reduced affinity for such relaxed forms of PAI-1. The binding of SomB is largely governed by hydrophobic interactions between residues in the 3_{10} helix of the somB domain and the area comprising hE-s1A-hF in PAI-1 (Fig. 2B; Zhou et al. 2003). Among the few polar interactions in the complex, there is a central salt bridge between Arg101 of PAI-1 and Asp22 of SomB, and hydrogen bonding between the hydroxyl of Tyr27 of SomB and Asp138 in hF of PAI-1. Hydrogen bonding is also observed between side chains Gln123, Asp125, and Arg131 of PAI-1 with the main chain of Phe13, Tyr28, and Arg131 in SomB, respectively, and between Glu23 of SomB and the main chain of Thr120 of PAI-1. Residues Phe98, Val124, Ile 135, Ile136, and Trp139 of PAI-1 form a hydrophobic cleft covering a large portion of the binding interface with Tyr27, Tyr28, and an inserted Leu24 side chain of SomB contributing considerably to the overall binding energy as predicted in Jensen et al. (2002). Upon insertion of the RCL, the expansion of sA pushes s1A and s2A outward through the plane formed by hE and hF (Fig. 2A,C). During this structural rearrangement of the primary VN-binding site, several side chain positions are reoriented to become incompatible with SomB binding. Thus, Thr120 flips from an inward-pointing buried position to an outward position. This forces Lys122 to point downward parallel to s1A, placing its positively charged side chain in the middle of the hydrophobic patch where Leu24 in SomB inserts in active 14-1B. Apart from adding potential main chain clashes between s1A and a docking SomB, the sliding out of s1A and s2A causes drastic changes to the hydrophobic cleft. Phe98 has been forced out toward a location that was the center of the binding pocket in active PAI-1. This in turn results in a side chain flip of Trp139 causing a complete loss of the solvent-shielded hydrophobic patch. All these changes are probably sufficient to account for the large reduction in primary affinity for VN.

Ligand-binding cavity for drug targeting

A binding cavity for a small peptide has previously been shown to exist in the flexible joints region of both latent and the cleaved substrate forms of PAI-1 (Jensen et al.

Figure 2. Structural comparison between rcc14-1B and active 14-1B complexed with SomB. (A) Overlay of cleaved 14-1B (gray) with active 14-1B (red) in complex with SomB (green). The PAI-1 structures are aligned by main-chain atoms s5A and s6A. (B) Close-up on the binding interface in the complex. Important side chains are labeled, and hydrogen-bonding interactions are shown in green. (C) Close-up of same area in cleaved 14-1B (gray) aligned by hE and hF. Residue numbers for PAI-1 are in black and those for SomB are in blue.

2006). This pocket was shown by modeling to be able to accommodate the peptide, and this location was confirmed by mutagenesis. It was also shown that there was preferential binding of peptide to relaxed compared with active conformations of 14-1B. This binding pocket may thus serve as a target area both for neutralizing the inhibitory activity of PAI-1 and for using PAI-1 as a “shuttle” molecule for small ligands. It was previously shown in Jensen et al. (2006) that the cavity in active 14-1B was highly distorted, in agreement with highly reduced binding of ligands compared with the relaxed form. A comparison between the cavity in active 14-1B PAI-1 (Fig. 3A) and in the present cleaved structure of 14-1B (Fig. 3B) highlights these differences. The cavity in cleaved 14-1B is deep and resembles the cavity shown in latent PAI-1 in Jensen et al. (2006). The presence of a “latent-like” cavity makes the high-resolution structure of rcc14-1B a good candidate for structure-based modeling and design of PAI-1 binding ligands targeting this cavity.

Materials and Methods

Expression and purification

A pQE-30 vector (Qiagen) with the cDNA sequence corresponding to residues 1–379 from human PAI-1 with the four-

point mutations N150H-K154T-Q319L-M354I was constructed by standard methods and verified by sequencing. The final expressed construct includes an N-terminal extension (MRGSH₆GSAV...) consisting of a hexa-Histidine-tag and a GS-linker followed by the PAI-1 sequence with the numbering Val1-His2-His3-Pro, etc. PAI-1 was expressed and purified in quantities needed for crystallography by the method described in Jensen et al. (2002), with the exception of using *Escherichia coli* SG13009 cells (Qiagen) instead of the BL21 strain. The full-length protein, including N-terminal extension, was confirmed by mass spectroscopy to give the exact theoretical mass of

Figure 3. Close-up comparison of the ligand-binding cavity in the flexible joints region of active 14-1B (1DVM) and rcc14-B (3CVM, present structure). (A) Active 14-1B; (B) cleaved 14-1B. The cavity boundaries are outlined by broken lines. Residues surrounding the cavity are labeled.

44.22 kDa. Sample purity better than 95% was estimated by SDS-PAGE analysis. PAI-1 protein concentration was determined by optical density at 280 nm using the extinction coefficient of 0.8 l/g-cm⁻¹. The PAI-1 protein used throughout this work is the stable 14-1B variant containing the four mutations.

Formation of reactive center cleaved 14-1B

Prior to PAI-1 cleavage with protease, the presence of full inhibitory activity of the protein was confirmed from the complete band shift upon complex formation with urokinase monitored by SDS-PAGE. RccPAI-1 was made by incubation with 1.1- to 1.5-fold excess of trypsin (Sigma), 10–15 min at 37°C in 20 mM Tris pH 7.4, 500 mM NaCl. The proteolytic reaction was subsequently quenched by adding a molar excess of the irreversible trypsin inhibitor TLCK (N- α -p-tosyl-L-lysine chloromethylketone hydrochloride; Sigma). RccPAI-1 was separated from traces of trypsin-PAI-1 covalent complex and excess inactivated trypsin by two rounds of size-exclusion chromatography through a 200 mL Superdex75 column (Pharmacia) in 20 mM Tris pH 7.4, 300 mM NaCl, and 1 mM CaCl₂. Purity of the resulting sample was estimated to be better than 95% by SDS-PAGE. The final sample for crystallography was prepared by concentrating to 8 mg/mL using 10-kDa cutoff spin concentrator columns (Amicon).

Crystallization and data collection

Initial screens were carried out using Hampton research 96-well crystal screens in 96-well single drop plates (Corning). Sitting drops (1 μ L of protein stock + 1 μ L of reservoir solution) were incubated at 20°C whereupon needle-like clusters appeared after 1–2 wk under several conditions. Conditions containing 4.5 M ammonium acetate, pH 7.5–8.5, were optimized by hanging-drop vapor-diffusion in 24-well plates with silanized cover slips (Hampton). Crystals suitable for diffraction (up to 50 $\times$ 200 μ m) were grown in 2- μ L drops against 600 μ L of 0.1 M Tris pH 8.5, 2.5 M ammonium acetate reservoir solution. Crystals appeared within a week and grew to mature size within 1–2 wk, predominantly as single rectangle-like box shapes. Crystals were harvested for data collection by 0.05–0.2 mm nylon loops (Hampton) and snap-frozen in liquid nitrogen. No cryoprotectant was needed as ammonium acetate at concentrations >2 M acts as a cryoprotectant. Data collection was performed at 1.0 Å wavelength, 100 K at the ID-line 22, SER-CAT, Advanced Photon Source, Argonne National Laboratory. HKL2000 (Otwinowski and Minor 1997) was used at the beamline to validate crystal diffraction quality during crystal screening prior to data collection.

Data processing and phasing

Preliminary indexing showed that all studied crystals belonged to the same space group *P*21 with similar space parameters (Table 1). The best single crystal reflection data set was indexed and integrated using XDS from the XDS program package (Kabsch 1993) to well below 2 Å. Scaling with XSCALE (XDS package) of the 305 frames (152.5°) devoid of noticeable radiation damage resulted in a >98% complete 2 Å data set with an average redundancy above 3, $I/\sigma(I)$ above 2.5 in the high-resolution shell, and an overall R_{merge} of 12.7%. The final

structure factor file was prepared by XDSCONV (XDS package) and subroutines in the CCP4 program package (<http://www.ccp4.ac.uk/index.php>). Isomorphous molecular replacement with 9PAI as a model was carried out using PHASER (CCP4) (McCoy et al. 2007), placing 2 molecules in the asymmetric unit with high individual Z-scores >20 and favorable packing. The estimated solvent content of ~64% was in the high end of what was predicted by Matthew's coefficient calculations, suggesting three molecules per asymmetric unit. The following initial rounds of simulated annealing and rigid body refinement in PHENIX (Adams et al. 2002) yielded initial R-factors of 31.6% and 36.3% working and test set, respectively, proving a good starting point for the subsequent model building.

Model building and validation

Extensive model building was performed by hand using COOT (Emsley and Cowtan 2004), with each round followed by refinement using PHENIX applying noncrystallographic symmetry, and in later rounds automatic water picking while gradually optimizing crystallographic-geometric weighting and solvent mask. During final rounds of model building and refinement, hydrogen atoms were added to the structure using REDUCE (through MOLPROBITY interface), and the MOLPROBITY (Davis et al. 2007) online interface (<http://molprobity.biochem.duke.edu/>) was used to guide proper geometry and all intra-atom interactions in the final structure. The >99% complete final model, including several hundred well-defined water molecules, was finally refined to acceptable R-factors of 22.8% and 28.2% working and test set, respectively (Table 2). The final structure exhibited good agreement with ideal geometry (Table 2) and was validated with highly acceptable scores by MOLPROBITY (Table 2). All structure figures were prepared using SWISS-PDBVIEWER 3.7 and POV-RAY 3.6. Protein–protein interaction surfaces were analyzed by online PDBSUM tool (<http://www.ebi.ac.uk/pdbsum/>).

Table 2. Structure refinement and validation statistics

Number of model atoms 12,801 (5985 hydrogen atoms)	
Refinement	
R factor	0.228
R_{free} (3%) ^a	0.282
RMS deviations from ideal geometry	
Bond lengths (Å)	0.006
Bond angles (deg)	0.81
Dihedral angles (deg)	13.4
Average atomic displacement parameters (Wilson B-factor)	
Protein (no hydrogen)	25.5 (33.9)
Solvent (821 H ₂ O)	43.8
Validation (molprobity)	
All-atom clashscore ^b	17.68
Bad rotamers	0.6%
Ramachandran outliers	0.1%
Ramachandran favored	97.5%
Residues with bad bonds	0.3%
Residues with bad angles	0.4%

^a R_{free} based on 2255 reflections.

^bClashscore is the number of serious steric overlaps (>0.4 Å) per 1000 atoms.

Protein Data Bank deposition

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (accession code 3CVM).

Acknowledgments

This work was supported by grant HL79430 from the NIH (National Institutes of Health). The Danish Research Council is acknowledged for funding J.K.J. with a two-year fellowship in the laboratory of P.G.W.G. Bernard Santarsiero is acknowledged for his continued support during data collection and processing. The staff at SER-CAT is acknowledged for their always-excellent beamline support.

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