Conformation of the Reactive Site Loop of α_1-Proteinase Inhibitor Probed by Limited Proteolysis†

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Received October 30, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: Elucidation of the reactive site loop (RSL) structure of serpins is essential for understanding their inhibitory mechanism. Maintenance of the RSL structure is likely to depend on its interactions with a dominant unit of secondary structure known as the A-sheet. We investigated these interactions by subjecting α_1-proteinase inhibitor to limited proteolysis using several enzymes. The P_1–P_{10} region of the RSL was extremely sensitive to proteolysis, indicating that residues P_3'–P_{13} are exposed in the virgin inhibitor. Following cleavage eight or nine residues upstream from the reactive site, the protein noncovalently polymerized, sometimes forming circles. Polymerization resulted from insertion of the P_1–P_8 or P_1–P_9 region of one molecule into the A-sheet of an adjacent proteolytically modified molecule. The site of cleavage within the RSL had a distinct effect on the conformational stability of the protein, such that stability increased as more amino acids insert into the A-sheet. We conclude that the A-sheet of virgin α_1-proteinase inhibitor resembles that of ovalbumin, except that it contains a bulge where two or three RSL residues are inserted. Insertion of seven or eight RSL residues, allowed by proteolytic cleavage of the RSL, causes expansion of the sheet. It is likely that the RSL of α_1-proteinase inhibitor and several serpins exhibits significantly more mobility than is common among other protein inhibitors of serine proteinases.

Human α_1-proteinase inhibitor (α_1PI)[1] is the best characterized member of a superfamily of proteins known as the serpins (Carrell & Travis, 1985). Many serpins are inhibitors of serine proteinases, although some, including human angiotensinogen (Doolittle, 1983) and hen ovalbumin (Hunt & Dayhoff, 1980) have no known inhibitory capacity. Serpins are found throughout the eukaryotic kingdoms, with the best characterized being the inhibitory serpins found in human blood that regulate proteinases involved in the hemostatic systems of coagulation, fibrinolysis, inflammation, and complement activation. Although a fair amount is known about the function of the human serpins, there have been several conflicting proposals regarding their mechanism of inhibition (Travis & Salvesen, 1983; Perlmutter et al., 1990; Skriver et al., 1991; Lawrence et al., 1990; Patston et al., 1991). This contrasts with a more complete knowledge of the mechanism of other active site directed inhibitors of serine proteinases, which is well described at both kinetic and structural levels.

The mode of inhibition of serine proteinases by most nonserpin protein inhibitors is known as the standard mechanism (Laskowski & Kato, 1980). These inhibitors bind to proteinases in a manner similar to good substrates, but much more tightly. Hydrolysis of the inhibitor, if it occurs, is very slow, and the modified[2] inhibitor is as strong an inhibitor as the virgin one. Thus, the reaction of proteinases with standard mechanism inhibitors is characterized as reversible tight binding inhibition. One of the key features shared by proteinase inhibitors that follow the standard mechanism is the similarity in conformation of the reactive site loop (RSL). X-ray crystallography has shown that the RSL is an extended rigid loop that is complementary to the substrate binding sites of a target proteinase. The RSL structure of the different classes of inhibitors is highly conserved despite the fact that the proteins themselves vary widely in overall structure (Laskowski & Kato, 1980; Read & James, 1986; Bode et al., 1986; Hubbard et al., 1991).

The interactions of α_1PI and other serpins with their target proteinases differ in some ways that suggest this class of inhibitors may not follow the standard mechanism [reviewed by Travis and Salvesen (1983) and Bode and Huber (1991)]. Notable deviations are (i) the binding between some serpins and their target proteinases is so tight as to suggest a covalent complex, and (ii) proteolytically modified serpins are inactive as inhibitors. The first deviation may represent an artifact of the denaturing conditions used to examine the complexes (Travis & Salvesen, 1983; Mast et al., 1991; Matheson et al., 1991). The inability of modified serpins to inhibit proteinases was explained when the crystal structure of a modified form of α_1PI was analyzed (Loebermann et al., 1984). In proteolytically modified α_1PI, the P_1 and P_1' residues of the RSL, connected before the cleavage occurred, are at opposite ends of the molecule, separated by 67 Å. The separation of these residues allows the formation of a five-stranded β-sheet to be completed. This completed sheet results in strong interactions that prevent the P_1 and P_1' residues from reuniting. This sheet (the A-sheet) is the dominant unit of secondary structure in modified α_1PI (Loebermann et al., 1984), modified antithrombin III (ATIII) (Mourey et al., 1990), modified α_1-antichymotrypsin (α_1ACT) (Baumann et al., 1991), and virgin

† This work was supported by NIH Grant GM-38860.

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[1] Abbreviations. α_1PI, α_1-proteinase inhibitor; α_1ACT, α_1-antichymotrypsin; ATIII, antithrombin III; RSL, reactive site loop; CA2, *Crotalus adamanteus* proteinase II; PP4, papaya proteinase IV; SV8, *Staphylococcus aureus* V8 proteinase; DCI, 3,4-dichloroisocoumarin; E-64 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate; TUG, transverse urea gradient.

[2] The nomenclature used to describe serpin forms follows that of Laskowski and Kato (1980) wherein "virgin" describes a native inhibitor that is fully active and has not reacted with a proteinase; and "modified" describes an inhibitor that has been proteolyzed somewhere in the RSL.

0006-2960/92/0431-2720$03.00/0

and modified ovalbumin (Stein et al., 1990; Wright et al., 1990).

Since molecular structures of virgin inhibitory serpins have yet to be described (ovalbumin is not a proteinase inhibitor), other methods must be used to determine whether serpins act in a manner consistent with the standard mechanism or whether they have a significantly different mode of action. As a starting point for determining their mechanism, we hypothesized that the serpin RSL is held in a rigid extended shape with a conformation similar to standard mechanism inhibitors (Mast et al., 1991), and this possibility is considered in recent reviews by Bode and Huber (1991) and Stein and Chothia (1991). This hypothesis is supported by the work of Madison et al. (1990), who showed that the effects of mutations introduced into the RSL of plasminogen activator inhibitor type 1 could be predicted by modeling it as a standard mechanism inhibitor. If serpins are standard mechanism inhibitors, the interaction between the RSL and the A-sheet, as well as other structural features responsible for maintaining the RSL in an active inhibitory conformation, must be defined. To define the length of the virgin RSL and its spacial relation to the A-sheet, we subjected α_1PI to limited proteolysis by a variety of enzymes with distinct substrate specificities. The results of these experiments are used to discuss the structure of virgin serpins and their mechanism of inhibition.

Materials and Methods

Reagents. 3,4-Dichloroisocoumarin (DCI), 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane (E-64), EDTA, Nonidet P-40 (NP-40), and the chromogenic substrate for chymotrypsin (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide) were obtained from Sigma Chemical Co., St. Louis, MO. The chromogenic substrates for trypsin (H-D-Pro-Phe-Arg-*p*-nitroanilide) and thrombin (H-D-Phe-pip-Arg-*p*-nitroanilide) were obtained from Helena Laboratories, Beaumont, TX. Ultrapure urea was obtained from Bethesda Research Laboratories, Gaithersburg, MD. Lactoperoxidase–glucose oxidase and electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, CA. $Na^{125}I$ (17.4 Ci/mmol) and [^{3}H]iodoacetic acid (200 mCi/mmol) were obtained from New England Nuclear, Boston, MA.

Proteins. Bovine trypsin (type XIII), bovine chymotrypsin (type VII), papain (type IV), *Staphylococcus aureus* V8 proteinase (SV8), and hen ovalbumin were obtained from Sigma Chemical Co., St. Louis, MO. Human neutrophil elastase (HNE) was a gift of Weislaw Watorek, University of Georgia. *Crotalus adamanteus* proteinase II (CA2) was a gift of Lawrence Kress, State University of New York. Thrombin was a gift of John Fenton, New York State Department of Health. Papaya proteinase IV (PP4) was a gift of David Buttle, Strangeways Laboratory. Purification of α_1PI (Pannell et al., 1974) and ATIII (Thaler & Schmer, 1975) was performed as previously described. α_1ACT was purified as described for C1-inhibitor (Salvesen et al., 1985) except that fractions from the DEAE-Sephacel column containing α_1ACT were pooled instead of those containing C1-inhibitor.

Peptides. The 16-mer peptide GTEAAGAMFLEAIPMY was synthesized on an Applied Biosystems 430A peptide synthesizer employing *tert*-butyloxycarbonyl amino acids and conditions recommended by the manufacturer. The C-terminal Tyr residue, which is not part of the natural RSL, was incorporated to allow spectrophotometric quantitation at 260 nm. The 9-mer RSL peptide MFLEAIPMC was purchased from Research Genetics, Huntsville, AL. The C-terminal Cys residue, which is not part of the natural RSL, was incorporated to allow labeling with [^{3}H]iodoacetic acid. Both peptides were purified by reverse-phase chromatography using a C_{18} column developed with a gradient from 0.1% trifluoroacetic acid to 90% acetonitrile/0.1% trifluoroacetic acid (5 mL min^{-1}), verified, and quantitated by amino acid compositional analysis and Edman degradation.

Amino Acid Sequence Analysis. Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer with on-line analysis of the phenylthiohydantoins using an Applied Biosystems 120A HPLC. Protein samples destined for sequence analysis were desalted into water on a Sephadex G-25 fast desalting column (Pharmacia). Samples were dried in a speed vac (Savant), dissolved in 0.1% trifluoroacetic acid, and applied to Porton protein or peptide sample support disks employing the modified cycles PI-BGN and PI-1 recommended by Porton Instruments.

Radiolabeling. α_1PI was radioiodinated using lactoperoxidase–glucose oxidase according to instructions provided by the manufacturer (Bio-Rad Laboratories). The purified 9-mer peptide (5 mg) was incubated in 200 mM Tris-HCl buffer, pH 8, containing 10 mM dithiothreitol for 60 min at 37 °C. The peptide was desalted by using a C_{18} Sep-Pak cartridge (Millipore) and freeze-dried from 90% acetonitrile/0.1% trifluoroacetic acid. The reduced peptide was dissolved in 100 μL of 200 mM Tris-HCl buffer, pH 8, and allowed to react for 30 min at 37 °C with 100 μCi of [^{3}H]iodoacetic acid and then for 30 min in the presence of 10 mM iodoacetic acid, followed by desalting as described above.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. The proteins were analyzed using continuous 5–15% linear gradient gels in the 2-amino-2-methyl-1,3-propanediol/glycine/HCl buffer system described by Bury (1981). All samples were boiled for 2 min in the presence of 50 mM dithiothreitol and 1% SDS prior to electrophoresis.

Nondenaturing Polyacrylamide Gel Electrophoresis. The proteins were analyzed by using the buffer system described for SDS–polyacrylamide gel electrophoresis except that SDS was omitted from all buffers and the samples were not boiled or treated with 50 mM dithiothreitol.

Transverse Urea Gradient (TUG) Polyacrylamide Gel Electrophoresis. A three-channel peristaltic pump was used to cast batches of 10 × 9 × 0.15 cm 7% polyacrylamide gels containing a continuous linear 0–8 M urea gradient (Goldberg, 1989) in the gel buffer system described above. The gel was rotated 90°, and a single sample containing approximately 50 μg of protein in a 150–300-μL total volume was loaded evenly across the top of the gel. Electrophoresis was performed at 23 °C with a constant current of 15 mA for 2 h. Proteins were visualized by staining with Coomassie blue.

Proteolytic Inactivation of α_1PI. The RSL of virgin α_1PI, or that of the other α_1PI used in this study, was cleaved by incubation with the various proteinases in 50 mM Tris-HCl and 150 mM NaCl, pH 8.0, for 30 min at 37 °C. Since proteinases sometimes cleave the RSL at more than one site, the reaction conditions that resulted in the initial cleavage event were optimized by analyzing cleavages with different proteinase dilutions using SDS–polyacrylamide gel electrophoresis. The samples containing the most dilute proteinase solution in which cleavage was observed were subjected to amino acid sequence analysis. The cleavage reactions were stopped by adding DCI (50 μM final concentration) to inhibit HNE, SV8, trypsin, and chymotrypsin (Powers & Harper, 1986), E-64 (50 μM final concentration) to inhibit papain and PP4, or EDTA (10 mM final concentration) to inhibit CA2.

Incorporation of the 16-mer RSL Peptide into the Serpins. The serpin (0.2–1.0 mg/mL) was incubated in the presence

of 100–200-fold molar excess of the RSL peptide for 15 h at 48 °C. The sample was then analyzed by gel electrophoresis. Alternatively, for determination of RSL cleavage sites, the peptide incorporated material was separated from excess free peptide by gel filtration chromatography employing Superose 6 (Pharmacia) equilibrated with 50 mM NH_4HCO_3. The serpin–16-mer complex was freeze-dried and redissolved in 0.1% trifluoroacetic acid (for direct sequence analysis) or in 50 mM Tris-HCl and 100 mM NaCl, pH 8 (for reaction with proteinases).

Measurement of the Rates of RSL Cleavage. Purified α_1PI–16-mer complex or virgin α_1PI was cleaved with increasingly dilute concentrations of either SV8, CA2, or PP4 for 30 min at 37 °C. After inhibiting the proteinases as described above, the samples were subjected to SDS–polyacrylamide gel electrophoresis. The relative rates of cleavage were determined by comparison of the amount of cleaved material at each proteinase dilution.

Incorporation of the 9-mer RSL Peptide into Cleaved α_1PI. α_1PI was cleaved with SV8, papain, CA2, or PP4, and the reactions were stopped as described above. A 100-fold molar excess of ^{3}H-labeled 9-mer was added to the samples, followed by incubation at 23 °C for 30 min. The samples were then subjected to nondenaturing polyacrylamide gel electrophoresis and autoradiography.

Electron Microscopy. Polymers were visualized by electron microscopy using negatively stained or rotary shadowed samples. For negative staining, a drop of protein solution at 30 μg/mL was applied to carbon film made hydrophobic by glow discharge. The film was washed with several drops of uranyl acetate and drained. To prepare the serpin polymers for rotary shadowing, a sample was sedimented through a 15–40% glycerol gradient in 0.2 M ammonium bicarbonate. The α_1PI polymers sedimented in a broad band centered around 10 S. The peak fraction was sprayed onto mica and rotary shadowed as described by Fowler and Erickson (1979). The proteins were observed in a Phillips EM 301 at initial magnification of 50 000.

Results

Proteolytic Sensitivity of the RSL of α_1PI. In one of the earliest investigations of serpin proteolytic inactivation, Johnson and Travis (1977) reported that the plant cysteine proteinase papain inactivated α_1PI by cleavage of the P_1–P_1' (Met_{358}–Ser_{359}) peptide bond. It is now recognized that serpin RSLs are rapidly cleaved by a number of proteinases that they do not inhibit [reviewed by Huber and Carrell (1989)]. In our studies of the proteolytic sensitivity of α_1PI, primary and secondary cleavage sites were carefully determined using the procedure described under Materials and Methods. When we repeated the work of Johnson and Travis (1977), we found that the initial cleavage of α_1PI by papain occurred at P_6–P_7 (see Figure 1). This primary cleavage was followed by secondary cleavage at the P_1–P_1' site, which implies that the hexapeptide Leu_{352}–Met_{358} (P_6–P_1) is excised by the action of papain. Therefore, some of the conclusions based on the use of papain-cleaved α_1PI to model a P_1–P_1' cleaved serpin (Mast et al., 1991; Carrell & Owen, 1985; Schulze et al., 1990; Hood & Gettins, 1991) should be reexamined.

The primary cleavage sites generated by SV8 proteinase (Potempa et al., 1986) and CA2 (Kress et al., 1979) are shown in Figure 1. We confirmed both these sites as primary cleavages, and no secondary cleavages were detected. Since Gly is present at P_{10} in the RSL of α_1PI, we used the recently described Gly-specific cysteine proteinase, PP4 (Buttle et al., 1990), to probe for cleavage. This enzyme cleaved the P_9–P_{10} bond, and no secondary cleavages were detected. Crude estimates of the rates of cleavage of P_9–P_{10} by PP4 and P_8–P_9 by CA2 indicated that each was cleaved about 10-fold slower than P_4–P_5 by SV8. Any model that explains the conformation of the RSL of α_1PI must take into account the observation that the Gly at P_{10} is available for recognition by PP4 but that the Gly at P_{15} is not (see Figure 1). Similarly, the Glu at P_5 is recognized by SV8 proteinase, but the Glu at P_{13} is not.

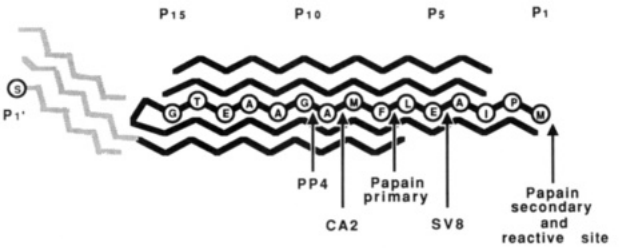


FIGURE 1: Diagram of modified α_1PI showing sites of proteolysis within the RSL. This schematic is an abbreviation of the structure of modified α_1PI, which is composed of three β-sheets and nine helices (Loebermann et al., 1984). Only components of sheets A (solid lines) and C (stippled lines), which bracket the RSL, are shown. The amino acid residues of the central strand of sheet A are labeled from P_1 to P_{15}, where P_1 fits the primary binding site of target proteinases. Bonds cleaved by the various proteinases used in this study are indicated by arrows.

Polymerization of α_1PI after RSL Cleavage. Evaluation of modified α_1PI revealed that the material cleaved with CA2 or PP4 migrated as a series of bands of decreasing mobility when analyzed by nondenaturing polyacrylamide gel electrophoresis, whereas material cleaved by papain or SV8 proteinase did not (Figure 2A). This behavior is consistent with polymerization of the cleaved material. The polymers are noncovalently assembled, since in SDS–polyacrylamide gel electrophoresis the material ran as a single band with the migration characteristic of modified α_1PI (data not shown). In some experiments, α_1PI was radioiodinated and incubated at low concentration (5 μg/mL) with CA2. Under this condition, very little polymerization was observed, indicating that the polymerization event is concentration-dependent. The stability of the polymers was not formally assessed; however, they persisted at room temperature for at least 4 days.

Heat-Induced Polymerization of Virgin α_1PI. Virgin α_1PI also polymerized when heated at 48 °C for 15 h (Figure 2B). The polymers are noncovalently assembled, since in SDS–polyacrylamide gel electrophoresis the material ran as a single band with the migration characteristics of virgin α_1PI. NP-40, a nonionic detergent, greatly increased the rate of polymerization (Figure 2B). In the presence of 0.025% (v/v) NP-40, the material was completely converted to polymers after 2 h at 48 °C, while in the absence of NP-40 about 25% remained unpolymerized after 15 h. Virgin α_1PI did not polymerize at 37 °C even in the presence of NP-40.

Conformational Stability of Polymerized α_1PI. We have shown that the conformational stability of serpins, thought to depend on the structure of the A-sheet (Huber & Carrell, 1989), can be readily analyzed by TUG–polyacrylamide gel electrophoresis (Mast et al., 1991). Virgin α_1PI undergoes an unfolding transition at 2–3 M urea, indicating the instability of the A-sheet in this form. After cleavage by SV8 or papain, α_1PI did not unfold during TUG–polyacrylamide gel electrophoresis (Figure 3), indicating that the A-sheet is stabilized by cleavage. The CA2-cleaved (both monomers and polymers) and heat-polymerized α_1PI also failed to unfold, indicating that the A-sheet is stabilized in these forms. PP4-cleaved material, on the other hand, showed transitions in the 6–7 M urea range, but the polymers did not dissociate (Figure 3). Therefore, this

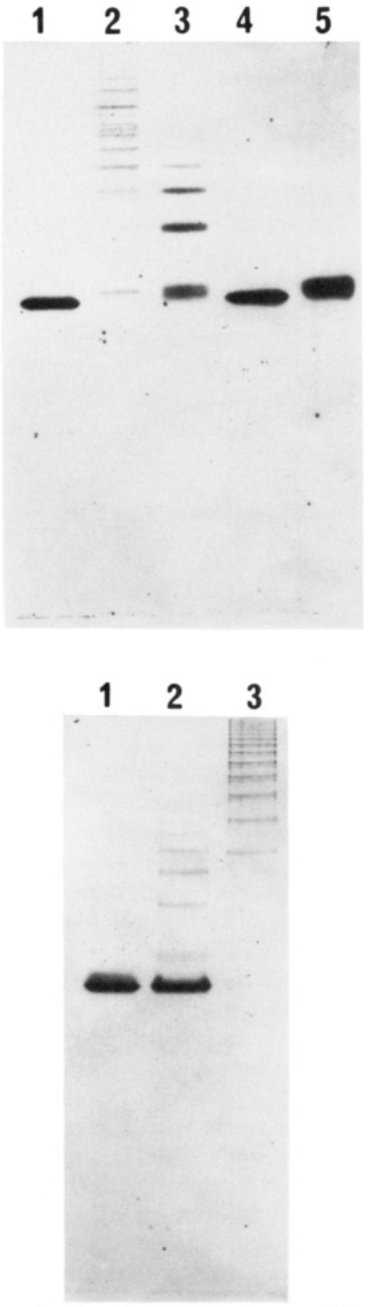


FIGURE 2: Polymerization of α_1PI derivatives. α_1PI polymers were visualized by nondenaturing polyacrylamide gel electrophoresis following inactivation by various proteinases (panel A, left or by heating (panel B, right) (see Materials and Methods for details). (Panel A) lane 1, virgin α_1PI; lane 2, α_1PI cleaved by CA2; lane 3, α_1PI cleaved by PP4; lane 4, α_1PI cleaved by papain; lane 5, α_1PI cleaved by SV8. (Panel B) lane 1, virgin α_1PI; lane 2, α_1PI heated at 48 °C for 15 h; lane 3, α_1PI heated at 48 °C for 2 h in the presence of 0.025% NP-40.

transition most likely represents unfolding of the A-sheet of the terminal molecule of the polymer species. The shape of the unfolding transition in TUG gels can be used to estimate the conformational stability, ΔG_f, of the unfolded state (Goldenberg, 1989). Using this method, we estimated ΔG_f for PP4 cleaved α_1PI to be 20 kJ mol^{-1}. Since the ΔG_f of virgin α_1PI was estimated to be 5 kJ mol^{-1} (Mast et al., 1991), the number of RSL residues inserted into the A-sheet influences the conformational stability of α_1PI. As the number of residues of the RSL that can be incorporated into the A-sheet increases, so does the stability of the sheet as a whole.

The Mechanism of Polymerization. In an attempt to define the specificity and mechanism of polymerization, we employed a synthetic 16-mer peptide corresponding to residues P_1–P_{15} of the RSL of α_1PI (see Materials and Methods). This peptide, which matches strand 4 of the A-sheet of α_1PI cleaved at P_1–P_1', is similar to the peptide that Schulze et al. (1990) showed could insert into the center of the A-sheet of virgin α_1PI. When α_1PI was cleaved by CA2 or PP4 in the presence of a 100-fold excess of the RSL peptide (Figure 4), or when the purified α_1PI–16-mer complexes were cleaved, polymerization did not occur, though cleavage of the RSL was confirmed by SDS–polyacrylamide gel electrophoresis and amino acid sequence analysis (data not shown). These data imply that polymerization is due to interactions between components of the A-sheet of adjacent RSL-cleaved α_1PI molecules as described in Figure 5. If this is true, then a peptide corresponding to P_8–P_1 should incorporate into α_1PI cleaved by CA2 or PP4 at 23 °C. This is shown in Figure 6 where the 9-mer incorporated into these forms but not into virgin α_1PI or into α_1PI cleaved by SV8 or papain. These data indicate that polymerization proceeds by a process where the P_1–P_8 region of one cleaved molecule may incorporate into the A-sheet of another cleaved molecule, but not into virgin α_1PI.

As with the proteolytically cleaved material, heat-induced polymerization of virgin α_1PI was blocked by the 16-mer RSL peptide (Figure 4). These data imply that this form of polymerization is also due to interactions between components of the A-sheet and RSL of adjacent α_1PI molecules; however, it is difficult for us to visualize how this may happen.

Electron Microscopy of the Polymers. Circular polymers were observed when the the CA2-cleaved material was examined by transmission electron microscopy (Figure 7). Circles were not detected in the PP4-cleaved for the heat-polymerized material (data not shown). The diameter of the circles ranged from 370 to 800 Å. Since the length of an α_1PI molecule is 67 Å, the circular polymers ranged from 6 to 12 units in length. Individual α_1PI molecules may be seen in some of the polymers (Figure 7).

Insertion of the 16-mer Synthetic RSL Peptide into α_1PI, α_1ACT, and ATIII. Schulze et al. (1990) showed that a synthetic peptide homologous to strand 4 of the A-sheet of modified α_1PI could be inserted into the virgin molecule at elevated temperatures causing a loss of inhibitory activity. We attempted to repeat this finding using a similar peptide.

synthetic S4A strand 16-mer (this study)	GTEAAGAMFLEAIPMY
natural S4A strand	GTEAAGAMFLEAIPM
synthetic S4A strand (Schulze et al., 1990)	*N*-acetyl TEAAGAMFLEAIVM

Our 16-mer peptide differs from that of Schulze et al. (1990) in that the natural Pro in position P_2 is included. The peptide of (Schulze et al., 1990) is also two residues shorter and is N-acetylated. Both peptides are designed to insert into the A-sheet of virgin α_1PI. Since the RSL peptide has a net charge of -2 at neutral pH, we were able to visualize incorporation as an anodal shift in nondenaturing polyacrylamide gel electrophoresis. Following incubation with excess 16-mer peptide as described under Materials and Methods, α_1PI, ATIII, and α_1ACT showed a shift in mobility consistent with peptide insertion, whereas ovalbumin did not (Figure 8). N-terminal sequence analysis of purified serpin–16-mer complexes indicated the formation of equimolar complexes of α_1PI, ATIII, and α_1ACT with the 16-mer. Unfolding transitions were not observed in TUG–polyacrylamide gel electrophoresis of these three serpins after insertion of the 16-mer, indicating that the A-sheet was stabilized (Figure 9). Titration with appropriate target proteinases revealed that inhibitory activity of all three serpins was completely abolished following peptide

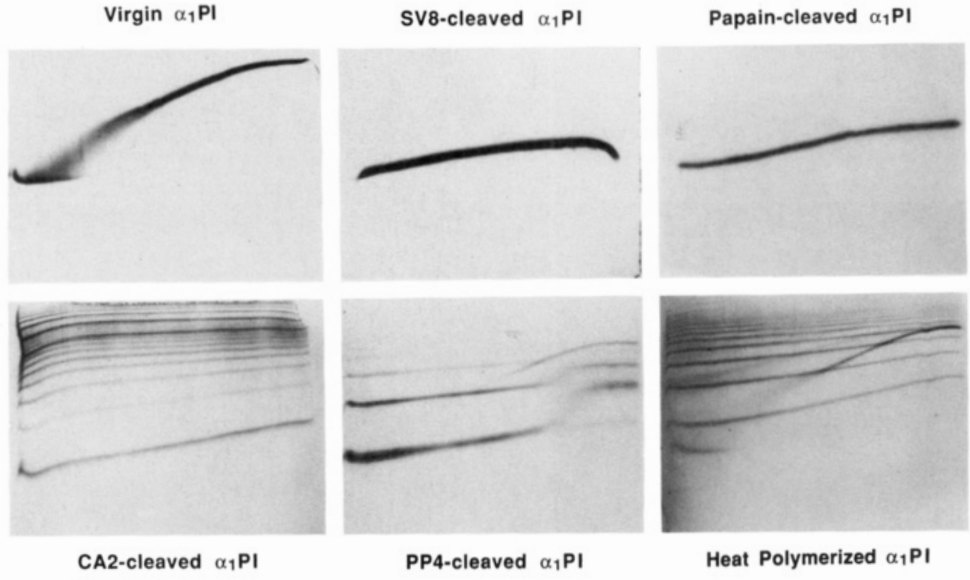


FIGURE 3: Conformational stabilities of α_1PI derivatives. α_1PI derivatives were analyzed by TUG–polyacrylamide gel electrophoresis as described under Materials and Methods. Each panel represents a single gel, with the direction of migration from top to bottom and the urea gradient (0–8 M) from left to right.

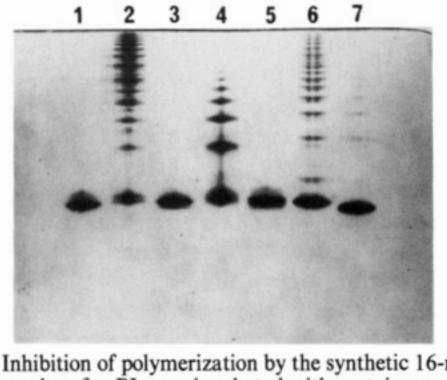


FIGURE 4: Inhibition of polymerization by the synthetic 16-mer RSL peptide. Samples of α_1PI were incubated with proteinases or heated for 15 h to induce polymerization. Lane 1, virgin α_1PI; lanes 2 and 3, α_1PI with CA2; lane 4 and 5 α_1PI with PP4; lanes 6 and 7, 48 °C for 15 h. In lanes 3, 5, and 7, the incubations were performed in the presence of 0.5 mM 16-mer RSL peptide.

insertion (data not shown). These results differ from those obtained by Schulze et al. (1990), who found that their peptide required acylation for insertion into α_1PI and would not insert into α_1ACT. Possible causes of these differences include the somewhat different structures of the peptides as well as the different conditions used for insertion. The ability of the peptide to insert into serpins other than α_1PI probably results from the high degree of homology of residues P_9–P_{15} of serpin RSLs, since residues P_1–P_8 are not well conserved. The inability of the peptide to insert into ovalbumin is expected since it possesses a stable four-stranded A-sheet into which its own putative RSL does not insert after cleavage (Wright et al., 1990).

RSL Cleavage after 16-mer Insertion. Following formation and purification of the α_1PI–16-mer complex, studies were performed to determine whether proteolytic sensitivity of the RSL was affected. Amino acid sequence analysis showed that CA2, PP4, and SV8 all cleaved at the same site within the RSL before and after 16-mer insertion (Figure 1). Proteinases which α_1PI normally inhibits (trypsin and HNE) cleaved the RSL of the α_1PI–16-mer complex at the reactive center P_1–P_1' peptide bond. Additionally, when SDS–polyacrylamide gel electrophoresis was used to compare the rates of cleavage of the virgin and 16-mer incorporated material, no difference

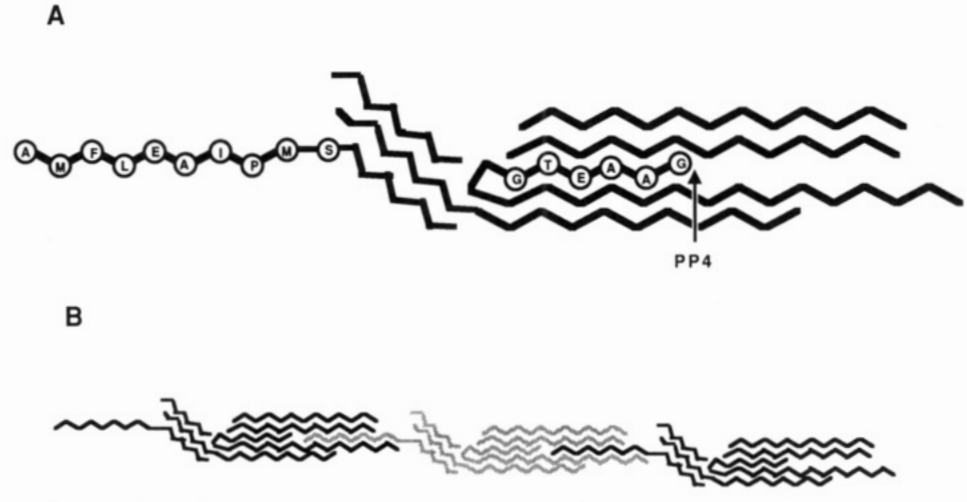


FIGURE 5: Probable mechanism of proteinase-mediated polymerization. (Panel A) Cleavage by PP4 at the P_9–P_{10} bond results in a modified form of α_1PI in which residues P_1–P_9 protrude as a "tail" from the C-sheet. Contrast this with P_1–P_1'-modified α_1PI shown in Figure 1, where no tail is produced. The tail of one molecule may insert into the unoccupied space in an adjacent modified molecule (panel B), in an ordered manner that produced head-to-tail polymers. If the polymer reaches a sufficient length, it may circularize. The example above is of PP4-modified α_1PI, but cleavage of P_8–P_9 by CA2 would result in a similar effect.

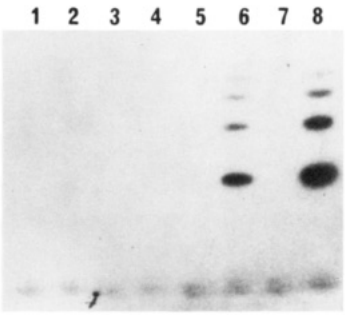


FIGURE 6: Incorporation of the synthetic 9-mer RSL peptide into α_1PI derivatives. Modified forms of α_1PI were generated by various proteinases. The proteinases were inhibited as described under Materials and Methods, and the mixtures were made 0.1 mM in ^{3}H-labeled 9-mer RSL peptide. Samples were run in nondenaturing polyacrylamide gel electrophoresis, and the gel was impregnated with 1 M sodium salicylate (Chamberlain, 1979), dried, and fluorographed for 3 days. Lane 1, virgin α_1PI; lane 2, SV8-cleaved α_1PI; lane 4 papain-cleaved α_1PI; lane 6, CA2-cleaved α_1PI; lane 8, PP4-cleaved α_1PI. Lanes 3, 5, 7, and 9 represent control experiments in which the respective proteinase used in the previous lane was inhibited (using DCI, EDTA, or E-64, as appropriate) before mixing with α_1PI.

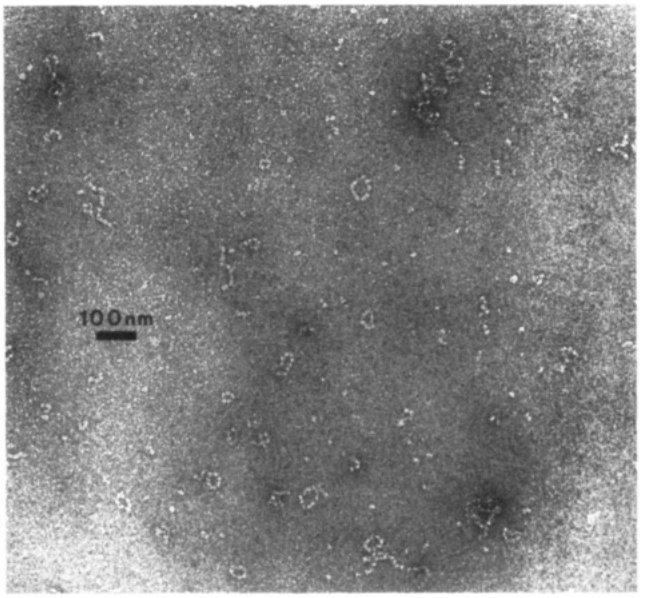
could be detected between the two. Though our assay system is not precise enough to detect less that a 2-fold difference in rates, no change in cleavage rates was detected following 16-mer insertion. These data argue for little change in the conformation of the RSL following 16-mer insertion, at least in the P_4–P_{11} region.

Discussion

Polymerization of RSL-Cleaved α_1PI. It is well established that serpins undergo a large change in conformational stability upon cleavage of the RSL. The central structures involved in this change are the RSL and the A-sheet into which the RSL inserts after cleavage. We show that α_1PI spontaneously forms noncovalent polymers at 23 °C when the RSL is cleaved either 9 or 10 residues upstream from the reactive site. The probable mechanism is presented in Figure 5. We presume that SV8-cleaved α_1PI fails to polymerize because the residues P_1–P_4 do not form sufficiently strong interactions with the A-sheet of an adjacent molecule. As previously reported by Schulze et al. (1990), virgin α_1PI also noncovalently polymerizes at elevated temperatures.

Although the polymers apparently form via the intermolecular insertion of portions of the RSL into the A-sheet of another molecule, each type of polymer has unique characteristics. The CA2-cleaved material was the only polymer to have circles visible in the electron micrographs. This observation explains why these polymers did not continually increase in size with increased time of incubation, since circle formation would terminate additional polymerization. CA2-cleaved α_1PI is thus analogous to α-crystallin, a protein found in the lens of the eye, that is capable of forming micellar-type polymers (Augusteyn & Korenz, 1987). The PP4-cleaved material formed fewer and smaller polymers than the CA2-cleaved material. The reason for this is not known, but the polymers formed may be too small to form circles. The heat-polymerized material formed increasingly larger polymers with increasing time of incubation. This observation is consistent with lack of circle formation and suggests that heat-induced polymers have characteristics somewhat different from the polymers generated by RSL cleavage. Though we can imagine how CA2- or PP4-induced polymers form (Figure 5), it is difficult to visualize how heat-induced polymers form without major reorganization of the α_1PI structure.

We speculate that the aggregation of the clinically important Z-mutant of α_1PI, seen as inclusion bodies in liver cells (Sharp,



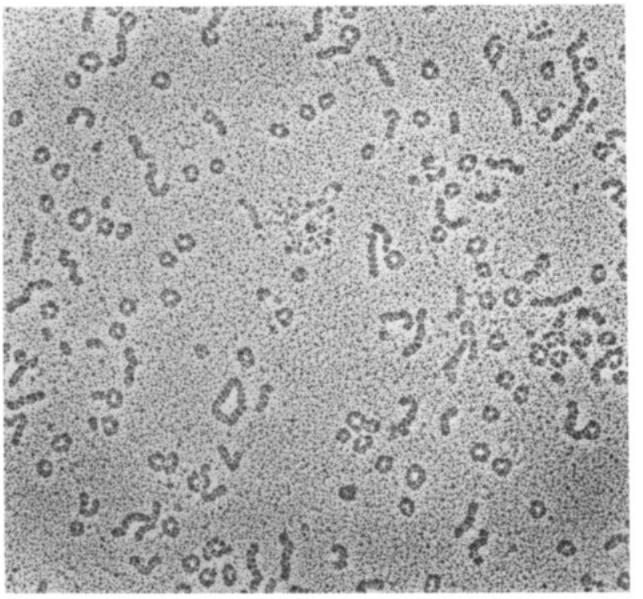


FIGURE 7: Electron microscopy of CA2-modified α_1PI. Samples of CA2-modified α_1PI were prepared and photographed as described under Materials and Methods. Portions of each image containing the most circles were enlarged by a factor of 120 000. (Panel A, top) Negative stain. (Panel B, bottom) Rotary shadowed image. Both images are at the same magnification, but the shell of metal exaggerates the apparent thickness of the strands in panel B. Dimensions and substructure are more accurately visualized in panel A. The micrographs were provided by Dr. H. P. Erickson, Department of Cell Biology, Duke University Medical Center. The scale bar represents 100 nm.

1971) or as high molecular weight forms in the plasma (Cox et al., 1986), may be due to polymerization. The Z-α_1PI mutant contains a Glu–Lys substitution at position 342 and is thus unable to form the salt bridge between Lys_{240} and Glu_{342} (Yoshida et al., 1976). The aggregates of Z-α_1PI may actually be polymers of the type described here, whose formation is favored at 37 °C due to the absence of the salt bridge, a normal component of the A-sheet.

The Interaction between the RSL and the A-sheet. Since P_9–P_{10} is susceptible to cleavage by PP4 and P_8–P_9 to cleavage by CA2, these residues must be exposed in the RSL of virgin α_1PI. This is compatible with the suggestion of Loebermann et al. (1984) that the reactive site of α_1PI is located near the position occupied by Ser at P_1' in RSL-cleaved α_1PI and that

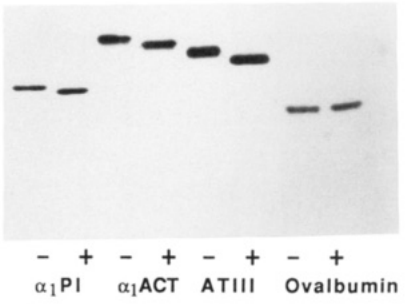


FIGURE 8: Insertion of 16-mer synthetic RSL peptide into various serpins. The serpins were incubated for 15 h at 48 °C in the presence (+ lanes) or absence (− lanes) of 0.5 mM 16-mer RSL peptide. Samples were analyzed by nondenaturing polyacrylamide gel electrophoresis. Insertion of the peptide, visualized as an anodal shift, is evident in α_1PI, α_1ACT, and ATIII, but not in ovalbumin.

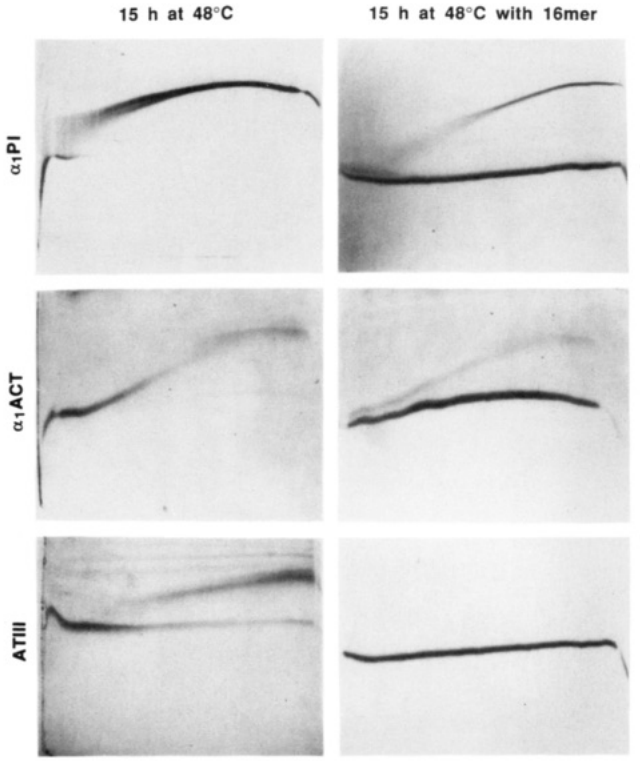


FIGURE 9: Conformational stabilities of serpins with or without inserted 16-mer RSL peptide. Samples of serpins treated as described in the legend for Figure 8 were examined by TUG gel electrophoresis. Each panel represents a single gel, with the direction of migration from top to bottom and the urea gradient (0–8 M) from left to right. Samples incubated without peptide are on the left, the samples incubated with peptide are on the right. Note that peptide-incorporated material fails to unfold.

much of the central strand of the A-sheet seen in the modified molecule must be extracted to create the RSL of the virgin molecule. Just how much of the RSL is exposed and how much may remain in the sheet bears directly on the inhibitory mechanism. If a portion of the RSL is inserted into the A-sheet in virgin α_1PI, then our data on the proteolytic susceptibility of the P_9–P_{10} bond indicate an upper limit for insertion of about three residues (Figure 10). Further insertion would bring this position of the loop too close to the sheet, prevent access of PP4 to the P_{10} amino acid, and lead to an inactive inhibitor as has been shown for ATIII by Carrell et al. (1991).

It is generally agreed that data obtained with one serpin can be used to understand structure–function relationships of the superfamily as a whole (Huber & Carrell, 1989). Since mutations in the P_{10}–P_{15} region change some serpins from

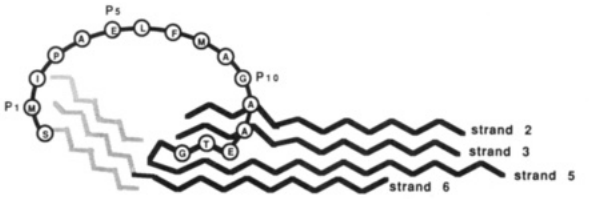


FIGURE 10: Proposed conformation of the RSL of virgin α_1PI. The rationale for this conformation is described in the text. This schematic is a derivative of that shown in Figure 1, except that residues P_1–P_{12} have been extracted from the A-sheet and joined to residue P_1' and that strands 2 and 3 of the A-sheet have been moved inward from the position they occupy in P_1–P_1'-modified α_1PI. Though dawn here in a rigid extended conformation, the loop probably has more flexibility than the RSL of standard mechanism inhibitors. This flexibility may derive from movements of some of the upstream RSL residues in and out of the A-sheet, so that the serpin RSL can be thought of as a dynamic, rather than static, structure.

inhibitors into substrates (Caso et al., 1990; Perry et al., 1991; Devraj-Kizuk et al., 1988; Skriver et al., 1991), the interplay between this region and the A-sheet is crucial in maintaining an active inhibitor. Mutations in this region affect inhibitory function by either (i) preventing insertion of the RSL into the A-sheet following proteinase binding (Skriver et al., 1991) or (ii) altering the conformation of an RSL which is partially inserted into the A-sheet before proteinase binding (Perry et al., 1991).

TUG gel data indicate that the protein does not unfold below 8 M urea when eight or more RSL residues are incorporated into the A-sheet (SV8-, papain-, and CA2-cleaved α_1PI). However, when seven residues are incorporated (PP4-cleaved α_1PI), the protein unfolds at 6–7 M urea. Carrell et al. (1991) recently presented data in which they examined the incorporation into virgin ATIII of peptides corresponding to residues P_{14}–P_9 and P_{14}–P_2 of the RSL of this serpin. Heat stability analysis, analogous to TUG gel analysis, indicated that both peptides increased the conformational stability of ATIII but that P_{14}–P_9 peptide-inserted ATIII was intermediate in stability between virgin and reactive site modified ATIII or P_{14}–P_2-inserted ATIII. These data complement our findings on the relative stabilities of PP4-modified α_1PI compared with α_1PI modified by other proteinases. In essence, PP4-modified α_1PI may be thought of as an analogue of the binary complex between ATIII and the P_{14}–P_9 peptide.

It is logical to conclude that the conformational stability of the molecule increases as more amino acids are inserted into the A-sheet. Native inhibitory serpins are conformationally unstable, typically unfolding around 2–3 M urea (Mast et al., 1991), suggesting that they have only a few residues inserted into the A-sheet (Figure 10). If the RSL was completely removed from the A-sheet, we would expect a conformational stability similar to virgin ovalbumin. In ovalbumin, the presence of Arg at P_{14} prevents insertion of the RSL into either the virgin or modified forms (Wright et al., 1990; Stein et al., 1990), yet it does not unfold in TUG gels (Mast et al., 1991). Consequently, it is likely that the characteristic conformational instability of the inhibitory serpins is caused by the presence of the P_{12}–P_{15} residues in the A-sheet of the virgin molecule. It seems that the insertion of the RSL into the A-sheet is energetically favored and that this process is already at work in the virgin molecule, a possibility considered by Schulze et al. (1990), Perry et al. (1991), and Carrell et al. (1991).

Huber and Bode (1991) and Stein and Chothia (1991) have hypothesized that the A-sheet of virgin α_1PI resembles that of ovalbumin in having a portion of strands 3 and 5 within hydrogen-bonding distance. We are now able to address this possibility using data obtained with the 9-mer RSL peptide.

The 9-mer RSL peptide readily incorporated into PP4- and CA2-cleaved α_1PI, without heating, but failed to incorporate into virgin α_1PI. Consequently, it is likely that, in the virgin molecule, the A-sheet does indeed adopt a shape simlar to the ovalbumin structure, except for the region into which residues P_{12}–P_{15} may be inserted (Figure 10). Insertion of the 9-mer is prohibited. Presumably the interactions between strands 3 and 5 of the A-sheet (Figure 10) are weaker in the PP4- and CA2-cleaved material than in the virgin molecule, allowing insertion of the 9-mer. Therefore, the transition from a virgin to a modified serpin, resulting in the insertion of at least seven residues of the RSL into the A-sheet, is accompanied by the separation of strands 3 and 5 and expansion of most of the A-sheet.

Conformation of the RSL. In the virgin molecule, the RSL would prefer to insert completely into the A-sheet; however, the tendency to insert is opposed by its connection to the C-sheet. Avron et al. (1991) considered that the tendency of the RSL to insert into the A-sheet could be opposed by a tendency of the loop to form an α-helix of the type seen in virgin ovalbumin, resulting in a rigid extended loop similar in shape to that of standard mechanism inhibitors. We feel this is unlikely since insertion of the synthetic 16-mer, which should result in expulsion of the RSL from the A-sheet, does not alter the proteolytic susceptibility of the α_1PI RSL to any large extent. If the RSL adopted a helical conformation when free from the A-sheet, its proteolytic susceptibility would change.

Much current evidence points to a standard mechanism interaction between serpins and their target proteinases in which a target proteinase is bound in a tight, but reversible, complex where peptide bond scission is greatly hindered. The data and ideas presented here suggest how a rigid extended RSL may be attained by opposing interactions between the A- and C-sheets. However, the work of Potempa et al. (1988), which we have been unable to falsify (J. J. Enghild, A. E. Mast, and G. Salvesen, unpublished data), showed that α_2-antiplasmin inhibits trypsin and chymotrypsin at adjacent reactive sites, such that P_1 is Arg_{357} for trypsin but Met_{358} for chymotrypsin (α_1PI numbering). It is difficult to reconcile this crucial observation with the rigid and highly superimposable RSLs of the standard mechanism inhibitors, and we are led to the conclusion that the RSL must possess enough flexibility to offer alternate reactive sites to different proteinases. The region around P_1–P_1' must possess rigidity in the complex with a target proteinase, or that proteinase would treat the region as a substrate. Therefore, serpins seem to maintain a RSL that is rigid and flexible. Possibly, the binding of a target proteinase allows the somewhat flexible virgin RSL to adopt a rigid conformation by moving further into the A-sheet. Since the RSL is connected to strand 1 of the C-sheet (Figure 10), such movement requires a compensatory movement, either by this strand or by a rotation of the portion of the molecule composed of sheets B and C around an axis through the B-sheet. Either of these compensations would be predicted to result in a significant conformational change upon proteinase binding.

Acknowledgments

We thank Alison Watta for technical assistance, Harold Erickson and the Duke University Comprehensive Cancer Center core facility for the electron micrographs, and Peter George, Edwin Madison, David Rubenstein, and Neil Tweedy for valuable suggestions.

Registry No. α_1-Proteinase inhibitor, 9041-92-3; serpin, 96282-35-8.

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Urea Denaturation of Barnase: pH Dependence and Characterization of the Unfolded State†

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Received October 22, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: To investigate the pH dependence of the conformational stability of barnase, urea denaturation curves were determined over the pH range 2-10. The maximum conformational stability of barnase is 9 kcal mol^{-1} and occurs between pH 5 and 6. The dependence of ΔG on urea concentration increases from 1850 cal mol^{-1} M^{-1} at high pH to about 3000 cal mol^{-1} M^{-1} near pH 3. This suggests that the unfolded conformations of barnase become more accessible to urea as the net charge on the molecule increases. Previous studies suggested that in 8 M urea barnase unfolds more completely than ribonuclease T1, even with the disulfide bonds broken [Pace, C. N., Laurents, D. V., & Thomson, J. A. (1990) *Biochemistry 29*, 2564-2572]. In support of this, solvent perturbation difference spectroscopy showed that in 8 M urea the Trp and Tyr residues in barnase are more accessible to perturbation by dimethyl sulfoxide than in ribonuclease T1 with the disulfide bonds broken.

In the 1960's, Tanford's group published a series of studies aimed at better characterizing the denatured states of proteins. The major conclusion was that proteins in 6 M GdnHCl with their disulfide bonds broken closely approach a randomly-coiled conformation (Tanford, 1968). The same seemed true in 8 M urea (Lapanje, 1969). Even with their disulfide bonds intact, proteins seemd to unfold as completely as possible given the restraints imposed by the disulfide bonds (Tanford, 1968). During the past five years, interest in the denatured states of proteins has been reawakened. Much of this was due to studies from Shortle's group suggesting that the denatured states of proteins are more complicated than previously thought (Dill & Shortle, 1991). Another contributing factor was the recognition that an intermediate folding state, now generally referred to as the "molten-globule" state, is formed by a number of different proteins under various conditions and has common characteristics: a secondary structure similar to the folded proteins and a tertiary structure similar to the unfolded protein (Kuwajima, 1989; Fink et al., 1991; Ptitsyn & Semisotnov, 1991; Dobson et al., 1991). Studies with NMR have been especially useful in providing information about the structure that may be present in unfolded proteins (Howard & Lian, 1984; Evans et al., 1991). For example, Evans et al. (1991) suggest "...a rather nonspecific clustering of residues in the thermally denatured form, rather than the persistence of well-defined elements of structure." In the summary of their recent review, Dill and Shortle (1991) state "There is now considerable evidence that even in strong denaturants such as 6 M GdnHCl and 9 M urea, some structure may remain in protein chains." The results presented here are consistent with both of these suggestions.

In a previous paper, we studied the pH dependence of the urea denaturation of ribonuclease A (RNase A) and ribonuclease T1 (RNase T1) (Pace et al., 1990). The pH dependence of both the conformational stability and the dependence of ΔG on urea concentration differed markedly for

† Supported by grants from the NIH (GM 37039), the Robert A. Welch Foundation (A-1060) and the Tom and Jean McMullin Professorship.

0006-2960/92/0431-2728$03.00/0