Conformational Studies on Plasminogen Activator Inhibitor (PAI-1) in Active, Latent, Substrate, and Cleaved Forms†

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ABSTRACT: Plasminogen activator inhibitor 1 (PAI-1), the primary physiological inhibitor of t-PA, is an unusual member of the serpin family of serine protease inhibitors, in that it spontaneously converts to a latent form. Latent PAI-1 has been reported to share characteristics with the cleaved form of other serpins. Here we examine the conformation of four forms of PAI-1, active and latent wild-type, together with a noninhibitory, substrate mutant that is cleavable at P_1-P_1', and its cleaved product. The circular dichroism spectra of active and latent PAI-1 showed differences consistent with decreased α-helix from 26% to 22% and increased β-sheet from 23% to 34% as active → latent. Active and substrate PAI-1 were less thermostable than latent PAI-1, which was 50% denatured at 70 °C. In contrast, cleaved PAI-1 was very stable, with little loss of structure at 100 °C. Cleaved PAI-1 was much more resistant to guanidinium chloride (Gdn-HCl), 50% unfolding requiring 4.5 M Gdn-HCl, while active, latent, and substrate forms of PAI-1 were 50% unfolded in 2−2.5 M Gdn-HCl. The differences in fluorescence emission maxima, latent 339 nm, active 336 nm, substrate 343 nm, and cleaved 333 nm, underline the contrast between latent and cleaved PAI-1. The conformational changes occurring on cleavage are clearly more profound than those seen on transition from active to latent PAI-1. The striking stability of the cleaved substrate mutant of PAI-1 toward thermal and Gdn-HCl induced unfolding suggests a strand insertion comparable with that in wild-type cleaved serpins, despite the presence of a bulky residue at position P_{12}, previously suggested to prevent insertion.

Plasminogen activator inhibitor type 1 (PAI-1) is a major regulatory component of the plasminogen−plasmin system. It is the principal inhibitor of the plasminogen activators t-PA and u-PA, with association rate constants of the order of 10^7 M^{-1} s^{-1} (Kruithof *et al.*, 1984; Thorsen *et al.*, 1988). PAI-1 is a glycoprotein of apparent molecular mass 48 kDa, consisting of 379 amino acids and containing no cysteine residues. The amino acid sequence derived from the cDNA nucleotide sequence shows that PAI-1 is structurally related to the serine protease inhibitor (serpin) superfamily (Ginsburg *et al.*, 1986; Ny *et al.*, 1986; Pannekoek *et al.*, 1986). Members of the family include α_1-proteinase inhibitor (α_1-antitrypsin), α_2-antiplasmin, antithrombin, heparin cofactor II, and C1-inhibitor. These proteins regulate the major protease systems of the body, including the fibrinolytic, coagulation, and complement cascades.

Serpins act as pseudosubstrates for their target serine proteases. They contain a reactive center peptide bond (P_1-P_1') that the protease recognizes as it would its substrate, and the interaction between the protease and the active inhibitor results in the formation of a tight, SDS-stable, equimolar complex (Travis & Salvesen, 1983). The serpins are known to undergo a conformational rearrangement upon cleavage of this peptide bond by the protease, resulting in a modified protein that is no longer inhibitory. Upon complex formation with the target protease, cleavage of the reactive center in the inhibitor and dissociation of the complex, the two amino acids at position P_1-P_1', which form the reactive center of the native inhibitor, appear located at opposite ends of the cleaved molecule, separated by 67 Å in the case of α_1-antitrypsin (Loebermann *et al.*, 1984). In addition, the loop that contains the reactive center residue P_1 forms a strand of β-sheet A. The inserted loop has been observed in the crystal structures of several cleaved serpins such as α_1-antitrypsin (Loebermann *et al.*, 1984) and α_1-antichymotrypsin (Baumann *et al.*, 1991). This capacity for insertion is thought to be crucial for inhibitory activity, since it does not occur in noninhibitory serpins such as plakalbumin, the cleaved form of ovalbumin (Wright *et al.*, 1990). On the basis of the structure of ovalbumin (Stein *et al.*, 1990) and on recent structures for intact, active serpins (Carrell *et al.*, 1994; Schreuder *et al.*, 1994; Wei *et al.*, 1994), it appears that the reactive center of the native active inhibitor is exposed in a loop out of the core of the protein.

The conformational differences between active and cleaved serpins have been studied by various methods, showing contrasting resistance to heat denaturation (Carrell & Owen, 1985; Gettins & Harten, 1988; Pemberton *et al.*, 1989), circular dichroism (CD) spectra (Bruch *et al.*, 1988; Powell & Pain, 1992), and fluorescence emission properties (Powell

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& Pain, 1992; Hervé & Ghélis, 1991; Koloczek *et al.*, 1991), and by NMR and FT-IR (Haris *et al.*, 1986; Perkins *et al.*, 1992). These studies are consistent with an increase in secondary structure content, which would stabilize the cleaved molecule. In addition, loop insertion has been further demonstrated by experiments in which incubation of native inhibitors with synthetic peptides, bearing the sequence of the loop, induced the conformational transition from native to cleaved forms (Schulze *et al.*, 1992; Carrell *et al.*, 1991; Mast *et al.*, 1992; Björk *et al.*, 1992).

PAI-1 is unique among the serpins in that it can exist in various conformations in addition to the active and cleaved forms. The inhibitor has been shown to be synthesized in its active form by endothelial cells in culture but, after release into the medium, it adopts a latent conformation that is inactive (Levin, 1986; Kooistra *et al.*, 1986). Latent PAI-1 can be reactivated to some extent by denaturation and refolding of the inhibitor (Hekman & Loskutoff, 1985). The crystal structure of latent PAI-1 has been solved (Mottonen *et al.*, 1992) and shows how part of the exposed loop that contains the reactive center is inserted in the β-sheet A of the molecule, resembling the structure of cleaved serpins. Conformational studies on PAI-1 have been made difficult by its latency. Previous studies have compared latent material with denaturant-reactivated PAI-1 (Boström *et al.*, 1990; Munch *et al.*, 1991). Some have used PAI-1 that was initially active but utilized buffers at neutral or slightly alkaline pH and relatively low salt concentrations (Dwivedi *et al.*, 1991; Seetharam *et al.*, 1992), conditions under which we have found that PAI-1 is not stable and would hence become latent during the studies. The production of preparations of active PAI-1 of high specific activity (80−100%) and the definition of conditions under which it is stable (Sancho *et al.*, 1994) have enabled us to undertake conformational studies on active and latent PAI-1. Construction of a PAI-1 mutant that acts predominantly as a substrate for t-PA (Audenaert *et al.*, 1994) has also allowed the study of PAI-1, cleaved at P_1-P_1'.

MATERIALS AND METHODS

PAI-1. Recombinant PAI-1 was expressed and purified in an active and stable form as previously described (Sancho *et al.*, 1994). Briefly, it was expressed in *Escherichia coli* and purified by cation-exchange chromatography in sodium acetate buffer, pH 5.6, with elution in buffer containing 1 M NaCl. Conversion to latent PAI-1 was achieved by diluting active PAI-1 in 5 mM sodium acetate buffer, pH 5.6, containing 0.01% Tween-20 and 1 M sucrose and incubating for 8 h at 37 °C. The solution was then dialyzed against 5 mM sodium acetate buffer, pH 5.6, containing 0.01% Tween-20 and concentrated in an Amicon ultrafiltration apparatus (YM2 membrane). A substrate mutant of PAI-1 and its cleaved derivative were as described (Audenaert *et al.*, 1994). Briefly, Ala at position 335 (P_{12}) was substituted with Pro. This PAI-1 mutant (PAI-1-P_{12}) behaved as a stable molecule with substrate properties toward the serine proteases t-PA, u-PA, plasmin, and thrombin. Its cleaved derivative was prepared by incubation for 2 h at 37 °C of PAI-1-P_{12} (2 mL of approximately 1 mg/mL) with 500 μL of Sepharose 4B-bound t-PA (1 mg of t-PA/mL of gel). Cleavage was confirmed by SDS−PAGE.

Heat Stability Experiments. Aliquots containing 35 μg/mL of PAI-1, in 20 mM sodium acetate buffer, pH 5.6, containing 1 M NaCl and 0.01% Tween-20, were heated at constant temperatures between 30 and 100 °C for 2 h. The samples were then cooled in ice and centrifuged for 5 min in a microfuge to precipitate denatured protein. The supernatants were carefully removed and stored at −70 °C until analyzed. Residual protein in the supernatants was determined by immunoelectrophoresis in agarose (Laurell, 1966) using a rabbit antiserum to PAI-1 (Booth *et al.*, 1987) and by ELISA for PAI-1 (Booth *et al.*, 1988). The percentage of PAI-1 antigen remaining in the supernatants was calculated from standard curves obtained from controls kept at 0 °C.

Circular Dichroism. Circular dichroism (CD) was measured at 20 °C, recording the data at 0.2 nm intervals, on a Jasco J-600 spectropolarimeter calibrated with (1*S*)-(+)-10-camphorsulfonic acid. Far-UV CD spectra (190−260 nm) were measured using a 0.02 cm path length cell. The PAI-1 concentration was 0.2 mg/mL in 5 mM sodium acetate, pH 5.6, containing 200 mM NaCl and 0.01% Tween-20. For near-UV CD measurements (260−320 nm) a 0.5 cm path length cell and protein concentrations of approximately 1 mg/mL were used. Ellipticity values are expressed as mean residue ellipticity $[\theta]_{mrw}$, deg·cm^2·dmol^{-1}, with a mean residue mass for PAI-1 of 116 Da. The protein secondary structure was estimated from the CD spectra using the CONTIN analysis (Provencher & Glöckner, 1981).

In order to study the unfolding transitions of PAI-1, samples (0.1 mg/mL) were incubated for 15 min with increasing concentrations (0−6 M) of guanidinium chloride (Gdn-HCl) and then subjected to far-UV CD analysis and, in the case of active and latent PAI-1, to fluorescence spectroscopy. The concentrations of Gdn-HCl (ultrapure grade, GIBCO/BRL) were checked by refractive index measurements (Nozaki, 1972).

Fluorescence Spectroscopy. Fluorescence spectra were monitored with a Perkin Elmer LS 50 spectrofluorimeter. The excitation wavelength was 290 nm, and fluorescence emission was recorded between 300 and 400 nm. The sample compartment was thermostated at 20 °C. The protein concentration was 0.1−0.2 mg/mL in 5 mM sodium acetate, pH 5.6, containing 200 mM NaCl and 0.01% Tween-20.

Differential Scanning Calorimetry. Samples were scanned in a Microcal MC2-D instrument over the range 20−100 °C, with an increase of 60 °C/h. Sample buffers were used for reference and for instrument equilibration. Protein concentrations were in the range 0.75−1.5 mg/mL.

FT-IR Spectroscopy. FT-IR spectra were recorded on a Perkin-Elmer 1750 spectrometer equipped with a TGS detector as described by Perkins *et al.* (1992). The non-enhanced absorbance FT-IR spectra, obtained after subtraction of the background H_2O absorption, were analyzed by the Perkin-Elmer CIRCOM program (Lee *et al.*, 1990) in order to obtain the proportions of secondary structure present in the protein.

RESULTS

Characterization of PAI-1. PAI-1, expressed and purified in an active and stable form, and its latent derivative were assessed by a chromogenic assay for u-PA, by which active PAI-1 had a specific activity that was 85−100% of its theoretical maximum, while that of latent PAI-1 was less than 3%. The active material could be converted to the 110 kDa t-PA−PAI-1 complex after incubation with excess t-PA,

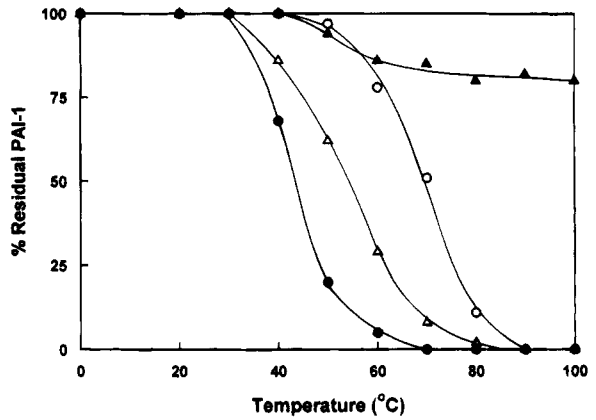

FIGURE 1: Heat stability of PAI-1. Duplicate aliquots containing 35 μg/mL PAI-1, active (●), latent (○), substrate mutant (△) and cleaved (▲) were incubated at constant temperatures from 0 to 100 °C for 2 h. After centrifugation, residual PAI-1 was assayed in duplicate by rocket immunoelectrophoresis and ELISA as described in Materials and Methods. The residual PAI-1 is expressed as percentage of a control kept at 0 °C. The data shown are from a single experiment on all four forms and represent typical data from 3−5 experiments.

unlike the latent material, which could not be converted to complex. By all these criteria the active material was judged to be 85−100% active (Sancho *et al.*, 1994). Where possible, the activity of the active preparation was checked after spectroscopic measurements such as CD and fluorescence; in all cases the activity was essentially unchanged.

The PAI-1-P_{12} mutant had a specific activity of <0.15% of the theoretical maximum and reacted primarily as a substrate, yielding >90% of the cleaved (at P_1-P_1') derivative after incubation with immobilized t-PA (Audenaert *et al.*, 1994). All physical measurements were made on several preparations of the four forms of PAI-1; all the data were reproducible from batch to batch.

Heat Stability. Heat stability was assessed by incubation of aliquots of PAI-1 at different temperatures for 2 h, followed by measurement of residual soluble antigen by ELISA or rocket immunoelectrophoresis, both methods giving comparable results. Active and substrate PAI-1 were the least thermostable forms, showing 50% precipitation at 43 and 55 °C, respectively (Figure 1). Latent PAI-1 was more stable than active or substrate PAI-1, with 50% precipitation at about 70 °C. The cleaved material was very stable, 80% of the original concentration of PAI-1 remaining in solution even at 100 °C.

The heat stability profiles were confirmed by differential scanning calorimetry, where no transition was observed for cleaved PAI-1. Transitions were observed for the other forms, T_m = 68 °C for latent PAI-1 and in the range 43−52 °C for active and substrate forms. Thermally-induced unfolding transitions were also confirmed by CD, measuring the change in ellipticity at 225 nm of samples heated at increasing temperatures; this method showed that the cleaved material was more thermostable than the other three forms (data not shown).

Circular Dichroism. The CD spectra of active and latent PAI-1 in the wavelength region from 190 to 260 nm are shown in Figure 2 (top panel). Both forms displayed a broad negative band between 205 and 250 nm and a positive CD band at approximately 195 nm. The two spectra showed different molecular ellipticities at peaks and troughs, and the crossover point with the base line was shifted from 202 nm

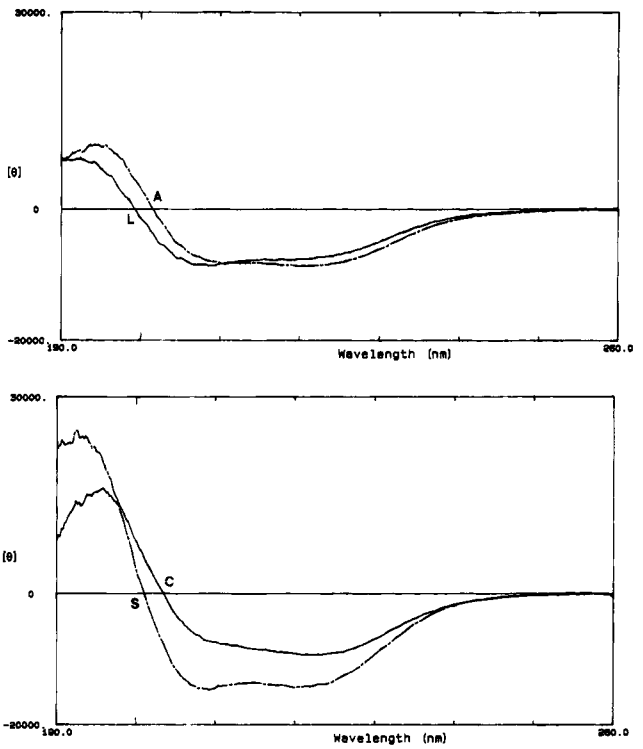

FIGURE 2: Circular dichroism on PAI-1. Spectra were recorded at 20 °C on PAI-1 in 5 mM acetate buffer, pH 5.6, containing 200 mM NaCl and 0.01% Tween-20. The protein concentrations were 0.1−0.2 mg/mL. Data were averaged from 2−4 scans for each form of PAI-1. The upper panel shows spectra for active (A) and latent (L) forms, while the lower panel shows substrate (S) and cleaved (C) forms of PAI-1.

in active PAI-1 to a shorter wavelength (199 nm) for latent PAI-1. The difference between these spectra was confirmed *in situ*, by heating active PAI-1 to 60 °C and then reanalyzing the spectrum, which was identical to the latent form.

The CONTIN procedure (Provencher & Glöckner, 1981) was applied to averaged data from 2−6 analyses, the molar amplitude between runs being within 5%, while the curve-fitting procedure has an error ±1%. The calculated secondary structures were 26% α-helix and 23% β-sheet for active PAI-1 and 22% α-helix and 34% β-sheet for the latent form. Some differences were also observed between the CD spectra of active and latent PAI-1 in the 260−310 nm region (not shown), where positive peaks at 265 and 292 nm could be assigned to phenylalanine and tryptophan contributions, respectively. The sizes of the near-UV bands were decreased in the latent form compared with the active PAI-1, indicating small differences in the overall folding of the polypeptide chain.

The spectra of the substrate mutant and its cleaved product are shown in Figure 2, lower panel. The crossover points with the base line were 204 nm for cleaved and 201 nm for the substrate form. Compared with the other forms of PAI-1, the spectrum of the substrate form had a much more typical α-helix pattern with the distinct characteristic minima at 208 and 222 nm and maximum at 193 nm. Analysis of this spectrum indicated 46% α-helix with 20% β-sheet. The spectrum of the cleaved product indicated 31% α-helix and 28% β-sheet, showing that cleavage at P_1-P_1' caused a marked change in secondary structure.

Fluorescence Spectroscopy. The differences in the tryptophan environment of active and latent PAI-1 were also studied by fluorescence spectroscopy. Excitation of the

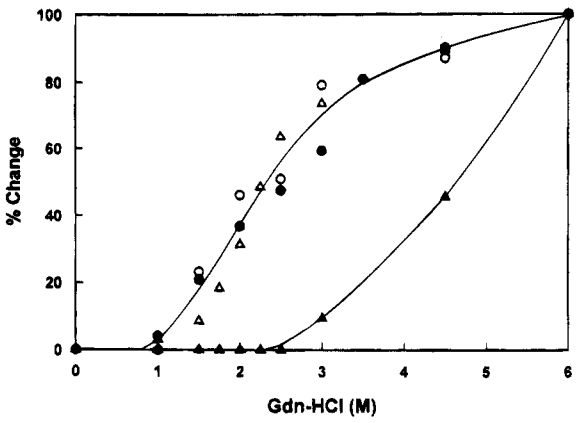

FIGURE 3: Denaturation by Gdn-HCl, monitored by CD. Changes in structure were calculated from the changes in ellipticity at 225 nm and presented as the % of maximum change, 100% corresponding to total unfolding of all forms in 6 M Gdn-HCl. Data are shown for active (●), latent (○), substrate mutant (△) and cleaved (▲) PAI-1. Data were taken from four scans for each preparation.

samples was at 290 nm, which selectively excites Trp residues. There was a shift of the λ_{max} of fluorescence emission from 336 nm in the active form to 339 nm in latent PAI-1. This is indicative of a greater degree of exposure to the solvent of one or more of the Trp residues in the latent form. This clearly confirms that differences exist between the tertiary structures of active and latent PAI-1. The fluorescence maxima for substrate and cleaved PAI-1 were 343 and 333 nm, respectively, indicating the marked change in the environment of the Trp residues that occurs on cleavage.

Fourier Transformed Infrared Spectroscopy (FT-IR). FT-IR spectroscopy was performed in order to provide additional information on the secondary structure of active PAI-1. The total amount of secondary structure present in active PAI-1 was calculated to be 28% α-helix, 41% β-sheet, and 27% turns. The concentrations of the other forms of PAI-1 were not high enough to obtain comparable data, and attempts to increase the concentration of PAI-1 to that required resulted in extensive aggregation.

Denaturation by Gdn-HCl. The far-UV CD spectra of active, latent, substrate, and cleaved PAI-1 were measured in the presence of increasing Gdn-HCl concentrations (0−6 M). The changes in structure were calculated in terms of changes in the θ_{225} values. When these values were plotted versus the concentration of Gdn-HCl (Figure 3), little difference was observed in the overall stability of the active, latent, and substrate forms, all three exhibiting 50% unfolding at 2−2.5 M Gdn-HCl. In marked contrast, cleaved PAI-1 required 4.5 M Gdn-HCl for 50% unfolding. All forms were totally unfolded in 6 M Gdn-HCl.

The fluorescence emission spectra of solutions containing active or latent PAI-1 in the presence of various Gdn-HCl concentrations were also recorded in order to monitor the unfolding of PAI-1. The spectrum of active PAI-1 in the absence of Gdn-HCl showed a maximum at 336 nm. At low concentrations, Gdn-HCl induced a slight increase in intensity without a shift in the maximum. At higher concentrations (1−3.5 M), the wavelength of maximum emission was gradually shifted to 356 nm, characteristic of fully exposed Trp side chains. In the case of latent PAI-1, there was no increase in intensity induced by low concentrations of Gdn-HCl as had been seen for the active form. However, a large red shift of the maximum emission toward the maximum value of 356 nm did occur at higher Gdn-HCl concentrations, indicating complete unfolding of the molecule. For both active and latent forms, the major changes occurred in the range 1−3 M Gdn-HCl, where changes in the far-UV CD were also observed. Both forms of PAI-1 were completely unfolded at 6 M Gdn-HCl.

DISCUSSION

The data presented in this study provide structural information on four forms of PAI-1, as analyzed by stability to heat and to Gdn-HCl denaturation and by changes in secondary structure and in the environment of aromatic side chains. These comparative studies on fully active and latent PAI-1, together with evidence for a much more marked conformational change in cleaved PAI-1, give new insights into the structure−function relationships in this inhibitor. The conformation of fully active PAI-1 is reported for the first time and shows several points of contrast with latent PAI-1, the X-ray structure of which has been solved (Mottonen *et al.*, 1992). The study of active PAI-1 has previously been impossible because of its tendency to adopt a so-called latent conformation (Hekman & Loskutoff, 1985). Our recent expression of recombinant PAI-1 in *E. coli* and its purification in high yield, together with definition of conditions of pH and ionic strength under which active PAI-1 is relatively stable (Sancho *et al.*, 1994), have allowed this analysis of its conformation.

Analysis of the far-UV CD (190−260 nm), which is dominated by conformationally-dependent contributions of the polypeptide backbone (Greenfield & Fasman, 1969), showed a decrease in α-helical content (from 26% to 22%) upon conversion from active to latent PAI-1, accompanied by an increase in β-sheet structure (23% to 34%). These values are comparable with those obtained from the analysis of active PAI-1 by FT-IR, where α-helix and β-sheet contents were calculated as 28% and 41%, respectively. The estimates are in good agreement with those for other members of the serpins, where the total amount of secondary structure present ranged from 25% to 37% α-helix, 31% to 45% β-sheet, and 13% to 25% β-turn, as determined by FT-IR studies (Perkins *et al.*, 1992). The increase in β-sheet content upon conversion from active to latent is consistent with the structure of latent PAI-1 (Mottonen *et al.*, 1992), which shows how part of the reactive center loop is inserted in β-sheet A. Our data are in contrast with earlier studies, where the α-helical content appeared to rise upon transition from active to latent PAI-1 (Boström *et al.*, 1990). Moreover, we observed distinct differences between the secondary structure of the two forms of PAI-1 by CD, not detected previously (Dwivedi *et al.*, 1991; Seetharam *et al.*, 1992). These discrepancies may reflect different sources of PAI-1 and the use of reactivated rather than naturally active PAI-1 (Boström *et al.*, 1991). A further important difference is that earlier studies used conditions that do not favor the retention of activity by PAI-1, maintaining the protein at room temperature in buffers of high pH and low ionic strength (Dwivedi *et al.*, 1991; Seetheram *et al.*, 1992), which would result in generation of the latent conformation during the time course of the experiments (Sancho *et al.*, 1994). In this study it was possible to confirm that active PAI-1 retained its activity after CD and fluorescence measurements.

Differences were also observed between the CD spectra of active and latent PAI-1 in the 260−310 nm region, where

the contributions of aromatic amino acid side chains are predominant (Strickland, 1974). Tryptophan fluorescence was used as an additional probe for conformational differences between the active and latent forms of PAI-1. One of the four Trp residues of the PAI-1 molecule is located at the end of strand 3A. This region has been proposed to form a flexible joint between two large portions of the serpins, allowing insertion of the loop (Stein & Clothia, 1991). Thus, its environment is likely to be affected by the transition from active to latent PAI-1. There was a clear difference in the fluorescence emission maximum of the latent form (339 nm) relative to that of active PAI-1 (336 nm), confirming differences in tertiary structure. The shift in emission maximum indicates a more hydrophobic environment for the Trp residues in the active form of the inhibitor. The data are consistent with the proposal that active PAI-1 has a more compact conformation than latent PAI-1, in which the Trp residues are more exposed to the solvent. These findings are in broad agreement with the studies of Vaughan *et al.* (1993), in which PAI-1 that was partially active was analyzed.

Much of the structural information on serpins relates to cleaved forms, since cleaved α_1-antitrypsin was the first structure to be solved (Loebermann *et al.*, 1984). The insertion of part of the reactive center loop in cleaved serpins is well documented. The discovery of a similar insertion in latent PAI-1 (Mottonen *et al.*, 1992) has led to the view that the conformation of latent PAI-1 is broadly comparable to that of cleaved serpins (Munch *et al.*, 1991; Carrell *et al.*, 1991). In the present study we have taken advantage of a mutant of PAI-1 (PAI-1-P_{12}, in which the alanine at position P_{12} is substituted by a proline). This mutant behaves as a substrate of t-PA and u-PA rather than as an inhibitor (Audenaert *et al.*, 1994) and has allowed us to generate cleaved PAI-1 in quantities suitable for conformational analysis.

The spectrum of the substrate mutant showed a much more typical α-helix pattern than the other forms. Analysis of this spectrum by the CONTIN procedure indicated a high α-helical content (46%) with 20% β-sheet. This is reminiscent of the increased α-helical content seen in wild-type PAI-1 after treatment with SDS, which caused it to adopt a substrate conformation (Urano *et al.*, 1992). Analysis of the spectrum of cleaved PAI-1 indicated 31% α-helix and 28% β-sheet. While the precise values of the proportions of α-helix and β-sheet may be subject to errors in the determination of protein concentration or in the fitting of the spectra to those of the reference set of proteins, the distinct shapes of the spectra point to significant differences in the secondary structure pattern as a result of cleavage at P_1-P_1'.

Comparison of the fluorescence maxima of active, latent, and intact substrate mutant confirmed the observation, originally made on the naturally occurring substrate form of PAI-1, that the structure of substrate PAI-1 more closely resembles latent PAI-1 rather than active PAI-1 (Declerck *et al.*, 1992). More interestingly, upon cleavage of PAI-1-P_{12} at the P_1-P_1' position, a large shift in fluorescence maximum was observed, from 343 nm (intact) to 333 nm (cleaved). This change is in the opposite direction to that observed during transition from the active (λ_{max}, 336 nm) to the latent (λ_{max}, 339 nm) conformation. Taken together, these data are indicative of relatively important structural differences between the latent and cleaved forms of PAI-1, in contrast to the previous emphasis on the stability of latent PAI-1, which suggested a conformation comparable to those of cleaved serpins (Munch *et al.*, 1991; Carrell *et al.*, 1991).

A marked increase in resistance to heat denaturation is characteristic of cleaved serpins (Carrell & Owen, 1985; Gettins & Harten, 1988; Pemberton *et al.*, 1989). Cleaved PAI-1 showed this typical heat stability, with no thermal transition detectable by differential scanning calorimetry up to 100 °C (data not shown). The slight loss of soluble antigen at 60−80 °C probably reflects aggregation rather than heat-induced denaturation, in view of the stability demonstrated by calorimetry. Latent PAI-1, while more heat-stable than active and substrate forms, was more labile than the cleaved form. Our data are broadly consistent with those previously reported (Munch *et al.*, 1991) and agree with the changes in secondary structure observed by circular dichroism upon heat denaturation of active and latent PAI-1 (Lawrence *et al.*, 1994).

Gdn-HCl-induced unfolding of the protein resulted in a large red shift of the fluorescence to a λ_{max} of 356 nm, typical of Trp side chains that are fully exposed to the aqueous environment (Eftink & Ghiron, 1976). The transitions observed by CD occurred over the same range of Gdn-HCl concentrations, indicating complete unfolding of the polypeptide chain. Active, latent, and substrate forms were 50% unfolded at a concentration of 2−2.5 M Gdn-HCl. However, cleaved inhibitor was found to be much more stable, requiring 4.5 M Gdn-HCl for 50% unfolding, consistent with previous studies on other serpins (Bruch *et al.*, 1988; Hervé & Ghélis, 1991).

All these experimental approaches suggest that the conformational changes that occur upon cleavage and which stabilize the structure take place only incompletely in latent PAI-1, despite insertion of the loop in β-sheet A. Physical studies of several other serpins (Bruch *et al.*, 1988; Schulze *et al.*, 1990; Hervé & Ghélis, 1990; Perkins *et al.*, 1992; Powell & Pain, 1992) also indicate more profound differences between the native and cleaved forms of serpins than those observed between active and latent PAI-1.

The substrate mutant used in this study was designed on the basis of the correlation between the presence of a bulky residue at positions in the region P_{10} to P_{14} and the behavior of the serpins as substrates. This is well established for naturally-occurring variants of the serpins, including antithombin (Devraj-Kisuk *et al.*, 1988; Molho-Sebatier *et al.*, 1989; Perry *et al.*, 1989; Ireland *et al.*, 1991) and C1-inhibitor (Levy *et al.*, 1990; Skriver *et al.*, 1991). The behavior as substrates of serpins with Thr or Ser in the P_{10} position (Levy *et al.*, 1990; Perry *et al.*, 1991) is particularly interesting, since these residues occur in this position in wild-type serpins, Thr in heparin cofactor II and Ser in PAI-1. It has been proposed that the importance of bulky residues in this region lies in the subsequent failure of insertion, as demonstrated for the noninhibitory substrate serpin, ovalbumin, and for the substrate variant of antithrombin A384P (P_{10}) (Carrell *et al.*, 1991). Our heat stability data, using a similar substrate mutant but in PAI-1, are suggestive of insertion of the reactive center loop upon cleavage. The cleaved P_{12} mutant of PAI-1 (A335P) shows similar heat stability to that reported for cleaved PAI-1 prepared from wild-type PAI-1 (Munch *et al.*, 1991). This finding suggests that failure of insertion after cleavage is not the primary reason for substrate behavior among the serpins. More detailed studies on the heat stability of a series of substrate mutants (Audenaert *et al.*, 1994) and

their cleaved derivatives will further improve our understanding of this question.

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