The active conformation of plasminogen activator inhibitor 1, a target for drugs to control fibrinolysis and cell adhesion

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Background: Plasminogen activator inhibitor 1 (PAI-1) is a serpin that has a key role in the control of fibrinolysis through proteinase inhibition. PAI-1 also has a role in regulating cell adhesion processes relevant to tissue remodeling and metastasis; this role is mediated by its binding to the adhesive glycoprotein vitronectin rather than by proteinase inhibition. Active PAI-1 is metastable and spontaneously transforms to an inactive latent conformation. Previous attempts to crystallize the active conformation of PAI-1 have failed.

Results: The crystal structure of a stable quadruple mutant of PAI-1(Asn150→His, Lys154→Thr, Gln319→Leu, Met354→Ile) in its active conformation has been solved at a nominal 3 Å resolution. In two of four independent molecules within the crystal, the flexible reactive center loop is unconstrained by crystal-packing contacts and is disordered. In the other two molecules, the reactive center loop forms intimate loop–sheet interactions with neighboring molecules, generating an infinite chain within the crystal. The overall conformation resembles that seen for other active inhibitory serpins.

Conclusions: The structure clarifies the molecular basis of the stabilizing mutations and the reduced affinity of PAI-1, on cleavage or in the latent form, for vitronectin. The infinite chain of linked molecules also suggests a new mechanism for the serpin polymerization associated with certain diseases. The results support the concept that the reactive center loop of an active serpin is flexible and has no defined conformation in the absence of intermolecular contacts. The determination of the structure of the active form constitutes an essential step for the rational design of PAI-1 inhibitors.

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Introduction

Plasminogen activator inhibitor 1 (PAI-1), a member of the serpin family of serine proteinase inhibitors, is the main physiological inhibitor of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). These serine proteinases convert plasminogen, an inactive zymogen, to the active enzyme plasmin, which degrades fibrin clots. Epidemiological studies have shown that elevated circulating levels of PAI-1 are associated with coronary heart disease and possibly atherosclerosis [1]. These findings have led to considerable interest in the development of drugs that specifically inhibit PAI-1. Such drugs may have a wider clinical application as the fibrinolytic system is also involved in ovulation, wound healing, angiogenesis, tissue remodeling and neoplasia [2].

Serpins bind to their target enzymes through a highly mobile peptide loop containing the reactive center or scissile bond (denoted P1–P1′). The active form of a serpin is apparently a metastable folding intermediate. Following attack of the reactive center by a target enzyme, conformational strain is released and the serpin–proteinase complex is trapped, probably at the acylenzyme step [3,4]. The complex dissociates very slowly, yielding an irreversibly inactivated cleaved serpin. The cleaved form has undergone a dramatic conformational change in which the N-terminal end of the cleaved reactive center loop has been inserted into the major β sheet (β sheet A), forming a new β strand [5]. A similar loop insertion is believed to occur in the formation of the inhibited complex [3]. PAI-1 is a prototype among serpins due to its ability to convert spontaneously, without cleavage, to an inactive conformation similar to the cleaved form; there is complete insertion of the intact reactive center loop into β sheet A together with release of the first strand of β sheet C [6]. This form of the serpin is termed ‘latent’ as activity of the inhibitor can be restored by denaturation and renaturation. Other serpins can fold into a latent conformation [7] under mild thermal

stress [8], and there is now evidence that latency has a role in the senescence and pathology of antithrombin [9]. In PAI-1, the spontaneous transition to latency may ensure sufficient turnover to allow a rapid physiological response to changing circumstances. In the circulation, most PAI-1 will exist in a tight complex with the adhesive glycoprotein, vitronectin. In such a complex *in vitro*, the lifetime of active PAI-1 is doubled from about two hours to four hours under physiological conditions [10].

Here, we describe the structure of a recombinant mutant of PAI-1 that is more stable in its active form than wild-type PAI-1 [11]. Attempts to crystallize active wild-type PAI-1 have so far been unsuccessful. The structures of latent PAI-1 [6] and two forms of cleaved PAI-1, by itself [12] and with a bound reactive center loop peptide [13], are already known. The addition of our structure of active PAI-1 will make it possible to analyze the full extent of the conformational transitions in PAI-1 without relying on homology modeling.

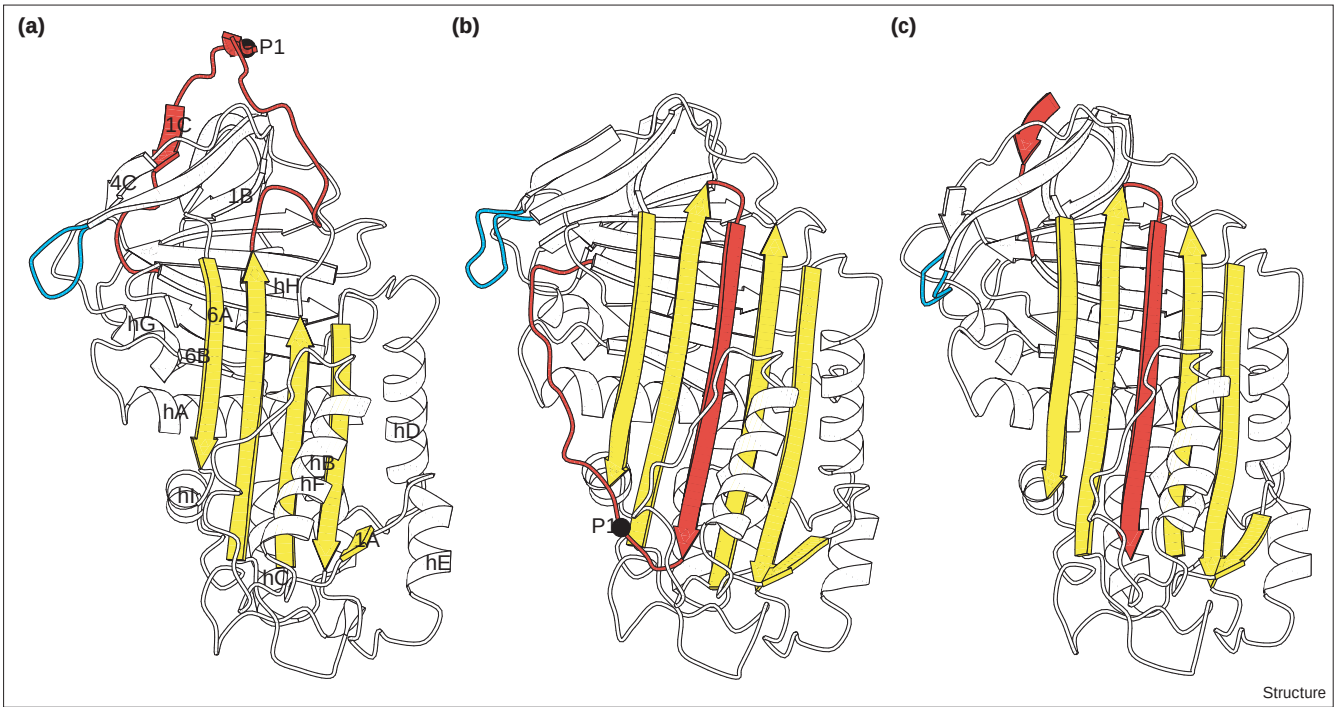
Results and discussion

Comparison with other active serpins

Since the determination of the first serpin structure, that of cleaved α_1-antitrypsin [5], many crystal structures have been solved of serpins in various conformations. Intact, active serpins have been the most difficult to crystallize, but structures are now available for intact forms of α_1-antichymotrypsin [14], antithrombin [15,16] and α_1-antitrypsin [17]. In its general features (see Figure 1), as expected, active PAI-1 resembles other active serpins.

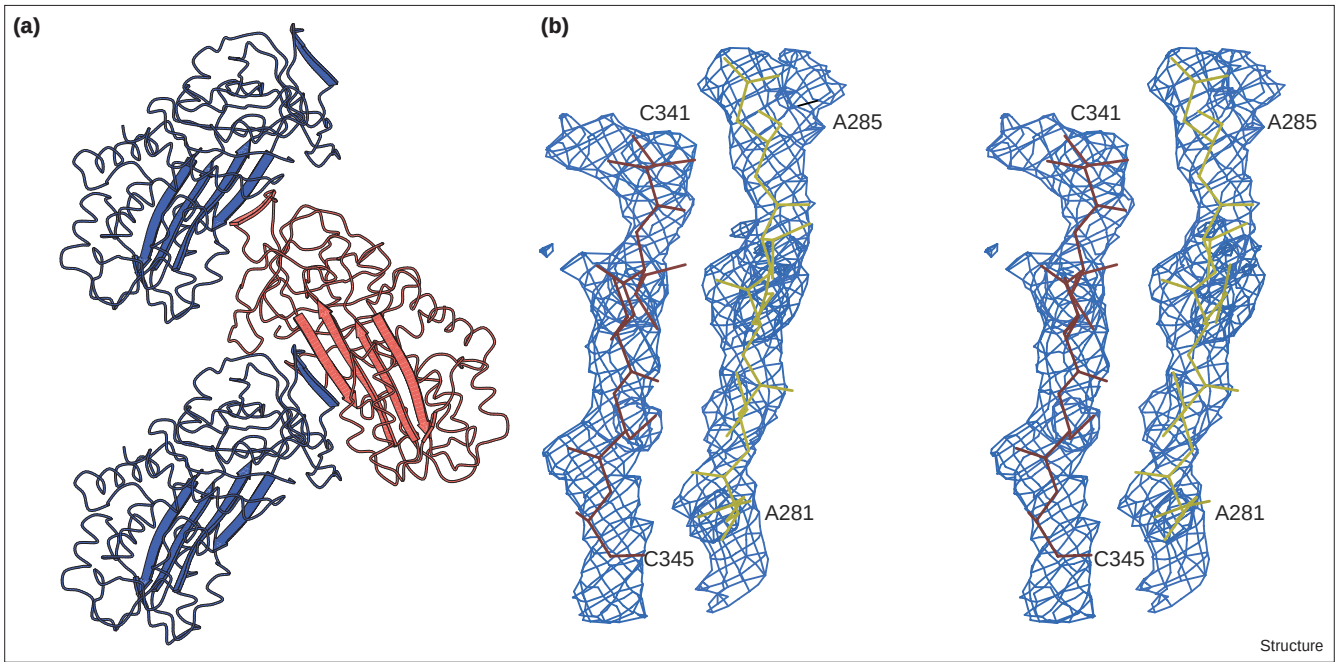
The unusual flexibility of the reactive center loop is shown by its markedly different conformations in the structures of active serpins. In PAI-1, the reactive center loop also shows evidence of mobility, and its conformation in the crystal appears to be significantly influenced by packing contacts. In two of the four independent PAI-1 molecules in the crystal (molecules B and D, discussed below), there are no packing contacts around the loop and it is disordered. However, in the other two molecules the C-terminal portion of the loop is tethered by crystal-packing contacts and is probably induced to adopt an extended conformation. The four molecules have been restrained to be almost identical (root mean square [rms] deviations on all atoms of less than 0.3 Å), with only a few residues at the N termini and in the reactive center loops being allowed to differ significantly. As the A and C molecules are nearly identical (rms deviation of 0.15 Å on all atoms), have the lowest overall temperature factors and have complete reactive center loops, the A

Figure 1



Schematic illustration of the three conformations of PAI-1. The major β sheet (β sheet A) is highlighted in yellow, the reactive center loop is in red and the gate region is shown in cyan. (a) The active conformation of a stable mutant of PAI-1 (this work). (b) The latent conformation of PAI-1 [6]. (c) The cleaved conformation of PAI-1 [12]. In both the latent and cleaved forms of PAI-1, the reactive center loop is inserted into β sheet A to form a new β strand, 4A. (The figure was prepared using the program MOLSCRIPT [40].)

Figure 2



Infinite chain of PAI-1 molecules in the crystal lattice. **(a)** Molecules A (red) and C (two copies in blue) are related by a pseudo-crystallographic 2_1 screw axis. The reactive center loop of one molecule forms a new strand '7A' at the edge of β sheet A of the other molecule. The β strands involved in the interaction are highlighted as broad arrows. (The figure was prepared using the program MOLSCRIPT [40].) **(b)** As a result of the interaction illustrated in (a), density for the reactive center loop is reasonably clear in the A and C molecules. Electron density, after averaging, is shown for the interaction between β strand 6A of molecule A (residues A281–A284, yellow) and the reactive center loop of molecule C (C341–C345, red). For clarity, only electron density contours within 2.5 Å of an atom in the figure are shown. (The figure was prepared using the program XtalView [41].)

molecule is used for discussion. The reactive center loop of PAI-1 could not dock to a proteinase without altering the conformation seen in the crystal, indicating that it must be flexible. The only active serpin that has crystallized in a conformation that could dock to a proteinase is α_1-antitrypsin [17]. As seen in other active serpins, the reactive center loop of PAI-1 appears poised to enter a gap between β strands 3A and 5A.

A new mode of serpin polymerization

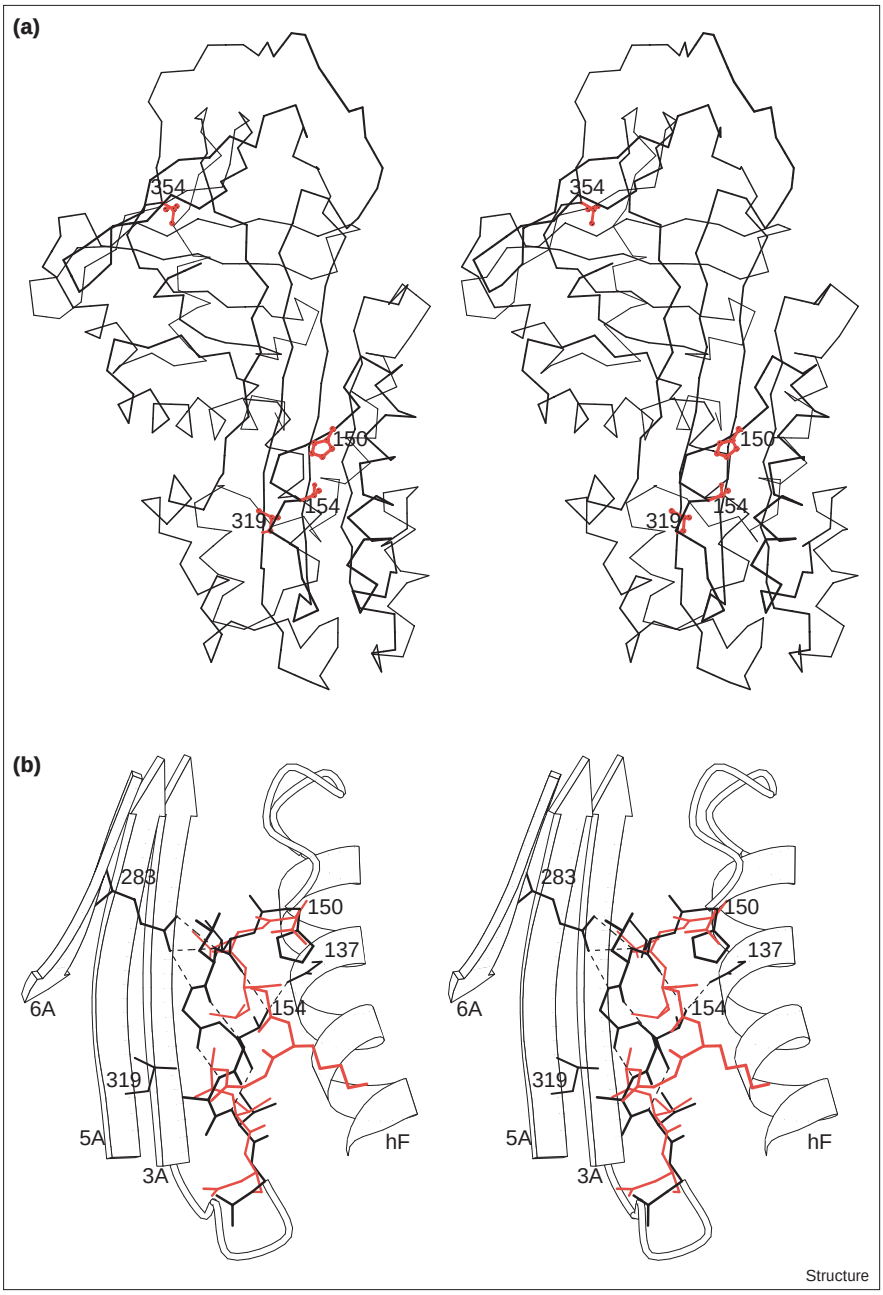
The four PAI-1 molecules in the P1 unit cell display pseudo C2 symmetry. The two unique molecules in the pseudo C2 cell (molecules A and B) form an improper dimer, which is related to a second dimer (molecules C and D) by a 2_1 screw axis. The interaction between this pair of dimers is mediated primarily by an intimate contact between the A and C molecules, in which the reactive center loop of one molecule adds a β strand to the edge of β sheet A of the other molecule. Repeated application of the screw symmetry generates an infinite chain of A and C molecules, shown in Figure 2.

This infinite chain of molecules may be relevant to the formation of pathological polymers of serpin molecules [18], as found in the liver disease associated with the Z variant of α_1-antitrypsin [19]. The mode of polymerization differs from those proposed earlier, however, involving the insertion of the reactive center loop of one molecule into another molecule as an edge strand of β sheet C or the central strand of β sheet A [18]. The interaction in the stable mutant of PAI-1 is reversible, as the crystals can be dissolved to give active PAI-1 (data not shown), but in other serpins such polymers could be more stable. Furthermore, the interaction illustrates the propensity of the reactive center loop to enter into a β sheet, which is central to the serpin mechanism of inhibition.

Factors influencing the latency transition

Although PAI-1 is unusual in the speed of its transition to latency, the relevant structural differences from other serpins are likely to be very subtle. Other inhibitory serpins appear to exist in a metastable state, but have a larger barrier to latency so that one must wait much longer or subject them to harsher conditions. Nonetheless, an increase of only 4 or 5 kcal/mol in the activation energy barrier to latency would make PAI-1 stable for months under physiological conditions. The quadruple mutant has a half-life of 145 h at 37°C [11], so it is sufficiently stable at room

Figure 3

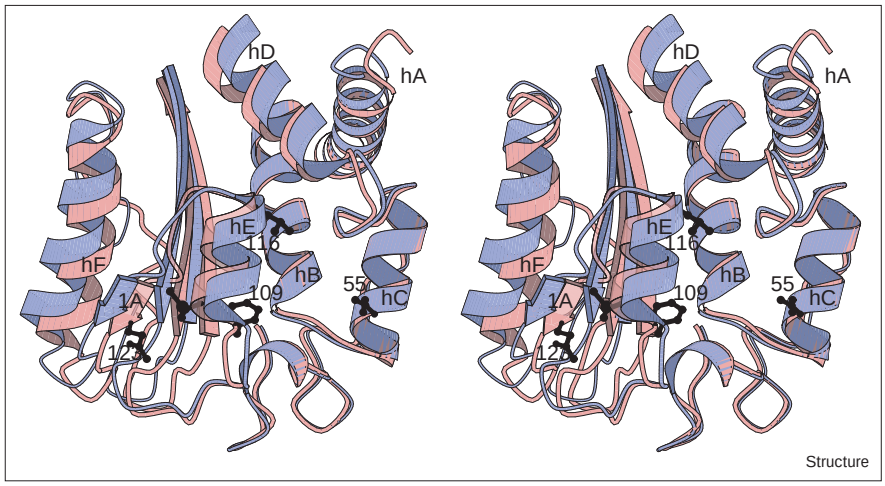


Mutations that increase stability in the quadruple mutant of PAI-1. (a) Stereo overview of the structure showing the location of the four mutations with their sidechains highlighted in red. (b) Close-up stereoview of the three mutations associated with a change in conformation that forms a 3_{10} helix in the loop connecting helix F to β strand 3A. The active form of the stable PAI-1 mutant is shown in black, while the residues for this loop from latent PAI-1 [6] are shown in red. The latent structure has been overlaid by superimposing residues 128–150 and residues 158–162. In other serpins latency or cleavage has no significant effect on the conformation of this loop. (The figure was prepared using the program MOLSCRIPT [40].)

temperature to crystallize in the active form. This amounts, however, to an increase of less than 3 kcal/mol in the energy of the transition state to latency.

As one would expect, all four mutations (see Figure 3) are located in parts of the serpin molecule where movement is known to occur during the transition from the active to the latent form. Only one of the mutations (Met354→Ile) reverses sequence differences between PAI-1 and the majority of inhibitory serpins. Met354→Ile is in β strand 1C, which forms the distal hinge of the reactive center loop and also has to be unraveled in the latency transition. The reverse mutation to methionine at the equivalent residue (Val366→Met) in C1 inhibitor results in polymerization and other evidence of reactive loop insertion [20]. In PAI-1, a methionine may have evolved at this position to give a conformationally labile molecule compatible with its function, whereas in C1 inhibitor, which has a higher concentration and longer half-life in plasma, the same mutation has adverse consequences [21].

Figure 4



Conformational changes in the region of the vitronectin-binding site that are caused by the latency transition. Latent PAI-1 [6] (shown in purple) has been overlaid on active PAI-1 (pink) by superimposing the large rigid fragment defined by Stein and Chothia [42]. Ball-and-stick models indicate the location of mutations that affect vitronectin binding without affecting inhibitory activity [29]. Helix E and β strand 1A have also been shown to be important in vitronectin binding (see text for discussion). (The figure was prepared using the program MOLSCRIPT [40].)

The other three mutations are close to one another, clustered around the site where β sheet A opens to accept the reactive center loop [21]. Interestingly, non-additive effects on stability were found when various single, double and triple combinations of the four mutations were evaluated [11]. The stabilizing effects of either Asn150→His or Gln319→Leu are only fully achieved when combined with Lys154→Thr; on its own, Asn150→His is actually slightly destabilizing. The prediction that this reflected complex structural interactions is now borne out by the structure. The loop connecting helix F with β strand 3A (which includes residues 150 and 154) contains a turn of 3_{10} helix, unlike any other serpin of known structure including latent and cleaved PAI-1. This conformation is only possible in the presence of Lys154→Thr, as the new threonine sidechain is buried and hydrogen bonded (Figure 3b). The other two mutations presumably also favor the 3_{10} conformation, either through burial of hydrophobic surface (Gln319→Leu) or through avoidance of steric conflict (Asn150→His). The new conformation of the loop allows hydrogen bonds to be formed to Glu283 in β strand 6A. These hydrogen bonds would have to be broken in the process of inserting the reactive center loop as a new strand between β strands 3A and 5A and, together with increased rigidity of the 3_{10} helix, may account for a large part of the increased barrier to latency.

When the structure of latent PAI-1 was determined, it was found that the loop from His185 to Pro200 (β strands 3C to 4C, termed the 'gate') was poorly ordered [6]. It was suggested that lack of order in this loop made it easier for the C-terminal portion of the reactive center loop to move to its final position in the latent form [22]. This argument is intuitively attractive, and the gate may move more readily in PAI-1 than in other serpins. However, our structure does not provide experimental support, as the gate region is well ordered. It was also suggested that an unusual position for the C terminus caused the gate to be displaced [22], but this must be a consequence of the latency transition as the C terminus of active PAI-1 occupies the same position seen in other serpins.

Vitronectin binding

The adhesive glycoprotein vitronectin binds to the active form of PAI-1, stabilizing it and altering its specificity. This interaction is specific for active PAI-1, as the affinity of vitronectin for latent, cleaved, proteinase-bound, and other forms of PAI-1 with the reactive center loop inserted into β sheet A is reduced by a factor of 100 or more [23]. The change in affinity may provide a mechanism for the release of inactive PAI-1 from the extracellular matrix, followed by clearance through uptake by the low density lipoprotein (LDL) receptor related protein, LRP [24].

Recent results suggest that the binding of PAI-1 to vitronectin competes with interactions mediating cell adhesion events. Active PAI-1 has been found to compete with α_v-integrins [25] and the uPA receptor [26] for binding to vitronectin. These results provide an explanation for the association of high levels of PAI-1 with increased risk of tumor metastasis [26] and also suggest a means by which coagulation and tissue remodeling could be coordinately regulated during wound healing [27]. Deficient PAI-1 expression in knockout mice has recently been shown to prevent tumor invasion and vascularization [28], supporting the proposed role of PAI-1 in metastasis.

Selection for random mutations of PAI-1 that reduce vitronectin binding, but not inhibitor activity, has identified a patch of five residues near the strand 1 edge of β sheet A [29]. The insertion of the reactive center loop has been presumed to alter this surface, inhibiting binding.

Indeed, comparison of the active and latent structures (Figure 4) reveals extensive rearrangements of the secondary structure elements in this region. Further structural studies will be required to determine the exact nature of the binding surface.

Binding of drugs that modulate PAI-1 activity

Björquist and coworkers [30] present evidence that small molecule PAI-1 inhibitors can bind in the vicinity of Arg76, Arg115 and Arg118. The site corresponds essentially with a pocket proposed as a good target for compounds that inhibit the polymerization of α_1-antitrypsin [31]. This site can be seen in Figure 4, above and behind helix E and between β sheet A and helix D. The same region has been implicated in the binding of heparin [18] and LRP [24], suggesting that it is a general site for serpins to bind ligands that influence, or are influenced by, the conformational transitions.

Our results show the profound structural changes that occur in this region on loop insertion (Figure 4). Conformational changes are seen in this region in other serpins and were thus expected to also occur in PAI-1 [30]. Drugs could modulate PAI-1 activity by preventing, or accelerating, the latency transition or by slowing or eliminating loop insertion on cleavage, thus converting PAI-1 into a substrate. Because all such drugs would bind, at least initially, to the active form of PAI-1 our structure provides an ideal starting point for structure-based drug design.

Biological implications

Plasminogen activator inhibitor 1 (PAI-1), a serine proteinase inhibitor from the serpin family, is being found to have a key role in a growing number of medically relevant pathways. Structural knowledge is essential for a detailed understanding of these processes, because PAI-1 and other serpins are exceptional in the degree to which conformational changes are required for function. Most serpins exist in a metastable state and their mechanism of proteinase inhibition exploits the energy released when the cleaved serpin refolds into its lowest energy state. The different serpin conformations can be distinguished by receptors, with further consequences for function.

PAI-1 is the physiological inhibitor of the fibrinolytic pathway, which in turn limits the coagulation pathway and hence prevents thrombosis. In keeping with this, the diurnal rhythm in levels of PAI-1 may account for the early morning occurrence of the majority of thromboses, and the rebound in PAI-1 levels after fibrinolytic treatment could contribute to the development of coronary restenosis. PAI-1 is therefore a promising drug target for the treatment of coronary heart disease. Useful drugs should bind to the active conformation of PAI-1, described here, but not necessarily to the latent or cleaved conformations determined in previous crystal structures.

Intriguingly, PAI-1 is now being identified as a key player in the link between coagulation and the cell adhesion pathways involved in tissue remodeling and metastasis. Active PAI-1 (but not its latent or cleaved forms) binds tightly to the adhesive glycoprotein vitronectin in the extracellular matrix. This binding localizes active PAI-1 at the site of its action in the fibrinolytic pathway, as well as doubling its half-life. It is now apparent, however, that, as a vitronectin ligand, active PAI-1 competes with α_v-integrins and the receptor for urokinase plasminogen activator. This potential to modulate cell adhesion to the tissue matrix opens further possibilities for rational drug design.

Materials and methods

Protein expression and purification

Mutant protein was expressed and purified as before [11], or was obtained from Molecular Innovations. Purified protein was concentrated to 5 mg/ml in 10 mM cacodylate pH 6.8, 0.25 M NaCl and 1 mM EDTA.

Crystallization

Crystals were produced by the hanging-drop method, equilibrating against 27–32% saturated ammonium sulfate, 0.25 M NaCl and 10 mM cacodylate pH 6.8 at room temperature.

Data collection

Data were collected from a single crystal on a Siemens multiwire area detector mounted on a Siemens rotating anode. Crystals were cryoprotected by transfer to 25% glycerol solutions and were cooled to 111K with an Oxford cryostream. The data were reduced using the program Xengen 2.0 [32] and programs from the Biomol package (University of Groningen).

Molecular replacement

Self-rotation and cross-rotation functions were computed with AMoRe [33]. Self-rotation functions indicated twofold symmetry parallel to the a axis. Cross-rotation functions, computed using latent PAI-1 [6] as a search model, showed four peaks. The AMoRe translation function was used to find solutions for this set of orientations, consistent with one improper dimer (A–B) related to another (C–D) by a twofold screw operation. (Molecule A is related to B by a 64° rotation and a 27 Å screw translation.) Rigid-body refinement was conducted with the program X-PLOR [34] using data from 10 to 5 Å resolution and allowing residues 90–175 (β strands 1A, 2A and 3A and associated loops and helices) to move as a rigid fragment relative to the rest of the molecule. This allowed β strand 3A to move into the position beside β strand 5A that was previously occupied by β strand 4A (the reactive center loop).

Refinement

Refinement was carried out initially with X-PLOR and later with CNS [35], using the maximum-likelihood refinement target MLI (MLF2 in [36]) and a correction for bulk solvent [37]. Rounds of refinement were alternated with rebuilding in the program O [38], inspecting the model in electron-density maps that had been averaged and solvent-flattened with the DEMON package [39]. Strict noncrystallographic symmetry (NCS) was maintained until it became obvious that the reactive center loops in molecules A and C differed from those in B and D, after which strong NCS restraints were imposed. Thermal motion parameters (B values) were refined only for groups of main-chain and sidechain atoms. The final model includes residues 7 to 379 in molecules A and C, and residues 7 to 332 (P15) and 348 (P2′) to 379 in molecules B and D. Statistics for the final model are summarized in Table 1.

Table 1

Structure and refinement statistics.

Space group	P1
Unit cell dimensions	
a, b, c (Å)	65.5, 74.9, 103.7
α, β, γ (°)	91.0, 93.3, 115.9
Resolution limits (Å)	24.0–2.99
Total observations	42,559
Unique reflections	25,369
Completeness	
overall (%)	71.2
3.12–2.99 Å shell (%)	19.7
R_{merge}*	
overall	0.102
3.16–2.99 Å shell	0.336
Protein atoms	11,658
R factor	0.247
R_{free}†	0.292
Rms deviations from ideal geometry	
bond lengths (Å)	0.009
bond angles (°)	1.51
dihedral angles (°)	22.6
improper angles (°)	0.70

*$R_{merge} = \Sigma(|F_j|-\langle|F|\rangle)/\Sigma|F_j|$. †Computed using 2576 reflections not used in the refinement and selected in thin shells of resolution.

Accession numbers

The atomic coordinates have been deposited with the Brookhaven Protein Data Bank with accession code 1b3k.

Acknowledgements

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