A redox-sensitive loop regulates plasminogen activator inhibitor type 2 (PAI-2) polymerization

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Plasminogen activator inhibitor type 2 (PAI-2) is the only wild-type serpin that polymerizes spontaneously under physiological conditions. We show that PAI-2 loses its ability to polymerize following reduction of thiol groups, suggesting that an intramolecular disulfide bond is essential for the polymerization. A novel disulfide bond was identified between C79 (in the CD-loop) and C161 (at the bottom of helix F). Substitution mutants in which this disulfide bond was broken did not polymerize. Reactive center loop peptide insertion experiments and binding of *bis*-ANS to hydrophobic cavities indicate that the C79–C161 disulfide bond stabilizes PAI-2 in a polymerogenic conformation with an open A- β -sheet. Elimination of this disulfide bond causes A- β -sheet closure and abrogates the polymerization. The finding that cytosolic PAI-2 is mostly monomeric, whereas PAI-2 in the secretory pathway is prone to polymerize, suggests that the redox status of the cell could regulate PAI-2 polymerization. Taken together, our data suggest that the CD-loop functions as a redox-sensitive switch that converts PAI-2 between an active stable monomeric and a polymerogenic conformation, which is prone to form inactive polymers.

Keywords: PAI-2/polymerization/redox/serpin

Introduction

Plasminogen activator inhibitor type 2 (PAI-2) is a serine protease inhibitor that belongs to the ov-serpin branch of the serpin superfamily. The molecular architecture of PAI-2 (Harrop *et al.*, 1999) resembles that of other serpins (Loebermann *et al.*, 1984; Stein *et al.*, 1990; Baumann *et al.*, 1991): it is built from nine helices (helix A–helix I) and three β -sheets (A–C), and has a mobile reactive center loop (RCL) that is supported by the dominant A- β -sheet (Figure 1). However, PAI-2 has additional features that distinguish it from the other better-studied serpins. PAI-2 has a 33 amino acid insertion that forms a loop between helices C and D. This so-called CD-loop is present only in a few ov-serpins, but in PAI-2 it is unusually long. The CD-loop is encoded by a separate exon (Ye *et al.*, 1989), indicating that it can be a separate functional domain. Another unique feature of PAI-2 is its inefficient secretion signal (Belin *et al.*, 1989; von Heijne *et al.*, 1991), which is

part of the mature protein and translocates only a fraction of PAI-2 to the extracellular compartments. The extracellular form of PAI-2 is recognized as an inhibitor of urokinase-type plasminogen activator (uPA), and may therefore be involved in regulating extracellular proteolysis and cell migration (Kruithof *et al.*, 1995). PAI-2 inhibits uPA by the established serpin inhibitory mechanism that involves RCL cleavage and insertion of the RCL into the A- β -sheet, whereby the covalently bound protease is translocated to the opposite pole of the serpin molecule (Lawrence *et al.*, 1995; Wilczynska *et al.*, 1995; Stratikos and Gettins 1997; Fa *et al.*, 2000; Huntington *et al.*, 2000). The function of the predominantly intracellular fraction of PAI-2 is an enigma. A role in protection of cells against apoptosis has been proposed (Kumar and Baglioni, 1991; Dickinson *et al.*, 1995), but so far no intracellular target(s) have been identified. PAI-2 can be cross-linked to membranes by tissue transglutaminase (Jensen *et al.*, 1993) and it binds to annexins (Jensen *et al.*, 1996). The anti-apoptotic activity, the interaction with annexins and the cross-linking are all linked to the CD-loop of PAI-2 (Jensen *et al.*, 1993; Dickinson *et al.*, 1995).

Serpin polymerization is the underlying cause of several diseases. In these cases, naturally occurring serpin variants carry mutations that affect the mobility of the RCL and/or the opening of the A- β -sheet, which makes them prone to polymerize. Spontaneous polymerization has been described for natural mutants of α_1 -antitrypsin, neuroserpin, C1-inhibitor and α_1 -antichymotrypsin, and it has been linked to diseases as diverse as liver cirrhosis, emphysema, dementia, angioedema or thromboembolism (Stein and Carrell, 1995; Risse *et al.*, 1998).

Most natural wild-type (wt) serpins are stable active monomers under physiological conditions, and polymerization can only be induced by treatment under mild denaturing conditions (Lomas *et al.*, 1992; Patston *et al.*, 1995). However, in that respect, PAI-2 is unique since it is the only wt serpin that spontaneously forms loop-sheet polymers under physiological conditions (Mikus and Ny, 1996). Polymerization of PAI-2 depends on protein concentration, and is linked to reduced secretion efficiency in an expression system designed for efficient secretion through the endoplasmic reticulum (ER) (Mikus and Ny, 1996). It has been proposed that serpin polymerization requires the formation of a polymerogenic intermediate (Dafforn *et al.*, 1999) where the RCL is partially inserted (Mast *et al.*, 1992; Lomas *et al.*, 1993; Fitton *et al.*, 1997), strand 1 of the C- β -sheet is released (Chang *et al.*, 1997) and a gap between strands 3 and 5 of the A- β -sheet is formed. Once such an intermediate is formed, polymerization can take place by the sequential insertion of the RCL from one molecule into the A- β -sheet of another (Lomas *et al.*, 1992; Li *et al.*, 1999). Since PAI-2

Fig. 1. Ribbon model of PAI-2. The model was built with co-ordinates from Brookhaven Protein Data Bank (accession No. 1BY7) using the Insight II program (Biosym. Technologies, San Diego, CA). The cysteines present in the X-ray structure are shown as red sticks, the CD-loop (not solved by the X-ray structure) is drawn schematically as a red line, and Cys79 is marked as a red sphere.

spontaneously polymerizes, it is likely that its conformation differs from that of the other wt serpins.

The X-ray structure of a PAI-2 deletion mutant reveals a unique configuration of amino acids at the breach region (located at the top of A- β -sheet), which is not present in other serpins (Harrop *et al.*, 1999). However, our mutational analysis has shown that this configuration is not the reason why PAI-2 spontaneously polymerizes (Wilczynska *et al.*, 2003). In this report, we provide evidence that the polymerogenic conformation of PAI-2 has an open A- β -sheet, which is stabilized by a disulfide bond that connects the CD-loop to the bottom of helix F. Based on these results, we propose that the CD-loop can function as a molecular switch that regulates polymerization of PAI-2 by sensing the redox status of cellular compartments.

Results

The effect of thiol group modifications on the ability of PAI-2 to polymerize

As shown previously (Mikus and Ny, 1996), purified wt PAI-2 spontaneously forms non-covalent loop-sheet polymers under physiological conditions. Consistent with this finding, purified wt PAI-2 that was incubated at 37°C for 16 h appeared mainly as polymers when analyzed by non-denaturing PAGE (Figure 2A, lane 1). However, when this pre-polymerized PAI-2 was reduced with dithiothreitol (DTT) and alkylated, the modified protein (defined as 'blocked' PAI-2) appeared as a monomer (Figure 2A,

Fig. 2. Influence of alkylation of thiol groups in wt PAI-2 on its polymerization and activity. (A) Polymerization of wt PAI-2 tested by non-denaturing PAGE followed by western blot (each lane contains 2 μ g of PAI-2). Lane 1, wt PAI-2 pre-polymerized in 50 mM Tris-HCl buffer pH 8.0, containing 0.14 M NaCl at 37°C for 16 h; lane 2, pre-polymerized PAI-2 modified by reduction with 100 mM DTT followed by alkylation with excess iodoacetamide (designed as 'blocked' PAI-2); lane 3, 'blocked' PAI-2 mixed with a 1.5 molar excess of uPA; lane 4, 'blocked' PAI-2 incubated at 37°C for 24 h; lane 5, 'blocked' PAI-2 as in lane 4 but incubated with uPA. The positions of monomers, dimers and trimers are marked with arrows; the bracket shows a gel region with PAI-2-uPA complex. (B) Inhibitory activities of native wt PAI-2 and the 'blocked' PAI-2 before (black bars) and after (dashed bars) incubation at 37°C for 24 h, tested by chromogenic activity assay.

lane 2). Blocked PAI-2 was active and could form a complex with uPA (Figure 2A, lane 3). Even after prolonged incubation under conditions that would lead to polymerization of the native inhibitor (pH 8.0, 37°C), blocked PAI-2 remained as a stable inhibitory active monomer (Figure 2A, lanes 4 and 5). Consistent with the above results, the inhibitory activity of native PAI-2 determined by chromogenic assay decreased by ~70% upon incubation at 37°C for 16 h, whereas the activity of the blocked PAI-2 remained stable under the same conditions (Figure 2B). Taken together, these data suggest that intramolecular disulfide bond(s) may be required to keep PAI-2 in a conformation that has the ability to polymerize spontaneously.

Identification of cysteine residues that are required for PAI-2 polymerization

PAI-2 exists in two allelic variants that contain either five or six cysteine residues (Bachmann, 1995). So far, only one disulfide bond that connects C5 (located in helix A) and C405 (located in strand 5 of B- β -sheet) (Figure 1) has been defined chemically (unpublished data in Huber and Carrell, 1989) and shown by X-ray structure (Harrop *et al.*, 1999). To identify the disulfide bond(s) that are required to keep PAI-2 in the polymerogenic conformation, we constructed single (C5S, C79S, C145S and C161S) and double (C5S/C79S, C5S/C145S and C5S/C161S) mutants of PAI-2. Similar results were obtained for the single and double mutants; thus only the data for single mutants are shown in Figure 3. The mutants were expressed and purified to homogeneity (Figure 3A, 'minus' lanes). All mutant proteins had an inhibitory activity similar to wt PAI-2 and they formed SDS-stable complexes with uPA (Figure 3A, 'plus' lanes). To study polymerizing ability, mutant proteins were analyzed by non-denaturing PAGE (Figure 3) before ('minus' lanes) and after ('plus' lanes) incubation at 37°C for 24 h. As shown in Figure 3B, wt PAI-2, as well as the C5S and C145S mutants, were partly polymerized already after the purification, and they formed more dimers, trimers and higher order polymers

Fig. 3. Polymerization and activity tests of PAI-2 mutants with a cysteine substituted by serine. (A) Purified PAI-2 mutants ('minus' lanes) and their complexes with uPA ('plus' lanes) analyzed by SDS-PAGE under reducing conditions and Coomassie Blue stained. The positions of intact and cleaved PAI-2, A-chain of uPA and the PAI-2-uPA complex are marked. (B) Non-denaturing PAGE analysis of wt PAI-2 and the mutants before ('minus' lanes) and after ('plus' lanes) incubation for 24 h at 37°C, followed by western blot and ECL detection. The positions of PAI-2 monomers (M), dimers (D), trimers (Tr) and tetramers (Te) are marked. (C) Relative inhibitory activities of wt PAI-2, and C79S and C161C mutants of PAI-2 assayed before (black bars) and after (dashed bars) incubation at 37°C for 24 h, tested by chromogenic activity assay. The activity prior to the incubation for each PAI-2 form was assumed to be 100%.

after incubation at 37°C. The same result was also seen for the double mutant C5S/C145S (data not shown). Mutant C161S (Figure 3B) and the double mutant C5S/C161S (data not shown) appeared on the gel as a mixture of monomers and dimers, and more dimers were formed following incubation at 37°C. However, with these mutants, no higher order polymers were formed even after prolonged incubation at 37°C. The mutants involving C79 (C79S, Figure 3B; and double mutant C5S/C79S, data not shown) always migrated as monomers, regardless of the incubation. As shown in Figure 3C, the activity of wt PAI-2 decreased by ~70% following incubation at 37°C due to formation of inactive loop-sheet polymers. However, despite differences in the oligomerization patterns, both the C79S and C161S mutants remained almost fully active after this treatment. To characterize further the dimers formed by the C161S mutant, molecular sieving experiments were performed (Figure 4). In agreement with results from non-denaturing PAGE, wt PAI-2 eluted from the column in peaks corresponding to inhibitory active monomers and inactive polymers. In contrast, the C161S mutant eluted in two peaks corresponding to monomers and dimers, but both forms had inhibitory activity. The dimers formed by the C161S mutant were therefore not formed by the loop-sheet mechanism, but presumably by intermolecular disulfide bonding between the free cysteines at position 79 in two

Fig. 4. Molecular sieving of wt PAI-2 and the C161S mutant. The PAI-2 proteins pre-incubated in 50 mM Tris-HCl pH 7.4 for 24 h at 37°C were analyzed on a Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column (1 × 120 cm) equilibrated with the above buffer. The positions of ovalbumin monomers (M) and dimers (D) are marked with arrows. The continuous line represents the OD at 280 nm; the dotted line corresponds to inhibitory activity measured by chromogenic assay.

PAI-2 molecules. Supporting this assumption, a mutant of PAI-2, where all cysteines except the cysteine at position 79 were substituted with serines, formed dimers similar to the C161S mutant (data not shown).

Identification of a disulfide bond that stabilizes PAI-2 in a polymeric conformation

The above data suggest the presence of an intramolecular disulfide bond between C79 and C161, which affects the folding properties of PAI-2 by linking the CD-loop to the bottom of helix F. If the disulfide bond rather than the conformation of the CD-loop is important for the polymerization to occur, mutations in the CD-loop should not influence the ability of PAI-2 to polymerize as long as the disulfide bond is intact. Supporting this assumption, substitution mutants K87E and F81K, which should dramatically change the conformation of the CD-loop, had no influence on the ability of PAI-2 to polymerize (Figure 5, lanes 1 and 2). Subsequently, we constructed a PAI-2 mutant (C5S/C145S/C161S/C405S) where all cysteines except those at positions 79 and 161 were substituted with serines. This mutant, denoted as 79cys/161cys, was active and formed a complex with uPA (data not shown). As shown in Figure 5, lane 3, the 79cys/161cys mutant formed higher order polymers after incubation at 37°C, but, after reduction and modification of the cysteines, remained in a stable monomeric form (Figure 5, lane 4).

To prove the existence of a disulfide bond between residues C79 and C161, wt PAI-2 and 79cys/161cys protein were digested with trypsin or chymotrypsin and analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The results of analysis of the chymotrypsin-digested PAI-2 proteins are shown in Table I. Peaks at the masses corresponding to the dipeptides connected by the

C5–C405 disulfide bond were detected in wt PAI-2, but not in the 79cys/161cys mutant that lacks the C5 and C405 residues. Dipeptides containing the C79–C161 disulfide bond were present in both wt PAI-2 and the 79cys/161cys mutant, but absent when the samples were reduced and blocked with a cysteine-specific probe before the analysis (data not shown). The same results were obtained by the MALDI-TOF analysis of the PAI-2 proteins digested with trypsin (data not shown). Taken together, the data clearly showed the presence of a disulfide bond between residues C79 and C161 in wt PAI-2 and in the 79cys/161cys mutant.

Fig. 5. Importance of the C79–C161 disulfide bond for PAI-2 polymerization. PAI-2 mutants were analyzed by non-denaturing PAGE followed by western blot. Lane 1, PAI-2 mutant with the K87E substitution in the CD-loop; lane 2, PAI-2 mutant with the F81K substitution in the CD-loop; lane 3, 79cys/161cys mutant; lane 4, 79cys/161cys mutant reduced with DTT and labeled with sulfhydryl-specific BODIPY.

The effect of cysteine substitutions on the conformation of the A- β -sheet of PAI-2

RCL peptide annealing experiments can be used to study the conformation of the A- β -sheet (Skinner, 1998). When a synthetic RCL peptide anneals into the A- β -sheet, it occupies the space where the RCL loop normally inserts (Skinner, 1998; Jankova *et al.*, 2001). After the annealing, serpins become inactive monomers, which are cleaved like substrates by their target proteases (Björk *et al.*, 1992; Chang *et al.*, 1996). To test if the polymerogenic conformation of PAI-2 has an A- β -sheet with a more open conformation than the non-polymerizing C79S and C161S substitution mutants, we performed annealing experiments with a synthetic peptide homologous to the RCL of PAI-2. The PAI-2 proteins were pre-incubated with different molar excesses of the RCL peptide for 6 h at 37°C, mixed with uPA and analyzed by SDS-PAGE followed by western blot analysis. As shown in Figure 6A (insert), the amount of cleaved PAI-2, which corresponded to the binary complex between wt PAI-2 and the RCL peptide, increased over time. For all PAI-2 forms studied, the amount of annealed RCL peptide increased with increasing concentrations of the peptide. However, about twice as much peptide annealed into polymerizing forms of PAI-2 (wt, C5S and C145S mutants; open symbols), as compared with the non-polymerizing mutants (C79S, C161S, C5S/C79S and C5S/C161S mutants; filled symbols) (Figure 6A). To measure the kinetics of the annealing reaction, samples of PAI-2 were incubated with a 100-fold excess of RCL peptide and the annealing was recorded over a time. As shown in Figure 6B, the annealing reaction

Table I. Dipeptides connected by disulfide bonds identified using MALDI-TOF mass spectrometry following digestion of PAI-2 with chymotrypsin

Disulfide-connected dipeptides (amino acid residues) ^a	Calculated mass (Da)	Wild type		79cys/161cys	
		Observed peak ^b (<i>m/z</i>)	Δ	Observed peak ^b (<i>m/z</i>)	Δ
C5/C405					
6–10/397–407	1576.826	1576.863	0.037	–	
5–20/396–407	1038.054	1038.121	0.067	–	
5–20/397–407	959.519	959.570	0.051	–	
5–20/400–407	780.915	780.981	0.066	–	
1–10/398–408	1211.630	1211.619	0.011	–	
5–11/400–408	927.984	927.983	0.001	–	
5–15/397–407	1238.678	1238.707	0.029	–	
2–11/400–412	1360.190	1360.121	0.069	–	
C79/C161					
72–81/159–184	2043.543	2043.465	0.078	–	
72–81/160–196	2615.784	2615.760	0.024	–	
77–81/150–172	1575.729	1575.690	0.039	–	
61–82/160–172	1940.375	1940.366	0.009	–	
72–95/150–172	2656.254	2656.210	0.044	–	
77–82/160–172	1104.498	1104.433	0.065	1104.436	0.062
77–95/150–172	2362.127	2362.087	0.040	2362.088	0.039
77–82/159–196	2443.659	2443.603	0.056	2443.724	0.065
72–82/160–172	1398.625	1398.696	0.071	1398.650	0.025
77–95/159–196	3164.598	–	–	3164.650	0.052
77–95/160–172	1825.376	–	–	1825.338	0.038
77–107/149–172	3085.472	–	–	3085.506	0.034

Δ is the difference between calculated and observed masses.

^aThe dipeptides on the MALDI-TOF spectra were mainly doubly charged, and the minority only migrated as singly charged. Therefore, only the doubly charged dipeptides are shown in the table. In that case, the masses of the dipeptides were calculated as $(M1 + M2 - 2 + 2H)/2$, where M1 and M2 were the theoretical masses of the corresponding single peptides.

^bMonoisotopic masses of single-charged peptides.

Fig. 6. Annealing of synthetic peptides into wt PAI-2 and the mutants. (A) Wt PAI-2 and the mutants were incubated with different molar excesses of a synthetic peptide homologous to RCL of PAI-2 (in 50 mM Na-phosphate buffer pH 5.9, with 20% glycerol) for 6 h at 37°C. As a control, a synthetic peptide homologous to the CD-loop was used. After the incubation, the PAI-2 samples were mixed with a 1.5 molar excess of uPA and analyzed by SDS-PAGE under reducing conditions, followed by western blot and ECL detection of PAI-2 antigen. The annealed PAI-2 was quantified as relative amount of the cleaved PAI-2 in relation to PAI-2 antigen in each lane. Inset: a western blot where the RCL peptide was annealed into wt PAI-2. Lane 1, wt PAI-2 pre-incubated without peptide and then reacted with uPA; lanes 2–6, wt PAI-2 pre-incubated with a 6-, 12-, 25-, 50- and 100-fold molar excess of the RCL peptide (respectively) and then reacted with uPA (*, cleaved PAI-2; **, PAI-2–uPA complex); lane 7, intact PAI-2. (B) Kinetics of annealing of the peptide homologous to RCL of PAI-2. The wt PAI-2 and the mutants were incubated with a 100-fold molar excess of the RCL peptide and samples were analyzed at different time points as described above. For (A) and (B), annealing of the RCL peptide into: wt PAI-2 (open circles), C5S (open squares), C145S (open triangles), C79S (filled circles), C161S (filled squares), C5S/C79S (filled upright triangles) and C5S/C161S mutants (filled inverted triangles). Annealing of the control peptide into wt PAI-2 is marked by diamonds.

was much faster for the polymerizing forms of PAI-2 than for the non-polymerizing C79S, C161S and C5S/C79S mutants. These data suggest that the A- β -sheet is significantly more open in wt PAI-2 and in the polymerizing mutants than in the non-polymerizing forms of PAI-2.

In other serpins, the extent of the A- β -sheet opening correlates with the volume of hydrophobic cavities on the serpin surface (Whisstock *et al.*, 2000). The *bis*-ANS is a conventionally used probe that binds to the hydrophobic cavities on protein surfaces with a resultant increase of its fluorescence intensity (Busby *et al.*, 1981; Desai *et al.*, 2002). As shown in Figure 7, the non-polymerizing C79S mutant bound about three times more *bis*-ANS probe than wt PAI-2, and the initial rate of *bis*-ANS binding to C79S mutant was about seven times higher as compared with wt

Fig. 7. Probing hydrophobic cavities in polymerizing and non-polymerizing forms of PAI-2 by *bis*-ANS binding. The *bis*-ANS was mixed with a monomeric wt PAI-2 and C79S mutant, and the fluorescence was measured by exciting at 370 nm and measuring emitted light at 520 nm. (A) Kinetics of *bis*-ANS binding. (B) Calculated rate of *bis*-ANS binding to PAI-2 (mean value of three experiments).

PAI-2. This suggests that the C79S substitution causes significant changes in the volume of hydrophobic cavities in the PAI-2 molecule. Taken together, the RCL peptide annealing experiments and the *bis*-ANS-binding experiments support the conclusion that wt PAI-2 spontaneously polymerizes because it has an open A- β -sheet stabilized by the C79–C161 disulfide bond. Elimination of this disulfide bond results in profound conformational changes in the PAI-2 molecule, including a closure of the A- β -sheet.

Polymerization of PAI-2 in different cellular compartments

PAI-2, which is naturally expressed by eukaryotic cells, is found mainly in the cytosol. However, a fraction of the PAI-2 protein can be directed to the secretory pathway where it is glycosylated and translocated to the extracellular environment (Genton *et al.*, 1987; Wohlwend *et al.*, 1987; Belin *et al.*, 1989). Since the environment in the cytosol is more reducing than in the organelles of the secretory pathway, we compared the polymerization of PAI-2 in the two intracellular compartments. The Semliki forest virus (SFV) expression system and CHO cells were used to express wt PAI-2 and a PAI-2 construct containing the signal peptide of PAI-1. In the latter construct, PAI-2 was engineered to enter the secretory pathway (Mikus and Ny, 1996). Cell extracts were analyzed by SDS-PAGE. Consistent with previous data, wt PAI-2 was non-glycosylated (Figure 8A, lane 1) and, therefore, is cytosolic. In contrast, PAI-2 directed through the ER was glycosylated (Figure 8A, lane 2). When analyzed on a non-denaturing gel, PAI-2 present in the cytosol was mainly monomeric (Figure 8B, lane 1), whereas PAI-2 that had been directed through the ER was highly polymerized (Figure 8B, lane 2).

Discussion

The serpins are the most widespread among protease inhibitors and they control proteases in many pathological processes, including complement activation, inflammation, coagulation, fibrinolysis, carcinogenesis, angiogenesis and apoptosis (Silverman *et al.*, 2001). There exist >250 serpins in nature, and the evolutionary success of this protein family apparently can be explained

Fig. 8. Polymerization of PAI-2 *in vivo*. Wt PAI-2 and PAI-2 fused with signal peptide derived from PAI-1 were overexpressed in CHO cells using the SFV expression system. Cell extracts in 50 mM phosphate buffer pH 5.9 were fractionated by SDS-PAGE (A) and non-reducing PAGE (B) followed by western blot and ECL. In both panels: lane 1, wt PAI-2; lane 2, PAI-2 fused with the external secretion signal.

by their unique ability for conformational change and structural mobility (Stein and Carrell, 1995). These properties allow the serpins to inhibit target proteases efficiently by translocation and, in some cases, to regulate their inhibitory activity by interaction with cofactors (Stein and Carrell, 1995). However, these remarkable folding properties make the serpins sensitive to mutations that lead to the formation of dysfunctional variants. In some naturally occurring pathological mutants, the RCL of one molecule inserts into the A- β -sheet of an adjacent molecule, resulting in spontaneous aggregation by so-called loop-sheet polymerization. Deposition of such aggregates within tissues or cellular compartments is linked to various forms of disease (Stein and Carrell, 1995).

With the exception of PAI-2, most natural serpins are stable active monomers under physiological conditions. These serpins require molecular destabilization by treatment under mild denaturing conditions before polymerization takes place (Dafforn *et al.*, 1999). There are two unique features that distinguish PAI-2 from other more well-studied serpins. It has an unusually long insertion between helices C and D (the CD-loop) and it spontaneously forms loop-sheet polymers under physiological conditions (Mikus *et al.*, 1993; Mikus and Ny, 1996).

Here we show that PAI-2 has a unique ability to regulate its conformation and thereby acquires the ability to polymerize. The polymerogenic conformation of PAI-2 has an open A- β -sheet, which is stabilized by a disulfide bond between C79, in the middle of the CD-loop, and C161, at the bottom of the serpin. Elimination of this disulfide bond causes A- β -sheet closure and abrogates polymerization, whereby PAI-2 becomes a stable inhibitory active monomer. The data suggest that the CD-loop of PAI-2 is a new functional domain that functions as a redox-sensitive molecular switch with the ability to convert PAI-2 between a stable inhibitory active monomeric conformation and a polymerogenic conformation that readily forms inactive polymers. Our finding that intracellular non-glycosylated PAI-2 exists mostly in monomeric form, whereas PAI-2 that is directed into the secretory pathway is highly polymerized, suggests that the redox status of the cell could be a regulator of PAI-2 polymerization.

The PAI-2 protein used in this study has five cysteine residues (Figure 1), but only two, C79 and C161, are conserved in PAI-2 in different species, implying that these cysteines may be of functional importance. The fact that polymerization of PAI-2 could be abrogated under reducing conditions (Figure 2) indicated that disulfide bond(s) may be important for the polymerization to occur. By mutational analysis, we could show that substitution of C5, C405 and C145 had no effect on polymerization, but substitution of C79 or C161 resulted in stable non-polymerizing monomeric forms of PAI-2. Subsequently, a disulfide bond between these residues was identified by MALDI-TOF mass spectrometry. The data therefore show that a disulfide bond, which connects the CD-loop to the bottom of helix F, is obligatory to keep PAI-2 in a polymerogenic conformation. Elimination of the disulfide bond by reduction, cysteine substitution or complete deletion of the CD-loop (S.Lobov, M.Wilczynska, F.Bergström, L.B.-Å.Johansson and T.Ny, unpublished data) converts PAI-2 into a stable monomeric conformation.

To test if the disulfide bond between C79 and C161 constitutes a conformational link between the CD-loop and the A- β -sheet, two different approaches were used. As shown by others (Elliott *et al.*, 2000), there is a correlation between the volume of hydrophobic cavities on the serpin surface and the extent of A- β -sheet opening. By using the dye *bis*-ANS that binds to and senses changes in protein surface hydrophobicity (Williams *et al.*, 1999; Desai *et al.*, 2002), we could show that elimination of the disulfide bond between C79 and C161 resulted in an increase in the volume of hydrophobic cavities (Figure 7). To test if the polymerogenic conformation with an intact disulfide bond has an A- β -sheet with a more open conformation than the non-polymerizing forms that lacks the disulfide bond, we performed RCL peptide annealing experiments. As shown in Figure 6, an external RCL peptide anneals into the A- β -sheet (in the place of the vacant strand 4A) much faster and to a larger extent in the polymerizing than in the non-polymerizing forms of PAI-2. Taken together, our data show that PAI-2's unique ability to polymerize is due to an unusually open A- β -sheet, and that this conformation is stabilized by the disulfide-linked CD-loop.

The crystal structure of an active PAI-2 mutant that lacks the CD-loop has been solved recently (Harrop *et al.*, 1999). According to our data, this structure should represent the stable non-polymerizing form of PAI-2. Indeed, *Escherichia coli* expressed and purified PAI-2 mutant lacking the CD-loop was active and stable in monomeric form (S.Lobov, M.Wilczynska, F.Bergström, L.B.-Å.Johansson and T.Ny, unpublished data). With the exception of the breach region, which is the place where insertion of the RCL begins during inhibition, there are no striking differences between the structure of PAI-2 and that of other serpins. However, in this region, the PAI-2 mutant has a partially opened A- β -sheet. The Z-mutation of α_1 -antitrypsin, which causes spontaneous polymerization of this serpin, maps to the breach region and results in molecular rearrangements similar to those found in PAI-2 (Lomas *et al.*, 1992). This molecular feature could, therefore, determine the ability of PAI-2 to polymerize. However, as we have shown (Wilczynska *et al.*, 2003), the breach region of PAI-2 has a minor effect on polymeriza-

Fig. 9. Three interconvertible forms of PAI-2. Under reducing conditions, when disulfide bonds cannot be formed, PAI-2 exists in stable monomeric form with a closed A- β -sheet. This form of PAI-2 resembles that of other native serpins. However, under oxidative conditions, when the disulfide bond between C79 (located in the middle of the CD-loop) and C161 (located at the bottom of helix F) can form, PAI-2 converts into a polymerogenic conformation with an open A- β -sheet. The gap in the A- β -sheet makes PAI-2 an acceptor for the RCL of another PAI-2 molecule, leading to spontaneous polymerization by the loop-sheet mechanism. Both the polymerogenic conformation of PAI-2 and the polymers can convert to the stable monomeric form following reduction.

tion and modifies rather than determines the polymerization ability of the serpin.

The finding that the CD-loop of PAI-2 functions as a molecular switch between the polymerizing and non-polymerizing conformations appears to be logical from a structural point of view. In general terms, helices D and E and the connecting loops have to move in relation to the rest of the serpin molecule before the A- β -sheet can open and allow insertion of the RCL (Stein and Chothia, 1991). It is therefore likely that a connection of the CD-loop with the bottom of helix F through the C79–C161 disulfide bond restricts the mobility of the A- β -sheet and stabilizes it in an open conformation. Consequently, the elimination of this disulfide bond would restore the conformational mobility of the PAI-2 molecule, leading to A- β -sheet closure with concomitant loss of the ability to polymerize.

Several hydrophobic cavities have been identified in serpins. Because it is a potential target for drugs neutralizing serpin activity and preventing serpin polymerization (Björquist *et al.*, 1998; Elliott *et al.*, 2000), a cavity located between strand 2 of the A- β -sheet, helix D and helix E has been studied extensively. In active PAI-1, this cavity binds various organic compounds, including bis-ANS (Egelund *et al.*, 2001). The cavity is also present in the PAI-2 mutant lacking the CD-loop, that according to our data represents the non-polymerizing form with a closed A- β -sheet. However, in a structure of the same PAI-2 mutant in complex with a RCL peptide, which has the A- β -sheet with an open conformation, this cavity is closed (Jankova *et al.*, 2001). Changes in the volume of hydrophobic cavities that correlate with A- β -sheet closure (Figure 7) may therefore relate to conformational rearrangements in the cavity located between strand 2 of the A- β -sheet, helix D and helix E.

Disulfide bonds are only present in a few extracellular serpins, and when present they often connect specific extensions to the main body of serpin molecule. Thus, disulfide bonds stabilize the N-terminal extensions in the C1 inhibitor (Simonovic and Patston, 2000) and in antithrombin III (Sun and Chang, 1989), and the short (6–7 amino acid residues) CD-loops found in ovalbumin, angiotensinogen and gene Y protein (Huber and Carrell, 1989). The disulfide bonds in C1 inhibitor stabilize the

protein in the active conformation (Simonovic and Patston, 2000), and the disulfide bond in antithrombin III is required for heparin activation of the serpin (Longas *et al.*, 1980). However, as far as we know, this is the first time that intramolecular disulfide bond formation has been linked to serpin polymerization. It is tempting to speculate that the CD-loop in PAI-2 is a novel redox-regulated molecular switch with a biological function. PAI-2 can be found as non-glycosylated cytosolic protein and as glycosylated protein that is secreted by the Golgi-dependent secretory pathway (Genton *et al.*, 1987; Wohlwend *et al.*, 1987; Ny *et al.*, 1989; von Heijne *et al.*, 1991). The relative distribution of the intracellular and secreted PAI-2 depends on cell type and differentiation state, and varies from almost all PAI-2 being secreted (Ye *et al.*, 1988) to the majority remaining intracellular (Genton *et al.*, 1987; Wohlwend *et al.*, 1987). This finding raises the question as to where and how the polymerization is regulated in the cell. Taking into account that the presence of the C79–C161 disulfide bond is obligatory for PAI-2 to polymerize, the polymerization should occur mainly in the organelles of the secretory pathway or outside the cell. In agreement with this, we found that PAI-2 that was glycosylated, and therefore must have passed through the ER, readily polymerized (Figure 8) (Mikus and Ny, 1996). In contrast, non-glycosylated PAI-2 that originates from the cytosol remained mainly in a monomeric form (Figure 8). However, growing evidence indicates that the intracellular redox status may be modulated in some pathological processes. For example, during a virus infection, virus-encoded redox-regulated proteins can catalyze the formation of the disulfide bonds that are required for the virion assembly in cytosol (Li *et al.*, 2002; White *et al.*, 2002). Therefore, the question remains open of whether the polymerization of PAI-2 can also occur in cytosol under certain conditions.

PAI-2 seems to have been selected by evolution as an inhibitor that can exist in three interconvertible forms: stable monomeric, polymerogenic and polymerized forms. The polymerogenic conformation of PAI-2 is stabilized by the unique redox-sensitive CD-loop. This form of PAI-2 can form loop-sheet polymers spontaneously, or convert to the stable monomeric form following reduction (Figure 9). The physiological function of PAI-2 remains

unclear, and it cannot be excluded that all three forms may have distinct biological roles.

Materials and methods

Construction and purification of PAI-2 mutants

Human PAI-2 was expressed in a thioredoxin reductase-deficient *E. coli* strain AD494(DE3) by pET15b vector (both from Clontech Laboratories, Inc., USA) encoding PAI-2 fused to a His tag at the N-terminus. Mutagenesis and purification were as described (Wilczynska *et al.*, 2003). Alternatively, wt PAI-2 and its fusion with the secretion signal of PAI-1 were expressed in CHO cells using the SFV system as described previously (Mikus *et al.*, 1993).

Reduction and alkylation of PAI-2

Purified wt PAI-2 (0.19 mg/ml) in 50 mM Tris-HCl buffer pH 8.0 with 0.14 M NaCl (TBS) was incubated with 100 mM DTT for 1 h at 37°C. Then, iodoacetamide was added to a final concentration of 113 mM, and the incubation continued for 30 min at room temperature in the dark. Finally, the PAI-2 protein (defined as 'blocked' PAI-2) was desalted on a NAP-25 column (Pharmacia, Uppsala, Sweden), which was equilibrated with TBS buffer.

Annealing of peptides to PAI-2

Different amounts of synthetic HPLC-purified peptides corresponding to the P2–P14 residues of the RCL of PAI-2 (TEAAAGTGGVMTG, the RCL peptide) or a control peptide consisting of residues 80–92 in the CD-loop of PAI-2 (GFMQQIQKGSYPD) were incubated with fully active, monomeric wt PAI-2 or its mutants (30 µg/ml in 50 mM Na-phosphate buffer pH 5.9, with 20% glycerol) at 37°C for 6 h. To study the kinetics, we used a 100-fold excess of peptide over PAI-2. Following incubation with peptide, PAI-2 samples were mixed with a 1.5 M excess of uPA and kept at room temperature for 10 min. After this incubation, sample buffer containing 100 mM DTT was added, and the proteins were analyzed by SDS-PAGE, followed by western blot and enhanced chemiluminescence (ECL) detection using a phosphorimager (GS-250 Molecular Imager™; Bio-Rad). The extent of the annealing was quantified as the relative amount of cleaved PAI-2 in each lane.

MALDI-TOF mass spectrometry analysis

Purified wt PAI-2, as well as non-labeled and BODIPY-labeled 79cys/161cys mutant of PAI-2 were supplied on a non-reducing 10% SDS-polyacrylamide gel where the pH of the resolving gel and the running buffer was adjusted to 8.3. The gel was stained with Coomassie Blue, and pieces with PAI-2 protein were excised, minced and destained further with three washes of 25 mM ammonium bicarbonate with 50% acetonitrile. Following vacuum drying, the gel homogenates were rehydrated with 25 mM ammonium bicarbonate containing 0.1 mg/ml trypsin or chymotrypsin. Following a 16 h digestion at 37°C, the peptides were extracted from the gel pieces with 5% trifluoroacetic acid (TFA)/50% acetonitrile, before being dried and reconstituted in the same solvent. Finally, the samples were mixed with CHCA (α -cyano-4-hydroxycinnamic acid) or sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and dried on the sample target. Mass spectra were acquired using MALDI-TOF mass spectrometry (Voyager DE-STR; Applied Biosystems, Boston, MA) in the positive-ion reflector mode with delayed extraction, using standard conditions.

Other methods

Chromogenic assay for PAI-2 inhibitory activity was according to Mikus *et al.* (1993). The labeling of PAI-2 with the sulfhydryl-specific derivative of BODIPY was carried out as described previously for PAI-1 (Wilczynska *et al.*, 1997). Bis-ANS binding to PAI-2 was performed as described (Wilczynska *et al.*, 2003). SDS-PAGE (10%) was run according to Laemmli (1970), and non-denaturing PAGE (10%) was performed with the same buffer system, except that SDS was not added. Following PAGE fractionation, the proteins were immunoblotted as described (Towbin *et al.*, 1979). PAI-2 antigen was detected using monoclonal antibodies MAI-21 (Biopool, Umeå, Sweden) and anti-mouse IgG conjugated to horseradish peroxidase (Promega, USA) by the ECL method (Amersham, UK). Protein concentration was determined by the bicinchoninic acid assay (Pierce, USA).

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