Crystal Structure of the Complex of Plasminogen Activator Inhibitor 2 with a Peptide Mimicking the Reactive Center Loop* 

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Lucy Jankova, Stephen J. Harrop, Darren N. Saunders, John L. Andrews, Kenneth C. Bertram, Alison R. Gould, Mark S. Baker, and Paul M. G. Curmi

From the Initiative in Biomolecular Structure, School of Physics, University of New South Wales, Sydney, New South Wales 2052, Australia, the Department of Biological Sciences, University of Wollongong, Northfields Ave., Wollongong New South Wales 2522, Australia, the Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria St., Darlington, New South Wales 2010, Australia, Biotech Australia, Roseville, New South Wales 2069, Australia, and Gynaecological Cancer Research Centre, Royal Women’s Hospital, Department of Obstetrics and Gynaecology, University of Melbourne, Melbourne, Victoria 3153, Australia

The structure of the serpin, plasminogen activator inhibitor type-2 (PAI-2), in a complex with a peptide mimicking its reactive center loop (RCL) has been determined at 1.6-Å resolution. The structure shows the relaxed state serpin structure with a prominent six-stranded β-sheet. Clear electron density is seen for all residues in the peptide. The P1 residue of the peptide binds to a well defined pocket at the base of PAI-2 that may be important in determining the specificity of protease inhibition. The stressed-to-relaxed state transition in PAI-2 can be modeled as the relative motion between a quasirigid core domain and a smaller segment comprising helix hF and β-sheet2 A to form the relaxed state. Attached to the RCL, the target protease moves to the base of the serpin, where it is partially unfolded, thus inhibiting its activity (4, 5). This mode of action, whereby one protein uses the increased stability of an altered conformer to partially unfold a second, covalently bound target protein, is unprecedented in biology.

Plasminogen activator inhibitor 2 (PAI-2) is a serine protease inhibitor that belongs to the ov-serpin branch of the serpin superfamily (6). PAI-2 is an effective inhibitor of urinary plasminogen activator (urokinase or uPA) and, to a lesser extent, an inhibitor of tissue-type plasminogen activator. PAI-2 has several features that distinguish it from the more widely studied serpins. PAI-2 lacks an N-terminal secretory signal but contains a relatively inefficient internal signal sequence (7).

The serpins are an unusual class of protein in that they fold into a metastable state (the relaxed state) that can usually undergo a major structural change to an extremely stable form (the relaxed state) on cleavage by a target protease (1). Most serpins are serine (or cysteine) protease inhibitors that act in a suicide fashion, using the stability of the relaxed state to trap the protease in a nonfunctional covalent serpin-protease complex. The target protease cuts the accessible reactive center loop (RCL) on the serpin to produce a covalent acyl interme-
serpin function. It was clear from the initial structure of cleaved α1-proteinase inhibitor (relaxed state) that the mechanism of protease inhibition was unusual (17). Subsequently determined structures of serpins in various states have led to models for the stressed to relaxed state (S → R) transition, which is central to protease inhibition (18, 19). The determination of the stressed state structure of a PAI-2 CD loop deletion mutant has contributed to the understanding of the mechanics of this transition, especially with regard to the conserved shuttle region (20).

We have determined the structure of the complex formed by the PAI-2 CD loop deletion mutant with a peptide mimicking the RCL at 1.6-Å resolution. The complex adopts the relaxed state, facilitating a high resolution analysis of the S → R transition in PAI-2. By comparing the Ramachandran plots of PAI-2 in the two states, we have identified key hinge regions that facilitate the transition. These are clustered into three regions coordinating the transition. In particular, we hypothesize that the binding of P14 threonine3 in the breach region of PAI-2 triggers the transition by altering the backbone torsion angles of conserved Gly320 (Gly192).4

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and PAI-2-Peptide Complex Formation—Recombinant human PAI-2 loop deletion mutant was cloned and expressed in Escherichia coli and purified as described by Jensen et al. (8). This mutant lacks the loop between helices hC and hD (residues 66–98, inclusive, in the PAI-2 gene sequence). In order to generate a relaxed form of the protein, a synthetic peptide with an acetylated N terminus (N-acetyl-Thr-Glu-Ala-Ala-Ala-Gly-Thr-Gly-Gly-Val-Met-Thr-Gly-Arg-Ohf (Auszep, Australia)), which mimics the RCL loop, was incubated with PAI-2 (1 mg/ml) at a 100-fold molar excess of the RCL peptide in 50 mM NaCl, 50 mM Tris, pH 8.0, at 37 °C for 48 h (21).

Crystallography—The PAI-2-peptide complex was dialyzed and concentrated to 10 mg/ml prior to crystallization using a vacuum dialysis system (Sartorius) against a buffer containing 5 mM Hepes, pH 7.5, and increasing concentrations of ethylene glycol (1% up to 25%). Crystals were grown by vapor diffusion using the hanging drop method. After several days, crystals appeared in 50 mM NaCl, 50 mM Tris, pH 8.0, at 37 °C, 45% relative humidity. The crystals were transferred to a mother liquor containing 25% polyethylene glycol (8000), 0.2 M Hepes, pH 7.5. Large crystals (600 × 500 × 250 μm3) appeared within 3–5 days, belonging to the orthorhombic space group P212121 with unit cell dimensions a = 93.14 Å, b = 104.16 Å, c = 41.34 Å. No. of measured reflections 313,356. No. of unique reflections 51,189. Completeness of data 95.1% (74.6%). R value 9.3 (3.0). Rfree 0.049 (0.22). Molecules in the asymmetric unit 1.

Structure Determination

The structure of a complex between a PAI-2 deletion mutant (where residues 66–98, which form the CD loop, have been genetically removed) and a peptide mimicking the RCL loop has been determined at 1.6-Å resolution. The data reduction and refinement statistics are given in Table I. The electron density maps showed unambiguous electron density for all residues of the protein with the exception of the N terminus (residue 1); the remains of the CD loop (residues 62–65 and 99–101); a loop before β-strand s4C (residues 216–219); and a portion of the endogenous RCL (residues 368–377). Clear density was observed for all 14 residues in the RCL peptide.

Description of the Structure

The structure of the PAI-2-RCL peptide complex is typical of that seen in relaxed state serpins. The prominent β-sheet A contains six strands, with the RCL peptide forming strand s4A in the center of the sheet (Fig. 1). The structure is similar to that of the complex formed between antithrombin and a P14–P3 RCL peptide (28).

Peptide Binding

Breach Region—in the breach region (19), P14 Thr of the peptide forms a hydrogen bond with the side chain of Tyr258.
**Crystal Structure of the PAI-2-RCL Peptide Complex**

Fig. 1. Structure of the complex between PAI-2 and a peptide mimicking the RCL. Shown is a schematic representation of the structure of the PAI-2-RCL peptide complex. The peptide mimicking the RCL is shown in blue, while a portion of the endogenous RCL can be seen at the top (both ends labeled RCL). The structure shows a six-stranded β-sheet A, which is typical of relaxed state serpins. α-Helices are shown in green with β-strands in red. All figures were made using the program SETOR (43).

(Tyr\(^{244}\)) (Fig. 2A), displacing the three water molecules seen in the stressed state structure of PAI-2 (20). The P14 Thr backbone forms two hydrogen bonds with Gly\(^{206}\) (Gly\(^{192}\)) and Arg\(^{294}\) (Arg\(^{283}\)), which may be important in initiating the S → R transition. The acetyl modification of the peptide N terminus mimics the peptide backbone by continuing the β-sheet hydrogen bonding to Asn\(^{363}\) (Asn\(^{353}\)). This chemical modification is essential for peptide binding (21).

**P1 Binding Pocket**—The structure shows that PAI-2 has a well-defined binding pocket for the P1 arginine residue of the inserted RCL peptide (Fig. 2B). The site is formed by the long loop that joins helix hI to β-strand s5A at the base of the molecule. The guanidinium group of the arginine is stabilized by a network of hydrogen bonds to the backbone carbonyl groups of Lys\(^{355}\) (Lys\(^{345}\)), Ala\(^{358}\) (Ala\(^{348}\)), and Asn\(^{347}\) (Asn\(^{337}\)) as well as a hydrogen bond to the side chain oxygen of Asn\(^{347}\) (Asn\(^{337}\)). An examination of the stressed state structure shows that the P1 binding pocket is essentially intact in both states, requiring only small backbone torsional changes between residues Ser\(^{344}\) and Leu\(^{349}\) and rotamer changes in Asn\(^{347}\) and Asp\(^{348}\) to accommodate the incoming arginine.

**Comparison with Other Serpin Structures**

An overlay of the PAI-2-RCL peptide structure with that of other relaxed state serpin structures shows considerable similarity (data not shown). Least-squares comparisons show that r.m.s. deviations between relaxed state serpin structures and the PAI-2-RCL peptide complex range between 1.0 and 1.4 Å, while the r.m.s. deviation between stressed state structures and the complex are ~2.0 Å (Table II). As a comparison, the stressed state PAI-2 structure has an r.m.s. deviation compared with other stressed state structures of 1.5–1.7 Å, and relaxed state structures range from 1.5 to 2.2 Å. The alignments of the PAI-2-RCL peptide complex with other relaxed state structures overlay most of the secondary structural elements with nearly all of the structural differences confined to the loops. There are, however, several regions of structural difference.

There is a significant structural rearrangement of the PAI-2-RCL peptide complex in the neighborhood of Trp\(^{294}\) (Trp\(^{275}\)), which is located at the C terminus of helix hI (Fig. 3A). Trp\(^{294}\) is conserved in many serpins, and an overlay of the structures of leukocyte elastase inhibitor (LEI) (30), ovalbumin (31), antithrombin (32), and the stressed state of PAI-2 shows that its side chain is located in a pocket formed by helix hH and β-strands s1B and s2B (Fig. 3A, red). Trp\(^{294}\) in the PAI-2-RCL peptide complex differs from this consensus, with the side chain adopting a different rotamer (Fig. 3A, green). The electron density and the B-factors for this region indicate that it may be strained compared with the neighboring regions (mean B-factor for residues Asp\(^{249}\)–Glu\(^{302}\)) in 31 Å\(^2\) compared with 18 Å\(^2\) for Asn\(^{243}–\)Ile\(^{246}\) (s1B) and 15 Å\(^2\) for Pro\(^{395}–\)Ser\(^{413}\) (s4B and s5B).

The altered structure of Trp\(^{294}\) in the PAI-2-RCL peptide complex appears to be due to a packing interaction that transmits a structural change over ~10 Å. The side chain of Tyr\(^{245}\) (Tyr\(^{231}\)) on β-strand s1B is at the surface of the molecule. An overlay of the stressed state and relaxed state structures of PAI-2 shows that the side chain of Tyr\(^{245}\) has moved significantly (1.4 Å), along with the backbone of β-strands s1B and s2B (Fig. 3A). The cause of this rearrangement appears to be a steric clash between Tyr\(^{245}\) and the salt bridge formed between Glu\(^{140}\) (Glu\(^{129}\)) and Arg\(^{143}\) (Arg\(^{132}\)) in a neighboring molecule. Although this structural rearrangement appears to be a result of the transmission of a distal packing interaction, the observed change indicates that there is some plasticity in this region of PAI-2.

**Comparison of the Relaxed and Stressed States of PAI-2**

A comparison of the relaxed state PAI-2-RCL peptide structure with the stressed state PAI-2 structure shows all of the general features of the S → R transition that have been obtained from comparisons of different serpins (18) or the same serpin at lower resolution (19). Given this, we will focus mainly on differences that are specific to PAI-2 and comparisons that are warranted by the higher resolution of our structures.

An important difference between stressed and relaxed state serpins is the existence of a cavity beneath β-sheet A in the stressed state, which originates between s2A, hD, and hE (20, 29, 33, 34). The closure of this cavity in the relaxed state is likely to contribute to its enhanced stability and thus to its mechanism of protease inhibition. In the stressed state of PAI-2, this cavity contains three ordered water molecules (20). It is also partially filled by Trp\(^{294}\) (Trp\(^{275}\)), which adopts two conformers, with the major one facilitated by the opening of the cavity (\(\chi_1 = 167^\circ, \chi_2 = -99^\circ\)). In the relaxed state, Trp\(^{294}\) is well ordered, adopting only the minor conformer seen in the stressed state (\(\chi_1 = 160^\circ, \chi_2 = 66^\circ\)), where the indole ring is flipped by 180°, allowing the closure of the cavity between β-sheet A and the body of the serpin.

Several changes occur in the position of helix hF in the PAI-2 S → R transition, which differ from those observed in other serpins. In LEI serpins, helix hF moves along with β-strands s3A and s2A. However, in PAI-2, helix hF appears to move upwards and closer to β-sheet A during the S → R transition (Fig. 3, B and C). A comparison of the PAI-2-RCL peptide complex with other relaxed state structures shows that the position of helix hF in relaxed state PAI-2 is typical; however, a comparison of stressed state PAI-2 with other stressed state structures shows that helix hF is different in PAI-2.

To preclude packing artifacts, we have examined crystal
contacts in the stressed state PAI-2 structure. Helix hF interacts with a region at the base of one other molecule in the crystal via salt bridges/hydrogen bonds between the side chains of Glu\textsuperscript{164} (Glu\textsuperscript{152}) and Lys\textsuperscript{168} (Lys\textsuperscript{156}) with the side chain of Arg\textsuperscript{138} (Arg\textsuperscript{127}) and the carbonyl of Glu\textsuperscript{345} (Glu\textsuperscript{323}), respectively. The loop connecting helix hF to β-strand s5A interacts
with a second molecule via van der Waals interactions between Glu187 (Glu175) and Ser189 (Ser175B) and between Pro221 (Pro207) and Arg229 (Arg215), respectively. None of these packing interactions appears to account for the difference in the orientation of helix hF with respect to β-sheet A in the stressed state of PAI-2. Thus, we believe that this is a genuine reflection of the structure of stressed state PAI-2 in solution.

The closure of the cavity below β-sheet A and the movement of helix hF appear to be correlated with a gross change in β-strands s2A and s3A. In the stressed state structure, both β-strands are relatively straight, while in the relaxed state structure, there appears to be a kink centered at residues Asn127 (Asn116) and Asn200 (Asn186) on β-strands s2A and s3A, respectively. The angle of the kink is 158° and 164° for β-strands s2A and s3A, respectively (cf. 178° for each strand in the stressed state).

Apart from these specific changes that occur on transition between the stressed and relaxed states, there is a general ordering of the structure in the relaxed, PAI-2-RCL peptide structure compared with the stressed state. This is reflected in the average temperature factors for all atoms being 20 and 38 Å² for the relaxed and stressed states structures, respectively. The stressed state also contains more regions that are disordered such as the loop joining helix hD to β-strand s2A.

Transition between the Stressed and Relaxed States

A least squares superposition of the stressed state and relaxed state structures of PAI-2 gives a remarkably good fit when most structural elements are included. Using LSQMAN (35) in the program O (25), the optimized r.m.s. deviation between the structures was 1.38 Å over 298 C-α atoms including all secondary structural elements with the exception of β-strands s1A, s2A, and s3A and helix hF. Visual examination of the superposition shows that all remaining secondary structure elements fit well with the exception of the following: helix hE, which is translated by ~2 Å and tilted by 8.3°; helix hD, which is tilted by 10.5°; and helix hH, which moves as a result of the distortion caused by Trp294 (Table III). Based on the above, PAI-2 can be partitioned into three segments: a quasi-rigid unit comprising helix hF and β-strands s2A and s3A; helix hE; and a quasirigid core formed by all remaining secondary structure elements. An optimized least-squares superposition of the quasirigid core of PAI-2 in the stressed and relaxed states results in an r.m.s. deviation of 0.85 Å over 250 C-α atoms (cf. 1.0–1.6 Å between different relaxed state serpins; Table II). Thus, the S → R transition can be viewed as the relative motion of two quasirigid domains, with an adjustment of helices hD and hE, which is similar to previous analyses (18, 19).
Protein structural transitions involving quasirigid units are often mediated by the existence of hinges. If the S → R transition of PAI-2 is a quasirigid two-domain motion as described above, then one would expect to find hinges in the following locations: the loop between helix hD and s2A; the loop between s2A and hE, the loop between hE and s1A, and the C terminus of s3A. These four hinges would be sufficient to allow the two domains to move in a quasirigid fashion.

In order to locate possible hinges in an unbiased fashion, we compared the Ramachandran plots of PAI-2 in the stressed and relaxed states and computed a Ramachandran distance, D, for each residue as follows.

\[ D = \sqrt{\phi_{\mathrm{a}} - \phi_{\mathrm{b}}^2 + (\psi_{\mathrm{a}} - \psi_{\mathrm{b}})^2} \]

A plot of Ramachandran distance versus residue number identified six regions undergoing large changes (Ramachandran distance > 50°; Fig. 4A). The key residues in these regions are Ala135, Ser136, Ser151, Glu152, Glu187-Gly188, Ala259-Gly260-Asp261, Lys289-Met300, and Arg294-Asn347. Fig. 4B depicts the transitions on the Ramachandran plot for all residues undergoing a large torsional change (Ramachandran distances >50°). Smaller transitions (Ramachandran distance > 30°) were observed for residues Asn127, Lys178, Asn200, Gly206, Lys207, Trp294, and Gly336. Gly314A forms part of the S1 binding pocket and the observed change is directly linked to the neighboring hairpin connecting hI and s5A. The transition in these residues appears to be the key to opening β-sheet A, and hence, initiating the S → R transition.

DISCUSSION

By determining the structure of the PAI-2-RCL peptide complex at high resolution, we are able to examine the structural transition that occurs in PAI-2 as the protein moves from the stressed to the relaxed state. A comparison of the two structures supports the simple model that the S → R transition involves the quasirigid motion of a small domain consisting of helix hF and β-strands s1A, s2A, and s3A, relative to a quasirigid core formed by the remaining secondary structure elements, as proposed by previous analyses (18, 19).

By analyzing large scale changes in the Ramachandran plot, we have been able to identify most of the hinge regions that facilitate the relative motion of the two quasirigid segments of PAI-2. These include segments linking both ends of helix E and the top of β-strand s3A. The hinge connecting helix hD to β-strand s2A undergoes a disorder-to-order transition, and it is linked to the neighboring hairpin connecting β-strands s1B to s2B.

The regions that are identified by analyzing the Ramachandran plot tend to cluster within PAI-2, indicating that these transitions are correlated. The hinge region between helix hE and β-strand s2A is adjacent to the two hinge regions in the top of β-strand s3A that straddle the loop between helix hF and β-strand s5A, which forms the P1 binding site for the inserted RCL peptide.

The transition at Arg294-Asn347 (Arg294-Asn347) is in the loop joining helix h1 and β-strand s5A. These residues form part of the P1 binding pocket (Fig. 2B) and are intimately linked with RCL insertion. The structural change is also coupled to that observed between helix hE and β-strand s1A (Fig. 4C). Fig. 5 shows this region in detail in both the stressed and relaxed states. Changes in Asn347 (Asn325) are directly linked to the binding of P1 Arg to the P1 pocket, where the Asn347 carbonyl group forms a hydrogen bond to the guanidinium moiety of P1 Arg. P1 binding also displaces the side chain of Asp348 (Asp326), which forms a hydrogen bond to the side chain of Ser344 (Ser322) in the relaxed state. The switch in Ser344 rotamer breaks the link with Ser136 (Ser125) (observed in the stressed state), altering the relationship between β-strand s2A and helix hE. Thus, there appears to be a coordination between the binding of P1 Arg to the loop between h1 and s5A and the transitions between s2A and hE.
Thus, a picture emerges of three regions that are critical for the $S \rightarrow R$ transition in PAI-2.

An examination of the structural details in each of these hinge regions shows that, for the most part, they appear to be passive, facilitating the transition but not initiating it. There is clear communication across the bottom of the molecule sensing the binding of P1 Arg to the P1 pocket; however, this event occurs after much of the transition has occurred. The question remains as to which event triggers the $S \rightarrow R$ transition.

Our hypothesis, based on these structures and the results of the accompanying paper (36), is that the binding of P14 Thr to the breach region is the critical event that triggers the $S \rightarrow R$ transition. Several pieces of evidence support this hypothesis. First, the breach site is open in the stressed state, and P14 Thr is the only residue in the RCL that can enter its relaxed state as part of $\beta$-strand s4A. On binding, P14 Thr forms two backbone hydrogen bonds with Gly$^{206}$ (Gly$^{192}$), and these bonds effect a torsional change within the residue resulting in the transition detected by the Ramachandran analysis. This glycine residue is highly conserved in all serpins (20, 37, 38). The backbone transition of Gly$^{206}$ is then propagated in the N-terminal direction along $\beta$-strand s3A, resulting in the opening of $\beta$-sheet A and the insertion of the RCL.

The transmission of the structural change in the N-terminal direction along $\beta$-strand s3A is due to the fact that Gly$^{206}$ is anchored in the C-terminal direction by the side chain of Trp$^{208}$ (Trp$^{194}$), which is also highly conserved (20, 37, 38). This pattern of anchoring a glycine-containing hinge by the binding of a neighboring aromatic side chain to the bulk of a protein has been observed in other protein systems where structural tran-
sitions occur, including the zymogen-to-active enzyme transition in serine proteases (39) and the open-to-closed state transition in the enzyme, rubisco (40).

The closure of the cavity beneath β-sheet A is likely to contribute to the enhanced stability of the relaxed state compared with the stressed state. In the stressed state, β-strands s1A, s2A, and s3A and helix hF appear to form an independent unit with its own hydrophobic core, which is separate from the main molecule. The closure of the cavity results in the expulsion of ordered, buried water molecules and the loss of side chain flexibility and disorder beneath β-sheet A. This movement of β-sheet A is in part due to the formation of a kink in β-strands s2A and s3A on transition to the relaxed state. The Ramachandran analysis identified two adjacent residues in β-strands s2A and s3A that are at the apex of the observed kink: Asn127 (Asn116) and Asn200 (Asn186).

Asn200 (Asn186) is critical to the shutter region of the protein. This buried polar region undergoes a structural rearrangement during the S → R transition (20). The altered hydrogen bonding pattern beneath β-sheet A, involving Asn200, is likely to be the cause of the closer interaction between the sheet and the underlying protein. Thus, the structure of the shutter region may be the cause of the closure of the cavity during the S → R transition and, hence, the enhanced stability of the relaxed state.

One of the unexpected findings upon determining the structure of the PAI-2-RCL peptide complex was the highly ordered nature of the P1 Arg binding site. The site is identically situated to that observed in other relaxed state serpin structures; however, in most of these structures, the P1 residue has a nonpolar side chain, and hence it does not appear to be rigidly held in position at the base of the serpin.

Given the structural order of the RCL peptide, we have docked the uPA structure (41) to the base of PAI-2 using only the coordinates of the RCL peptide residues P3–P1 and the uPA peptidic inhibitor. The resulting model of the complex differed from the recently reported structure of the α1-antitrypsin-trypsin complex only in the torsional orientation of the protease attached to the inserted RCL (4). It did show severe steric overlap between PAI-2 and residues 94–101, 161–194, and 213–231 in uPA. These regions include key portions of the activation domain of uPA that are disordered in the trypsin-
α₁-antitrypsin complex (4). Limited proteolysis studies of the PAI-1uPA complex demonstrate that many of these regions do become disordered upon complex formation (5). We conclude that the specific, well ordered P1 binding site on the base of PAI-2 may be important for the location and orientation of the target protease during inhibition. This site may contribute to the specificity of the serpin for its target.

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