Interaction Between the P14 Residue and Strand 2 of β-sheet B is Critical for Reactive Centre Loop Insertion in Plasminogen Activator Inhibitor-2.

Darren N. Saunders¹,², Lucy Jankova³, Stephen J. Harrop³, Paul M.G. Curmi³, Alison R. Gould⁴, Marie Ranson¹, Mark S. Baker¹,⁵

¹Dept. of Biological Sciences, University of Wollongong, Northfields Ave, Wollongong 2522; ²Cancer Research Program, Garvan Institute of Medical Research, St Vincent's Hospital, Darlinghurst 2010; ³Initiative in Biomolecular Structure, School of Physics, University of New South Wales, Kensington 2052; ⁴Biotech Australia Pty Ltd, 28 Barcoo St, Roseville 2069; ⁵Gynaecological Cancer Research Centre, Royal Women’s Hospital, 132 Grattan St, Carlton 3053, AUSTRALIA.

Corresponding Author:
Darren Saunders
Cancer Research Program
Garvan Institute of Medical Research
St Vincent's Hospital
384 Victoria St
Darlinghurst 2010
Australia
Phone: +61 292958341
Fax: +61 292958321
Email: d.saunders@garvan.unsw.edu.au

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SUMMARY

The molecular interactions driving reactive centre loop (RCL) insertion are of considerable interest in gaining a better understanding of the serpin inhibitory mechanism. Previous studies have suggested that interactions in the proximal hinge / breach region may be critical determinants of RCL insertion in serpins. In this study, conformational and functional changes in PAI-2 following incubation with a panel of synthetic RCL peptides indicated that the P14 residue is critical for RCL insertion, and hence inhibitory activity, in PAI-2. Only RCL peptides with a P14 threonine were able to induce the S→R transition and abolish inhibitory activity in PAI-2, indicating that RCL insertion into β-sheet A of PAI-2 is dependant upon this residue.

The recently solved crystal structure of relaxed PAI-2 (PAI-2:RCL peptide complex) allowed detailed analysis of molecular interactions involving P14 related to RCL insertion. Of most interest is the rearrangement of hydrogen bonding around the breach region that accompanies the S→R transition, in particular the formation of a sidechain hydrogen bond between the threonine at P14 and an adjacent tyrosine on strand 2 of β-sheet B in relaxed PAI-2. Structural alignment of known serpin sequences showed that this pairing (or the equivalent serine/threonine pairing) is highly conserved (~87%) in inhibitory serpins and may represent a general structural basis for serpin inhibitory activity.
INTRODUCTION

The recent discovery of the crystallographic structure of the serpin-protease complex (1) confirms that the serpin inhibitory mechanism is dependent upon insertion of RCL into β-sheet A and the conformational changes associated with the stressed to relaxed (S→R) transition. By hyperstabilising the serpin structure and translocating the protease to the opposite end of the serpin molecule, RCL insertion effectively crushes the protease against the body of the serpin. This in turn causes a striking loss of structure in the protease, making it susceptible to proteolytic degradation and preventing deacylation by distortion of the catalytic site (1).

Relative rates of RCL insertion and deacylation determine partitioning between the substrate and inhibitory pathways of the reaction mechanism proposed by Wright and Scarsdale (2). If RCL insertion and distortion of the protease active site can occur more rapidly than deacylation, the protease is kinetically trapped in a stable covalent complex and inhibited. However, if the rate of RCL insertion is decreased, or insertion is blocked completely, the substrate reaction predominates (2). Hence, the molecular interactions driving RCL insertion are of considerable interest in order to gain a better understanding of the serpin inhibitory mechanism. We have previously shown that incubation with synthetic RCL analogues could induce the relaxed conformation of plasminogen activator inhibitor type-2 (PAI-2). However, RCL peptides lacking both the P13 and P14 residues (ie 12mer and shorter) were not able to induce relaxed PAI-2 or affect inhibitory activity, indicating that interactions in the breach region are critical for RCL insertion in PAI-2 (3).

Several studies have shown that recombinant or naturally occurring point mutations in the proximal hinge region (particularly the P12 and P14 residues) have profound effects on the rate of RCL insertion and hence inhibitory activity of a range of serpins. The presence of large, charged residues at P14 in non-inhibitory ovalbumin (Arg) and angiotensin (Glu) may preclude RCL insertion by preventing burial of the side chains in the hydrophobic core underlying β-
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sheet A (4,5). Replacement of the P14 arginine residue of ovalbumin with serine greatly increased the rate of RCL insertion following cleavage of the P1-P1′ bond by elastase but was not sufficient to convert the molecule into an inhibitor, possibly reflecting additional defects in the serpin conformational change mechanism at sites other than the proximal hinge (6). Furthermore, mutation of the P12 or P14 residues of a number of serpins (eg α₁-PI, PAI-1, α₁-ACT) with either charged or conformationally restrictive proline residues converted the molecules from inhibitors into substrates. It is likely that this conversion results from a decreased rate of RCL insertion rather than complete blocking of insertion (7-10). The crystal structure of a P14 (Thr₃₄₅→Arg) α₁-ACT mutant, which behaves as a substrate, reveals that only the lower part of the RCL is buried, with the P16-P13 segment twisted so that the P14 arginine sidechain is directed towards solvent (11). Statistical comparison of inter-strand bonding in β-sheet A predicts that interactions around both P13 and P8 are critical determinants of RCL insertion and hence serpin inhibitory activity (12). These data indicate that interactions around the proximal hinge / breach region may be critical for initiation of RCL insertion, whereas interactions around the P8 residue (and underlying shutter region) may facilitate complete insertion and stabilise the relaxed form.

Synthetic peptides have been used to study RCL insertion and associated conformational changes in a number of serpins. Peptides corresponding to the P1-P14 section of α₁-PI, antithrombin and PAI-2 have previously been shown to induce the S→R transition and convert these molecules from inhibitors to substrates (3,13,14). Recently, the crystal structures of both antithrombin and PAI-1 complexed with synthetic RCL peptides confirmed that these peptides bind as strand 4 of β-sheet A, homologous to the position occupied by the RCL in the relaxed and latent forms of the molecules (15,16). In this study we used a panel of synthetic RCL peptides with mutations at P13 and P14, and the recently solved crystal structure of a PAI-2:RCL peptide complex (ie. relaxed PAI-2), to characterise the molecular interactions driving
RCL insertion in PAI-2. Furthermore, implications of these data for a general structural basis of serpin inhibitory activity were examined by structural alignment of all available serpin amino acid sequences.
EXPERIMENTAL PROCEDURES

Materials: Recombinant human PAI-2 was a kind gift from Biotech Australia (Sydney). Human two-chain urokinase plasminogen activator (uPA) was purchased from Serono (Sydney). SDS-PAGE analysis showed that uPA in this preparation existed mainly in the 55kDa form. SpectrolyseUK substrate was purchased from American Diagnostica (Sydney).

RCL Peptides: A panel of PAI-2 RCL analogue peptides was synthesised incorporating deletions or mutations at the P13 and/or P14 sites (Table 1). Peptide with a randomly scrambled sequence of the wild-type PAI-2 RCL was used as a control. Peptides were purified to >80% purity by RP-HPLC on a C18 column and characterised by mass spectrometry (Auspep, Melbourne). All peptides were acetylated at the N-terminus. PAI-2/RCL complexes were prepared as previously described (3).

Urea Denaturation & Fluorescence Spectroscopy: Fluorescence spectroscopy was performed on a fluorescent plate reader (Molecular Dynamics) at an excitation wavelength of 280nm. Fluorescence measurements were performed on 0.4µM PAI-2 (in 50mM Tris, 50mM NaCl, 8M urea) at an emission wavelength of 360nm. Data is presented as the fraction of protein denatured ($f_d$), calculated as $\frac{F_{\text{native}} - F}{F_{\text{native}} - F_{\text{denatured}}}$ where $F_{\text{native}}$ is the fluorescence emission (360nm) of native PAI-2, $F$ is the fluorescence emission (360nm) of the individual sample, and $F_{\text{denatured}}$ is the fluorescence emission (360nm) of completely denatured PAI-2 (17).

PAI-2 Activity Assay: Specific activity of PAI-2 following incubation with various RCL analogues was determined by inhibition of uPA mediated hydrolysis of 2mM carbobenzyo-L-γ-glutamyl-(α-t-butoxy)-glycyl-arginine-p-nitroanilide-diacetate (Spectrolyse UK assay; American Diagnostica, Sydney). Residual uPA activity following incubation of 20 IU uPA (100 µl) with 50 µl of PAI-2:RCL binary complex at 37°C for 30 min was calculated by
P14 is critical for RCL insertion in PAI-2. Reference to a standard curve of uPA activity. This value represents unbound uPA and hence the specific activity (IU/mg) of PAI-2 could be calculated as \( \frac{20 - \text{residual activity (IU)}}{\text{PAI-2 (mg)}} \). In order to detect formation of stable uPA:PAI-2 complexes (indicative of inhibition of uPA by PAI-2), samples of PAI-2 which had been incubated with various RCL peptides were reacted with equimolar amounts of uPA for 15 min at 25°C. Samples were then electrophoresed on 12% Tris/Glycine polyacrylamide mini-gels under non-reducing conditions.

**Molecular Modelling and Structural Alignments:** Graphical representations of protein crystal structures and measurement of molecular distances were performed using Swiss-PdbViewer (V3.5b3) (18). Coordinates for crystal structures of cleaved leukocyte elastase inhibitor (PDB# 1HLE (19)) and stressed PAI-2 (CD-loop deletion mutant) (PDB# 1BY7 (12)) were obtained from the Brookhaven Protein Data Bank (20). Structural determination of the binary complex between PAI-2 (CD-loop deletion mutant) and 14mer RCL peptide (PDB# 1JRR) is described in the accompanying paper (21). Alignment of the 225 serpin amino acid sequences available in the SwissProt and TrEMBL databases (releases 37 and 10 respectively) (22) was generated by ProDom99.2 (23) using PSI-BLAST with a profile built from the seed alignment of the PF00079 serpin entry of Pfam-A3.4. Sequences containing gaps or deletions at sites corresponding to either P14 or the top of s2B (Tyr 258 in PAI-2) were excluded from further analysis and pairwise frequencies of the remaining 198 serpin sequences were plotted.
RESULTS

P14 is Critical for Induction of Relaxed PAI-2 by RCL Peptide Insertion:
Intrinsic tryptophan fluorescence has previously been used to measure urea-induced denaturation of PAI-2. Incubation of PAI-2 with 14mer synthetic RCL peptide significantly increased resistance to urea denaturation, indicating induction of the relaxed conformation of PAI-2 following peptide insertion (3). Hence, measurement of conformational stability by intrinsic fluorescence spectroscopy provides a convenient method of detecting RCL insertion following incubation of PAI-2 with various mutated peptides. Figure 1 shows that PAI-2 incubated at 37°C for 48h in the absence of any peptides (control) was completely denatured in the presence of 8M urea. However, PAI-2 incubated with wild-type 14mer peptide was resistant to denaturation by urea, indicating peptide insertion and subsequent transition to the more stable relaxed conformation. Incubation of PAI-2 with RCL peptide in which only the P13 residue had been mutated (E13Q, E13K) also induced resistance to urea denaturation, indicating peptide insertion and transition to the relaxed state. Note that a small fraction (~15%) of PAI-2 incubated with P13 mutant RCL peptides was denatured (Figure 1). In contrast, incubation of PAI-2 with RCL peptides in which the P14 threonine was removed (13mer) or mutated to a valine (T14V) did not induce resistance to denaturation, indicating that these peptides were not able to insert and induce the relaxed conformation. Furthermore, peptides with mutations at both P14 and P13 (13K14V) did not induce resistance to urea denaturation, indicating that these peptides were not able to insert and induce the relaxed conformation (Figure 1). Interestingly, PAI-2 incubated with scrambled peptide exhibited higher fluorescence in 8M urea than PAI-2 alone. As the scrambled peptide has no effect on PAI-2 inhibitory activity (see below) and PAI-2 is completely unfolded in 8M urea (3), this result may be an artifact of an unusual interaction between denatured PAI-2 and the scrambled peptide. Regardless, it is clear that the scrambled peptide is unable to induce the relaxed conformation of PAI-2.
Effect of RCL Peptides on PAI-2 Inhibitory Activity:

We have previously shown (3) almost complete loss of uPA specific inhibitory activity of PAI-2 following incubation with 14mer RCL peptide. Despite this, cleavage of the P1-P1' bond by uPA was still detectable, indicating conversion of PAI-2 from an inhibitor to a substrate of uPA. Measurement of the uPA specific inhibitory activity of PAI-2 was used again here to confirm RCL insertion following incubation of PAI-2 with various mutated peptides. PAI-2 incubated at 37°C for 48h in the absence of any peptide (control) had a uPA inhibitory activity of 1.04x10^5 IU/mg (Figure 2). SDS-PAGE analysis confirmed the presence of a band corresponding to uPA:PAI-2 complex following incubation with uPA (Figure 3, lane 1). PAI-2 incubated with 14mer peptide had no detectable uPA inhibitory activity and failed to form stable complexes with uPA (lane 2), indicating insertion of the synthetic RCL peptide as strand 4 of β-sheet A.

Incubation of PAI-2 with peptides in which the P14 residue had been removed (13mer) or mutated to valine (T14V) had no detectable effect on inhibitory activity (Figure 2). Similarly, a band corresponding to uPA:PAI-2 complex was detectable in these samples (Figure 3, lanes 3 & 4), indicating normal inhibitory activity in PAI-2 incubated with these peptides. In contrast, PAI-2 incubated with peptides in which only the P13 residue had been mutated (E13Q, E13K) exhibited significantly reduced uPA inhibitory activities (~12% of control), indicating peptide insertion. Only very faint bands corresponding to uPA:PAI-2 complex were detectable in these samples (lanes 5 & 6). A band corresponding to a ~35kDa species was detected in all samples following incubation with uPA, most likely representing trace amounts of uncomplexed 33kDa uPA. Incubation of PAI-2 with peptide in which both P13 and P14 were mutated (13K14V) had no detectable effect on uPA specific inhibitory activity or formation of uPA:PAI-2 complex (lane 7). Similarly, incubation of PAI-2 with scrambled peptide did not significantly decrease inhibitory activity or affect the formation of uPA:PAI-2 complex (lane 8). In summary, removal or mutation of the P14 residue effectively prevented synthetic RCL peptide insertion into PAI-2, whereas mutation of the P13 residue had little detectable affect.
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*The Structure of PAI-2/14mer Binary Complex Confirms RCL Insertion into β-sheet A:*

The crystal structure of the binary complex formed between PAI-2 (C-D loop deletion mutant) and 14mer RCL peptide (ie. relaxed PAI-2) is shown in Figure 4C (21). The overall structure closely resembles that of relaxed LEI (19), with a root mean square (rms) deviation of structurally equivalent α-carbon (Cα) atoms of the two structures of 0.98 Å. The overall rms deviation of Cα atoms of stressed and relaxed forms of PAI-2 is 2.45 Å. However, comparison of rms deviations of individual residues (Figure 4D) shows that several distinct regions of the molecule shift noticeably to accommodate insertion of the RCL and transition to the relaxed state. Relatively high rms values (>2) are obtained for residues 120-205 (strands 1-3 of β-sheet A and helix F), 246-250 (s2B), 293-300 (C-terminal end of helix H), and 345-348 (bottom of s5A). Hence the most striking feature of the relaxed PAI-2 structure is separation of strands 3 and 5 of β-sheet A to accommodate the RCL peptide, which forms s4A, and movement of helix F towards the top of β-sheet A (Figure 4C).

Accompanying insertion of the RCL and transition to the R state is a separation of the top of strands 3 and 5 of β-sheet A and strand 2 of the underlying β-sheet B, known as the breach region (24). This separation allows the top of the RCL to be incorporated as strand 4 of β-sheet A. For example, the distance between Asp_{361} (s5A) and Phe_{204} (s3A) increases from 3.2 Å in stressed PAI-2 to 7.3 Å in relaxed PAI-2. Similarly, the distance between Gly_{206} (s3A) and Tyr_{258} (s2B) increases from 5.7 Å in stressed PAI-2 to 6.2 Å in relaxed PAI-2. A complete description of the structural differences between stressed and relaxed PAI-2 is provided in the accompanying paper (21).

*Structural Changes in the Proximal Hinge / Breach Region of Relaxed PAI-2:*

Interactions between the 14mer peptide and adjacent residues in the breach region of relaxed PAI-2 are shown in Figure 4E. A similar illustration showing interactions around this region of
relaxed LEI is presented in Figure 4F for comparison. The most prominent structural feature in this region of relaxed PAI-2 (and LEI) is the extensive rearrangement of hydrogen bonding upon insertion of the RCL. The three water molecules bound to this region in stressed PAI-2 (Figure 4A,B) are excluded and typical anti-parallel interstrand hydrogen-bonding is observed between all residues of the RCL (s4A) and adjacent residues in s3A and s5A, confirming that the peptide occupies the position that the endogenous RCL of PAI-2 would be expected to occupy following cleavage of the P1-P1’ bond.

The P14 residue (Thr_{367} in PAI-2, Thr_{345} in LEI) forms backbone hydrogen bonds with the adjacent glycine residue on s3A (Gly_{206} in PAI-2, Gly_{192} in LEI), while the P13 residue (Glu_{368}) bonds to Asp_{361} on s5A (21). Hydrogen bond rearrangements in the PAI-2/14mer complex involving residues immediately N-terminal to P14 (P15-P17) are difficult to interpret. Normally this region would be expected to form the turn between strands 4 and 5 of β-sheet A (refer to the structure of relaxed LEI, Figure 4F) but the insertion of a synthetic RCL analogue in place of the endogenous RCL prevents this from occurring. Hence, bonding in this region of the PAI-2/14mer complex is probably not entirely indicative of the situation in relaxed PAI-2 formed by target protease mediated cleavage of the P1-P1’ bond. In addition to backbone hydrogen bonds with Gly_{206}, the sidechain hydroxyl of P14 threonine in the RCL peptide forms a hydrogen bond with the sidechain hydroxyl of Tyr_{258} on strand 2 of β-sheet B (Figure 4E). This interaction is also present in the structure of relaxed LEI, where the sidechain of Thr_{345} is hydrogen bonded with sidechain of Tyr_{244} (Figure 4F).

**Comparison of Interactions Involving P14 and RCL Insertion in the Serpin Family:**

Alignment of all serpin sequences available in the SwissProt and TrEMBL databases showed that the pairing of a threonine at P14 and tyrosine at the residue on s2B corresponding to Tyr_{258} of PAI-2 is very highly conserved, occurring in ~69% of the 198 serpins analysed (Figure 5). However when only inhibitory serpins are considered, this frequency rises to 82%. The next most conserved pairing at this site (occurring in ~5% of the 198 sequences analysed) is a serine
at P14 and tyrosine on s2B. Other pairings that occur less frequently (<3%) include arginine / phenylalanine (ovalbumin), alanine / leucine (PEDF), glycine / phenylalanine (maspin), asparagine / leucine (47kDa HSP), and glutamate / leucine (angiotensinogen) (Figure 5). The residue on s3A (Gly_{206} in PAI-2) that forms backbone hydrogen bonds with P14 is also highly conserved across the serpin family, with a glycine occurring in ~74.6% of the serpins analysed. Alanine is the next most conserved residue at this site, occurring in ~20.2% of the sequences analysed. Interestingly, 47kDa heat shock protein and collagen binding protein, which are both non-inhibitory, have a proline residue at this site.
DISCUSSION

Several studies have addressed the role played by proximal hinge / breach region residues in driving the characteristic RCL insertion and conformational change accompanying the inhibitory activity of serpins. Recombinant or naturally occurring point mutations in the proximal hinge region (particularly the P12 and P14 residues) have previously been shown to have profound effects on RCL insertion and hence inhibitory activity in a range of serpins (4-6). Taken together, measurements of both conformational stability (Figure 1) and uPA inhibitory activity of PAI-2 following incubation with various synthetic RCL peptides (Figures 2 and 3) indicate that the P14 residue is a critical determinant of RCL insertion and inhibitory activity. The P13 residue did not appear to be a critical determinant of RCL insertion or inhibitory activity in PAI-2. Harrop et al. (12), based on statistical analysis of pairwise interactions in β-sheet A across the entire serpin family, have previously suggested that electrostatic interactions involving the P13 residue may be important in both stressed and relaxed serpins. Indeed, the presence of a negatively charged Glu sidechain at P13, adjacent to a positively charged Lys sidechain on s3A (Lys_{205}) would be expected to generate electrostatic interactions at this site in addition to the expected backbone hydrogen bonding. However, replacement of the P13 Glu with a Lys (E13K peptide), which might be expected to generate a strong repulsive force between P13 and the adjacent Lys_{205}, did not have any significant effect on peptide insertion (Figure 3).

The inability of RCL peptides in which the P14 residue was removed or mutated to induce conformational changes or block inhibitory activity in PAI-2 is open to several interpretations. Removal or replacement of the P14 residue may prevent synthetic RCL peptides from inserting into PAI-2 at all. Hence, only peptides with a threonine at P14 are able to insert into β-sheet A and block subsequent insertion of the endogenous RCL of PAI-2 upon interaction with uPA, converting PAI-2 into a substrate. Alternatively, RCL peptides in which the P14 residue was removed or mutated may still interact weakly with PAI-2, possibly only via partial insertion into β-sheet A. Upon interaction with uPA, the endogenous PAI-2 RCL may displace the weakly
bound synthetic peptide and allow complete RCL insertion, formation of a stable uPA:PAI-2 complex and inhibition of the protease. The absence of any detectable changes in conformation of PAI-2 following incubation with mutated RCL peptides supports the former mechanism (ie. complete lack of insertion). However, the later mechanism cannot be discounted as any conformational changes induced by partial insertion of mutated peptides may not be detectable by the methods used.

Definitive evidence for either mechanism would be provided by direct observation of a PAI-2/RCL peptide complex. Investigation of PAI-2/RCL complexes was attempted by electrospray mass spectrometry but this technique did not prove suitable as no evidence of any higher molecular weight species representing PAI-2/RCL complexes were detected. It is likely that any PAI-2/RCL complexes present were dissociated by the ionisation conditions required to achieve sufficient signal strength. Similarly, detection of RCL binding to PAI-2 using surface plasmon resonance analysis (BIAcore) was unsuccessful. Xue et al. (16) have previously demonstrated binding of a short RCL pentapeptide to PAI-1 using this technique, however the rate of binding of 14mer RCL peptide to PAI-2 under identical conditions appears to be much slower, making the reaction difficult to interpret.

The native RCL is held in position at the top of stressed PAI-2 (Figure 4A,B) by a network of conserved hydrogen bonds and sidechain electrostatic interactions in the proximal hinge / breach region (12). Residues P17-P14 (Glu364-Thr367) are stabilised by a series of backbone hydrogen bonds to the C-terminal end of β strand 3A (Lys207-Phe210). This network of hydrogen bonds is conserved in ovalbumin and α1-PI and is further stabilised by a balance of oppositely charged residues on the RCL proximal hinge and the top of strand 3 of β-sheet A (12,25,26). The top of strands 3 and 5 of β-sheet A (s3A, s5A) are held apart by steric interactions between two large aromatic residues on s3A and s2B (Trp208, Tyr258 respectively), with interstrand hydrogen bonding not commencing until approximately 4 residues down into
the β-sheet (Asp$_{361}$ and Phe$_{204}$). This separation is further stabilised by the sidechain of the conserved residue at P17 (Glu$_{364}$), which is held in position by hydrogen bonds to adjacent residues Lys$_{214}$ and Gln$_{311}$, forming the pivot about which the RCL turns to form s4A in the relaxed state. The gap between the top of strands 3 and 5 of β-sheet A in stressed PAI-2 is bridged by a cluster of 3 bound water molecules (12).

The recently solved crystal structure of the complex between PAI-2 and 14mer RCL peptide (21) (Figure 4C, E) allows a detailed examination of the mechanisms underlying the apparent importance of P14 for RCL insertion in PAI-2. The most striking difference in the structures of stressed and relaxed PAI-2 (formed by RCL peptide insertion) is the separation of strands 3 and 5 of β-sheet A to accommodate the RCL (which forms s4A) and exclusion of the 3 water molecules present in the stressed PAI-2 structure. Rearrangement of hydrogen bonding around the proximal hinge / breach region of relaxed PAI-2 may provide the best explanation of the apparent importance of P14 for RCL insertion. Typical anti-parallel interstrand hydrogen bonding is observed between the backbone of P14 and the adjacent residue on s3A (Gly$_{206}$) in the PAI-2/14mer complex. This bonding is also observed in the structure of relaxed LEI (19), with the P14 residue (Thr$_{345}$) forming backbone hydrogen bonds with Gly$_{192}$ on s3A.

Of most interest is the formation of a hydrogen bond in relaxed PAI-2 between the sidechain hydroxyl of P14 threonine and the sidechain hydroxyl of Tyr$_{258}$ on s2B (Figure 4E). Again, a similar arrangement is observed in relaxed LEI, with the sidechain of P14 (Thr$_{345}$) hydrogen bonded with the sidechain of Tyr$_{244}$ on s2B (Figure 4F). This is significant in light of experimental data indicating that replacement of the P14 threonine residue with a valine (T14V peptide) prevented RCL insertion (Figures 1-3). In contrast to threonine, the sidechain of valine does not contain a donor/acceptor hydroxyl and hence cannot form a hydrogen bond with the aromatic hydroxyl of Tyr$_{258}$. Whilst the Thr→Val replacement may alter sidechain mediated hydrogen bonding, it would not be expected to significantly affect backbone hydrogen...
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bonding. Hence, bonding interactions between the sidechains of P14 and the adjacent tyrosine residue on s2B appear to be the main determinant of RCL insertion in PAI-2 and LEI.

Another possibly significant interaction in the structure of relaxed PAI-2 is the close proximity of the sidechains of P13 glutamate in the RCL peptide and Lys\textsubscript{205} on s3A, indicating a possible electrostatic interaction between these two residues. This interaction is also apparent in the structure of relaxed LEI, where the sidechain of P13 (Glu\textsubscript{346}) is in close proximity to the sidechain of Lys\textsubscript{191}. Furthermore, analysis of interstrand bonding in β-sheet A across the serpin family indicated that P13 was a critical determinant of RCL insertion (12). However, replacement of the P13 glutamate in the RCL peptide with either an uncharged glutamine (E13Q) or oppositely charged lysine (E13K) had only a small effect on the ability of these peptides to insert into PAI-2 (Figures 1-3) suggesting that this interaction is not critical for RCL insertion in PAI-2.

A recent phylogenetic analysis of the serpin family (24) identified both the P14 residue and underlying tyrosine on s2B as highly conserved individually but did not examine the co-occurrence of these residues in various serpins. We have shown that the pairing of threonine at P14 with an adjacent tyrosine on s2B in the breach region is highly conserved in serpins, occurring in ~69% of all known serpins (Figure 5). However, when only inhibitory serpins are considered, the frequency of this pairing is ~82%, hence it is likely to represent an important structural basis for RCL insertion and inhibitory activity. The next most conserved pairing at this site is a serine at P14 with a tyrosine on s2B (occurring in ~5% of known serpins; Figure 5). From a structural perspective, there is little difference in these pairings, as both threonine and serine have sidechain hydroxyl groups that can act as hydrogen donor/acceptors in formation of a hydrogen bond with the aromatic hydroxyl of tyrosine. Hence the overall conservation of this interaction in inhibitory serpins is ~87%.

It should be noted however, that this interaction is clearly not the sole determinant of RCL insertion and serpin inhibitory activity. The presence of the Thr/Tyr pairing in some non-
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inhibitory serpins indicates that structural deficiencies in other aspects of the serpin mechanism (e.g. initial folding of the stressed form to provide the necessary energy for the $S \rightarrow R$ transition, or stabilisation of the RCL inserted/relaxed conformation) may be responsible for the lack of inhibitory activity in these molecules. Various alternative pairings occur at this site (albeit with much lower frequencies), however the majority of these appear in non-inhibitory serpins such as ovalbumin (Arg/Phe), PEDF (Ala/Leu), 47kDa HSP (Asn/Leu) and angiotensinogen (Glu/Leu). Interestingly, the P14/s2B interaction does not appear to be present in a few inhibitory serpins (e.g. $\alpha_2$-antiplasmin, C1 inhibitor, factor XIIa inhibitor, antithrombin III), suggesting that RCL insertion in these molecules may be facilitated by alternative mechanisms. Different bonding patterns around the proximal hinge / breach region (including P14) in these serpins may be involved in separation of s3A and 5A and/or anchoring of the top of the RCL during insertion. However as no crystal structures are available for the relaxed conformations of these molecules, it is not possible to analyse the precise nature of these interactions.

Along with P13, Harrop et al. (12) also identified P8 as an important determinant of RCL insertion and inhibitory activity in serpins. Therefore, further investigation of the mechanism of RCL insertion in PAI-2 could involve performing similar experiments to those described above using synthetic RCL peptides in which the P8 amino acid is replaced. Further examination of the importance of the interaction between P14 and adjacent residues in $\beta$-sheets A and B for serpin inhibitory function may be facilitated through the generation of site-directed mutants of PAI-2. For example, mutation of Thr$_{367}$ (P14) and/or Tyr$_{258}$ (on s2B) would allow more direct comparisons of inhibitory activity and conformational stability between wild-type and mutant PAI-2 than is feasible using the synthetic RCL peptides described here. This approach was recently used to demonstrate the importance of P14 in determining the rate of RCL insertion (and hence partitioning between the inhibitory and substrate pathways) in PAI-1 (27).

In summary, experimental evidence presented in this study indicates that the P14 residue is an important determinant of RCL insertion in PAI-2. Surprisingly, P13 does not appear to play a
critical role in this process in PAI-2. Previous studies have identified constraints on the size and charge of residues in the proximal hinge region (particularly P14) of various serpins imposed by the tightly packed, hydrophobic nature of the protein core underlying β-sheet A (4-11).

Interactions around the proximal hinge / breach region (particularly those involving P14) most likely influence inhibitory activity by determining the rate of RCL insertion into β-sheet A, and hence partitioning between the inhibitory and substrate pathways (2). Structural analysis of the breach regions in both relaxed PAI-2 and LEI indicate that a hydrogen bond between the sidechain hydroxyl of the P14 threonine and the aromatic hydroxyl on the sidechain of an adjacent tyrosine residue on s2B may be the main determinant of RCL insertion in these molecules. Furthermore, this interaction appears highly conserved in inhibitory serpins.
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REFERENCES


P14 is critical for RCL insertion in PAI-2.
PI4 is critical for RCL insertion in PAI-2.

FOOTNOTES

1Abbreviations used: plasminogen activator inhibitor-2 (PAI-2), reactive centre loop (RCL), \( \alpha \)-antiproteinase (\( \alpha \)-PI), plasminogen activator inhibitor-1 (PAI-1), \( \alpha \)-Antichymotrypsin (\( \alpha \)-ACT), urokinase plasminogen activator (uPA), leukocyte elastase inhibitor (LEI).
FIGURE LEGENDS

Figure 1. Urea denaturation analysis of PAI-2 incubated with mutant RCL peptides: Resistance to denaturation by 8M urea of PAI-2 incubated with 100x molar excess of various RCL peptides was monitored by intrinsic fluorescence emission at 360nm. Data is presented as (F\text{native} - F) / (F\text{native} - F\text{denatured}), where F\text{native} is the fluorescence emission (360nm) of native PAI-2, F is the fluorescence emission (360nm) of the individual sample, and F\text{denatured} is the fluorescence emission (360nm) of completely denatured PAI-2.

Figure 2. uPA inhibitory activity of PAI-2 incubated with mutant RCL peptides. PAI-2/RCL complexes were assayed for uPA specific inhibitory activity using the Spectrolyse UK assay. Residual uPA activity following incubation of uPA (20 IU) with PAI-2:RCL complex (50 µl) at 37°C for 30 min was calculated by reference to a standard curve of uPA activity and the specific activity (IU/mg) of PAI-2 was then calculated as described in section 3.2.4. Results presented as mean ± s.d. of triplicate measurements.

Figure 3. Formation of uPA:PAI-2 complexes following incubation of PAI-2 with mutant RCL peptides: SDS-PAGE analysis of uPA:PAI-2 complex formation following incubation of various PAI-2/RCL samples with uPA for 15min at 25°C. Samples loaded as follows: (1) no peptide; (2) 14mer; (3) 13mer; (4) T14V; (5) E13Q; (6) E13K; (7) 13K14V; (8) scrambled.

Figure 4. Bonding interactions around the proximal hinge / breach region of PAI-2: (A) Stick model (with α-carbon trace overlaid in blue) showing hydrogen bonding (green) between residues in the breach region of stressed PAI-2. Three water molecules (red spheres) maintain the hydrogen bonding network between the separated top ends of strands 3 and 5 of β-sheet A. (B) Ribbon diagram showing the sidechain positions (red) of the 2 large aromatic residues (Trp\text{208} and Tyr\text{258}) causing the separation of s3A and s5A (blue). The gap between the separated strands at the top of β-sheet A is bridged by a cluster of bound water molecules.
P14 is critical for RCL insertion in PAI-2.

(green spheres) (PDB ID# 1BY7 (12)). (C) The 1.9 Å resolution structure of the PAI-2:RCL peptide binary complex (ie. relaxed PAI-2) induced by insertion of 14mer synthetic RCL peptide (red) into β-sheet A (green). The synthetic RCL peptide inserts between s3A and s5A, forming s4A and filling the position that the RCL would be expected to occupy in the serpin:protease complex (PDB# 1JRR (21)). (D) Plot of the root mean square (rms) deviation (Å) of corresponding residues in stressed and relaxed PAI-2. Not shown are values for residues 61-101 (C-D loop), 117-118 (loop between s2A and helix D), 215-220 (s4C), 367-385 (RCL). (E) Stick model of relaxed PAI-2 showing hydrogen bonding (green) between the OH group on the sidechain of RCL peptide P14 (Thr367) and the aromatic ring of Tyr258 (on s2B). Carbon atoms are coloured black, nitrogen blue and oxygen red, with the α-carbon trace overlaid in light blue. (F) Stick model of relaxed LEI showing similar bonding between the sidechains of Thr345 (P14, on s3A) and Tyr244 (on s2B).

Figure 5. Frequency of adjacent amino acid pairs at the top of β-sheet A: Pairing of amino acid residues at P14 (Thr367 in PAI-2) and on the adjacent strand 2 of β-sheet B (corresponding to Tyr258 in PAI-2). Data compiled from alignment of serpin sequences in the SwissProt and TrEMBL databases (refer experimental procedures).
Table 1. Amino acid sequence and characteristics of synthetic RCL peptides. Peptides were synthesised with an N-terminal acetyl group. Sequences shown with P14 residue (N-terminus) at left and mutated or removed residue underlined.

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<thead>
<tr>
<th>NAME</th>
<th>1SEQUENCE</th>
<th>2M_R</th>
<th>DESCRIPTION</th>
</tr>
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<tbody>
<tr>
<td>14mer</td>
<td>Ac-TEAAAGTGGVMTGR-OH</td>
<td>1320</td>
<td>wild-type RCL (P14 to P1)</td>
</tr>
<tr>
<td>13mer</td>
<td>Ac_-EAAAGTGGVMTGR-OH</td>
<td>1219</td>
<td>wild-type RCL (P13 to P1)</td>
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<td>T14V</td>
<td>Ac_VEAAAGTGGVMTGR-OH</td>
<td>1318</td>
<td>P14 (Thr) mutated to Val</td>
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<tr>
<td>E13Q</td>
<td>Ac-TQAAAGTGGVMTGR-OH</td>
<td>1319</td>
<td>P13 (Glu) mutated to Gln</td>
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<tr>
<td>E13K</td>
<td>Ac-TKAAAGTGGVMTGR-OH</td>
<td>1319</td>
<td>P13 (Glu) mutated to Lys</td>
</tr>
<tr>
<td>13K14V</td>
<td>Ac-VKAAAGTGGVMTGR-OH</td>
<td>1317</td>
<td>P14 (Thr) mutated to Val and P13 (Glu) mutated to Lys</td>
</tr>
<tr>
<td>Scrambled</td>
<td>Ac-GAGAGTAEGRTVMT-OH</td>
<td>1320</td>
<td>Randomised sequence of wild-type RCL</td>
</tr>
</tbody>
</table>

1Determined by mass spectrometry.
FIGURE 1

Comparison of fluorescence intensity changes between native and denatured states with different peptides.

- no peptide
- 14mer
- 13mer
- T14V
- E13Q
- E13K
- 13K14V
- scrambled

The graph shows the relative changes in fluorescence intensity, with native states compared to denatured states.
Inhibitory Activity (IU/mg)

- Control
- 14mer
- 13mer
- T14V
- E13Q
- E13K
- 13K14V
- Scrambled

FIGURE 2
FIGURE 5
Interaction between the P14 residue and strand 2 of beta-sheet B is critical for reactive center loop insertion in Plasminogen Activator inhibitor-2
Darren N. Saunders, Lucy Jankova, Stephen J. Harrop, Paul M. G. Curmi, Alison R. Gould, Marie Ranson and Mark S. Baker

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