Analysis of the Plasma Elimination Kinetics and Conformational Stabilities of Native, Proteinase-Complexed, and Reactive Site Cleaved Serpins: Comparison of α_1-Proteinase Inhibitor, α_1-Antichymotrypsin, Antithrombin III, α_2-Antiplasmin, Angiotensinogen, and Ovalbumin†

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ABSTRACT: Proteinase inhibitors of the serpin superfamily may exist in one of three distinct conformations: the native form, a fully active protein with the reactive site loop intact; the proteolytically modified form in which inhibitory capacity is abolished; and the proteinase-complexed form, a stable equimolar complex between the inhibitor and a target proteinase. Here, the specificity and kinetics of the plasma elimination of different serpin conformations are compared. Proteinase-complexed serpins were rapidly cleared from the circulation. However, the native and modified forms were not cleared rapidly, indicating that the receptor-mediated pathways which recognize the complexes fail to recognize the native and modified forms. This result suggests that significant structural differences exist between modified and proteinase-complexed serpins. The structural differences were probed by using transverse urea gradient gel electrophoresis, a technique that allows comparisons of the conformational stabilities of proteins. With the exception of the noninhibitory serpins ovalbumin and angiotensinogen, the modified and proteinase-complexed serpins were both stabilized thermodynamically compared to the native forms. In addition, the proteinase component of the serpin–proteinase complex was usually thermodynamically stabilized. These data are used to compare the conformations of serpin–proteinase complexes with those of native and modified serpins; they are discussed in terms of a model whereby serpins inhibit proteinases in a manner similar to that described for other types of protein inhibitors of serine proteinases.

Blood contains members of a superfamily of homologous proteins called serpins that regulate the proteolytic cascades of coagulation, fibrinolysis, inflammation, and complement activation (Carrell & Travis, 1985). Some of the best characterized serpins, including α_1-proteinase inhibitor (α_1PI),[1] α_1-antichymotrypsin (α_1ACT), antithrombin III (ATIII), and α_2-antiplasmin (α_2AP), are inhibitors of serine proteinases. Others, including angiotensinogen and hen ovalbumin, have no known proteinase inhibitory capacity. The inhibitory serpins regulate proteolysis by forming tight, equimolar complexes with a target proteinase (Travis & Salvesen, 1983). Once formed, the complexes are rapidly removed from the circulation by cellular receptors (Pizzo, 1989), thereby preventing adventitious proteolysis of blood and tissue proteins.

On the basis of the known mechanism of inhibition of members of other proteinase inhibitor families (Laskowski & Kato, 1980), one would expect inhibition of proteinases by serpins to depend on the structure of a region known as the reactive site loop. It is likely that this loop, located near the C-terminus, offers a substratelike target. In doing so, it is exposed to attack by proteinases such as those from bacteria and snake venoms for which this region is a good substrate (Salvesen & Travis, 1989). Significantly, the loop of some serpins is also sensitive to certain host proteinases, the most intensively studied being human neutrophil elastase (HNE) (Brower & Harpel, 1982; Jordan et al., 1989). We can, therefore, recognize two types of interaction between proteinases and serpins. In the first case, the serpin inhibits the target proteinase with the formation of a stable serpin–proteinase complex. In the second, the serpin serves as a substrate and is catalytically inactivated.

The structural attributes that cause the loop to be a substrate for some proteinases and an inhibitor for others have yet to be elucidated. However, the results of proteolysis within the loop have been well documented. A single cleavage of any peptide bond in the reactive site loop results in the transition of most serpins to a thermodynamically stable structure (Carrell & Owen, 1985; Huber & Carrell, 1989). This contrasts with the native (unreacted) form which has a less stable structure. In the case of proteolytically modified α_1PI, the P_1 and P_1' residues of the reactive site, connected before the cleavage occurred, are at opposite ends of the molecule, separated by 67 Å (Loebermann et al., 1984). The separation of these two residues allows completion of a five-stranded β-sheet which is thought to be the source of new favorable interactions and the characteristic thermodynamic stability of modified α_1PI. The structure of proteinase-complexed serpins is unknown, very few studies having compared native, complexed, and modified conformations simultaneously.

Proteolytic modification of serpins, accompanied by a complete loss of inhibitory capacity, has been proposed to be

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[1] Abbreviations: α_1PI, α_1-proteinase inhibitor; α_1ACT, α_1-antichymotrypsin; ATIII, antithrombin III; α_2AP, α_2-antiplasmin; HNE, human neutrophil elastase (EC 3.4.21.37); PPE, pig pancreatic elastase (EC 3.4.21.36); DCI, 3,4-dichloroisocoumarin; E-64, 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane; SDS, sodium dodecyl sulfate; TUG, transverse urea gradient.

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a physiological "switch" which allows the proteinase/inhibitor balance of the plasma proteolytic cascades to be shifted to favor proteolysis (Carrell & Boswell, 1986). Moreover, it is now recognized that some proteinase-complexed serpins and modified serpins are able to modulate the function of certain cells. For example, α_1PI in complex with HNE and α_1PI modified following reaction with a mouse metalloproteinase are both powerful chemoattractants for neutrophils (Banda et al., 1988a,b). Furthermore, α_1PI–proteinase complexes mediate increases in expression of the α_1PI gene in human monocytes and macrophages (Perlmutter et al., 1988).

Previous studies have shown that serpin–proteinase complexes are rapidly removed from circulation by one of two distinct pathways, most likely mediated by selective receptors on hepatocytes (Gonias et al., 1982; Pizzo et al., 1988). We had previously thought that these receptors simply cleared serpin–proteinase complexes from the circulation, but the recent data on biological roles of serpin–proteinase complexes and modified serpins indicate that these receptors may be involved in cellular signaling pathways. If this is the case, the clearance receptors should also recognize modified serpins. We have investigaed this by comparing the kinetics and specificity of the circulatory clearance of proteinase-complexed and modified serpins. These studies suggested significant structural differences between the modified and proteinase-complexed forms. Transverse urea gradient gel electrophoresis was used in conjunction with plasma elimination studies to compare the conformational differences of native, modified, and proteinase-complexed serpins. Our results provide insight into the physiological relevance of modified serpins and raise questions about the structural relationship between modified serpins and serpin–proteinase complexes.

Materials and Methods

Reagents. 3,4-Dichloroisocoumarin (DCI), 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane (E-64), 1,10-phenanthroline, and heparin (210-6) were obtained from Sigma Chemical Co., St. Louis, MO. The chromogenic substrates for trypsin (H-D-Pro-Phe-Arg-*p*-nitroanilide), thrombin (H-D-Phe-pip-Arg-*p*-nitroanilide), chymotrypsin (methoxysuccinyl-Arg-Pro-Tyr-*p*-nitroanilide), and elastases (methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide) were obtained from Helena Laboratories, Beaumont, TX. Lactoperoxidase–glucose oxidase and electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, CA. Ultrapure urea was obtained from Bethesda Research Laboratories, Gaithersburg, MD. $Na^{125}I$ (17.4 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Iodobeads were obtained from Pierce, Rockford, IL.

Proteins. Bovine trypsin (type XIII), bovine chymotrypsin (type II), porcine pancreatic elastase (type IV) (PPE), papain (type IV), *Staphylococcus aureus* V8 proteinase, and hen ovalbumin were obtained from Sigman Chemical Co., St. Louis, MO. Human neutrophil elastase (HNE) was a gift of Dr. Weislaw Watorek, University of Georgia. *Crotalus adamanteus* proteinase II was a gift of Dr. Lawrence Kress, State University of New York. Thrombin was a gift of Dr. John Fenton, New York State Department of Health. Angiotensinogen was a gift of Dr. Duane Tewksbury, Marshfield Medical Research Foundation. Purification of α_1PI (Pannell et al., 1974), ATIII (Thaler & Schmer, 1975), and α_2AP (Wiman, 1980) was performed as previously described. α_1ACT was purified as described for human 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.

Protein Radiolabeling. Serpins were radioiodinated by using lactoperoxidase–glucose oxidase according to instructions provided by the manufacturer (Bio-Rad Laboratories). The proteinases were radiolabeled using Iodobeads according to instructions provided by the manufacturer (Pierce).

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. The proteins were analyzed by 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.

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 0–8 M urea gradient (Goldenberg, 1989) in the gel buffer system described above, but without SDS. The gel was rotated 90°, and a single sample containing approximately 50 μg of protein in 150–300-μL total volume was loaded evenly across the top of the gel. Electrophoresis was performed at 23 °C (±2° C) with a constant current of 15 mA for 2 h. Proteins were visualized by staining with Coomassie blue or by autoradiography, as appropriate. The objective of these experiments is to observe the folded and unfolded states of a protein under a continuously varying urea concentration gradient (Creighton, 1979). As pointed out by Goldenberg (1989), the method is simple, requires only small amounts of protein, and yields results consistent with those obtained with other biophysical measurements of conformational stabilities.

Proteolytic Inactivation of Serpins. The reactive site loops were cleaved by incubating the serpin (0.3–0.7 mg/mL) with appropriate proteinases under the following conditions. α_1PI was cleaved by papain, *S. aureus* V8 proteinase, or *C. adamanteus* proteinase II by incubating the proteinase with α_1PI in 50 mM Tris-HCl, pH 8.0, for 1 h at 37 °C. Papain was activated for 15 min with 5 mM dithiothreitol before use. The molar ratios used to obtain complete cleavage were 50:1 for α_1PI/papain and α_1PI/*S. aureus* V8 proteinase and 30:1 for α_1PI/*C. adamanteus* proteinase II. α_1ACT, ATIII, α_2AP, and ovalbumin were cleaved with HNE in 50 mM Tris-HCl, pH 8.0, for 30 min at 37 °C. The molar ratios used to obtain complete cleavage were 50:1 for α_1ACT/HNE and ATIII/HNE and 30:1 for α_2AP/HNE and ovalbumin/HNE. Angiotensinogen was cleaved in 50 mM sodium phosphate, pH 6.8, for 1 h at 37 °C. The molar ratio of angiotensinogen to *S. aureus* V8 proteinase was 10:1. The cleavage reactions were stopped by adding DCI (5 μM final concentration) to inhibit HNE and *S. aureus* V8 proteinase, E-64 (50 μM final concentration) to inhibit papain, or 1,10-phenanthroline (1 mM final concentration) to inhibit *C. adamanteus* proteinase II. Cleavage of the reactive site loop of the serpins under these conditions was analyzed by SDS–polyacrylamide gel electrophoresis, inhibitory assays (where appropriate), and amino acid sequence analysis.

Preparation of Serpin–Proteinase Complexes. Serpin–proteinase complexes for use in the mouse plasma elimination studies were prepared by mixing the inhibitor and proteinase in equimolar amounts for 15–30 min at 23 °C. Serpin–proteinase complexes for the TUG gels were made by mixing 50 μg of the serpin with 0.5 μg of radioiodinated proteinase.

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. Samples destined for

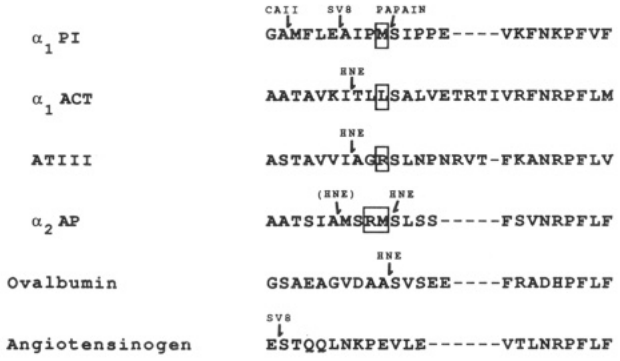

FIGURE 1: Location of cleavage sites. Each serpin was incubated with an inactivating proteinase as described under Materials and Methods. The locations of the *C. adamanteus* proteinase II (CAII) and *S. aureus* V8 proteinase (SV8) cleavage sites in α_1PI are taken from Kress et al. (1979) and Potempa et al. (1986), respectively. The location of the other sites was determined by protein sequence analysis as described under Materials and Methods. The HNE reactive site cleavage of α_1ACT has been previously reported by Morii and Travis (1983); the site of HNE reactive site cleavage of ATIII has been reported by Carrell and Owen (1985); the site of *S. aureus* V8 proteinase cleavage in angiotensinogen has been confirmed by Tewkesbury (1990). We found a different cleavage site in α_2AP by HNE to that reported by Sheih et al. (1987), and the site reported by these authors is in parenthesis. It is possible that cleavage at Ala-Met preceeds cleavage at Met-Ser. The P_1 residue of each of the inhibitory serpins is boxed [reviewed by Boswell and Carrell, (1986)]. In the case of α_2AP, Arg is P_1 for plasmin whereas Met is P_1 for chymotrypsin (Potempa et al., 1988). The sequence alignment is taken from Huber and Carrell (1989) and references cited therein.

sequence analysis were desalted on a G25 fast desalting column (Pharmacia) into 0.1% trifluoroacetic acid. The samples were concentrated in a speed vac (Savant) and applied to polybrene-treated precycled glass fiber filters in the 477A. The instruments were operated as suggested by the manufacturer.

Plasma Elimination Studies. This procedure has been described in detail elsewhere (Imber & Pizzo, 1981). In brief, approximately 1.0 μg of radioiodinated protein was injected alone or in the presence of unlabeled protein into the lateral tail vein of CD-1 mice. Blood samples of 25 μL were removed at timed intervals via retroorbital puncture. The initial time point, taken 5–10 s after injection, was considered to represent 100% radioactivity in the circulation. Each preparation was studied at least in duplicate.

Results

Proteolytic Cleavage of Serpins. Reaction conditions were established to determine the quantity of proteinase that caused complete inactivation of the inhibitory capacity of the serpins. These conditions (described under Materials and Methods) were used to prepare samples of reactive site cleaved serpins. Sites of cleavage, in each of the serpins, by the various inactivating proteinases are shown in Figure 1. We note that the primary cleavage sites in all serpins examined were in the reactive site region and that the reactive site modified serpins were quite resistant to further proteolysis, with the exception of α_1ACT. In the latter case, a secondary cleavage was observed to occur near the N-terminus (between Val-23 and Asp-24), and the extent of this cleavage increased with higher concentrations of HNE. Since ovalbumin and angiotensinogen are not proteinase inhibitors, it is not strictly correct to designate a region as the reactive site. However, since the primary proteolysis site within these serpins is in a region that aligns with the reactive site loops of other serpins (Carrell & Huber, 1989; Figure 1), this nomenclature is extended to the noninhibitory serpins for the purpose of comparison. Examination of portions of modified serpins by SDS–polyacrylamide gel

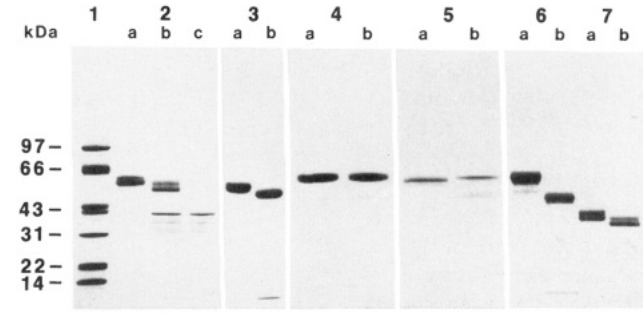

FIGURE 2: SDS–polyacrylamide gel electrophoresis of native and reactive site cleaved serpins. Cleaved serpins were prepared as described under Materials and Methods and run in 5–15% linear gradient SDS gels. Each pair of lanes contains unreacted serpin in lane a and cleaved serpin in lane b. (1) Molecular weight standards; (2) angiotensinogen (lane c, *S. aureus* V8 proteinase); (3) α_1PI (native or cleaved with papain); (4) α_1ACT; (5) ATIII; (6) α_2AP; (7) ovalbumin.

electrophoresis is shown in Figure 2. In some cases, the C-terminal fragment, approximately 4 kDa, released by proteolysis can be seen near the bottom of the gel. As expected, the remaining fragment of α_1PI, α_2AP, angiotensinogen, and ovalbumin migrated further into the gel than the native molecule. α_1ACT and ATIII, however, did not migrate as expected. Modified ATIII migrated more slowly than the native molecule. The reason for this behavior is unknown but also has been observed by other investigators (Asakura et al., 1989). Modified α_1ACT migrated at the same rate as the native form, but the rate of migration increased following cleavage at the N-terminal site.

Plasma Elimination Studies. Modified α_1PI was removed from the circulation more rapidly than the native form, but more slowly than the proteinase-complexed form (Figure 3, upper panel). To determine whether the position of cleavage within the reactive site loop affected the removal rate, we employed papain, *S. aureus* V8 proteinase, and *C. adamanteus* proteinase II, proteinases which cleave at different sites within the reactive site loop (Figure 1), to generate modified α_1PI. The rate of removal of modified α_1PI was found to be independent of the proteinase used to effect cleavage (Figure 3, upper panel). The mechanism of circulatory removal of modified α_1PI was investigated using competition studies. These studies are based on the principle that receptor-mediated removal of a radiolabeled ligand can be slowed by coinjection of a large excess of unlabeled ligand. Thus, the specificity of receptors may be determined by using different competing ligands. Removal of modified α_1PI was slowed slightly by a competing excess of itself or α_1PI–trypsin (Figure 3, middle panel). However, increasing the α_1PI–trypsin concentration failed to further decrease the rate of clearance. Moreover, the removal rate remained much faster than that of native α_1PI. Removal of the α_1PI–trypsin complex was drastically decreased when allowed to compete with itself (Figure 3, lower panel), but only slightly decreased in the presence of competing concentrations of modified α_1PI. These results were independent of the proteinase used to form α_1PI complexes, since the clearance of α_1PI–HNE was also only slightly decreased in the presence of competing concentrations of modified α_1PI (Figure 3, lower panel). We could not determine the mechanism responsible for the slightly accelerated clearance of modified α_1PI; however, our data suggest that this modified serpin is not recognized by the receptor responsible for the circulatory removal of α_1PI–proteinase complexes.

Modified α_1ACT (cleaved in the reactive site loop, or both the reactive site loop and the N-terminus), modified ATIII, and modified α_2AP were removed from the circulation of mice

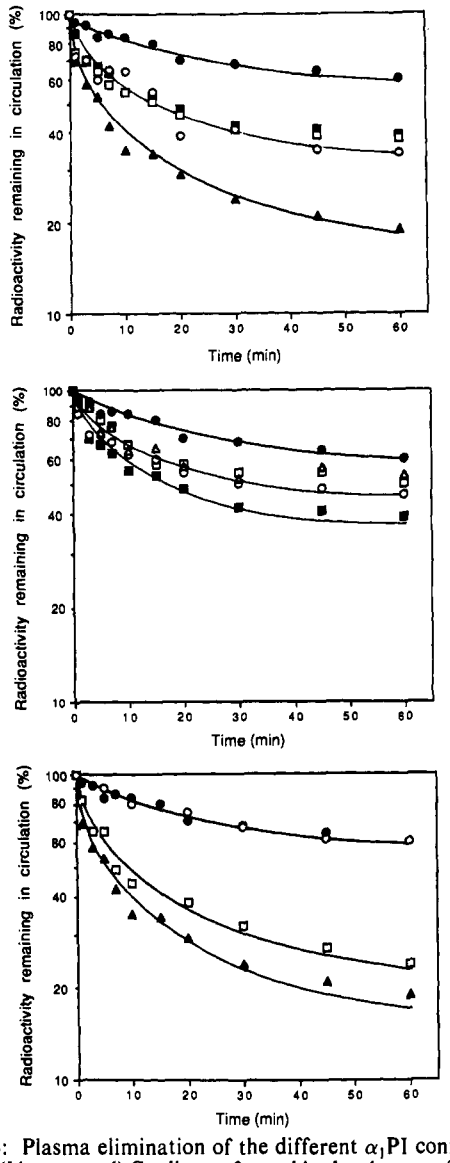

FIGURE 3: Plasma elimination of the different α_1PI conformations in mice. (Upper panel) Studies performed in the absence of competing ligands. Papain-cleaved α_1PI (■), *C. adamanteus* proteinase II cleaved α_1PI (□), *S. aureus* V8 proteinase cleaved α_1PI (○), native α_1PI (●), or α_1PI–HNE complex (▲) was injected into a mouse. (Middle panel) Studies performed examining the plasma elimination of papain-cleaved α_1PI in the presence of competing ligands. Papain-cleaved α_1PI was injected with a 1000-fold molar excess of itself (□), a 1000-fold molar excess of α_1PI–trypsin complex (○), or a 2500-fold molar excess of α_1PI–trypsin complex (△). Native α_1PI (●) and papain-cleaved α_1PI (■) are shown for comparison. (Lower panel) Studies performed examining the plasma elimination of α_1PI–proteinase (HNE or trypsin) complex in the presence of competing ligands. α_1PI–proteinase complex was injected with a 1000-fold molar excess of papain-cleaved α_1PI (□) or a 1600-fold molar excess of itself (○). Native α_1PI (●) and α_1PI–HNE complex (▲) are shown for comparison. The clearance of native α_1PI and α_1PI–proteinase complex was previously reported by Fuchs et al. (1982).

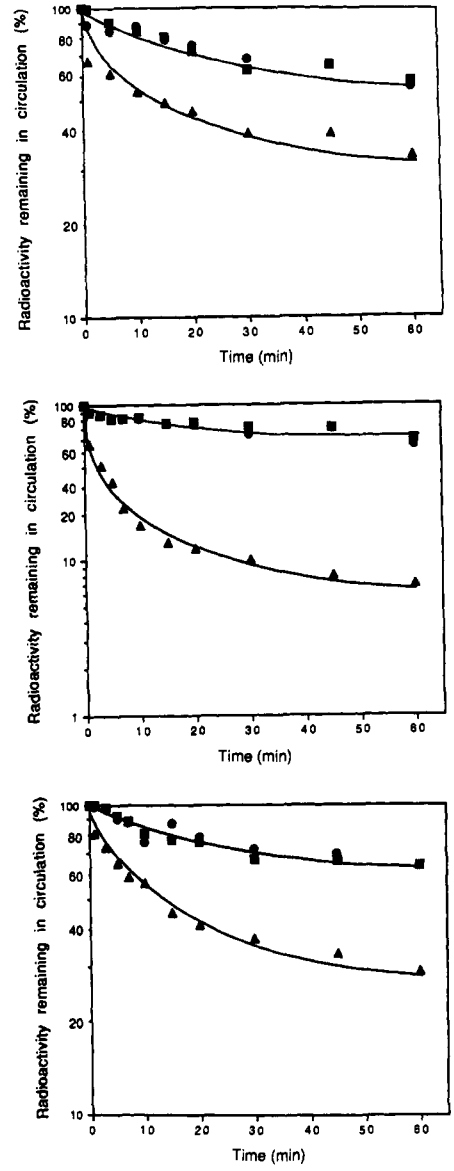

FIGURE 4: Plasma elimination of α_1ACT (upper panel), ATIII (middle panel), and α_2AP (lower panel) in mice. The native serpin is represented by (●), the modified serpin by (■), and the proteinase-complexed serpin by (▲). The clearance of native α_1ACT and α_1ACT–chymotrypsin complex was previously reported by Pizzo et al. (1988), the native ATIII and ATIII–thrombin complex by Shifman and Pizzo (1982), and native α_2AP and α_2AP–plasmin complex by Gonias et al. (1982).

at the same rate as the native protein. These results were in sharp contrast to those for serpin–proteinase complexes which were removed from the circulation much more rapidly than the native form (Figure 4). These data suggest that these modified serpins are not recognized by receptors that remove serpin–proteinase complexes from circulation and are consistent with the lack of a detectable receptor-mediated clearance of this serpin conformation.

Transverse Urea Gradient Polyacrylamide Gel Electrophoresis. The results of the mouse plasma elimination studies indicated that significant structural differences exist between proteolytically modified and proteinase-complexed serpins. TUG gels were chosen as a technique to probe these structural differences and to investigate the conformation of the serpin–proteinase complexes. In these gels, the protein migrates more slowly as it unfolds since the unfolded state occupies a larger hydrodynamic volume (Goldenberg, 1989). This results in the visualization of an unfolding transition in the area of the gel between the folded and unfolded states. The shape of the unfolding transition can be used to estimate the conformational stability [ΔG_f (Goldenberg, 1989); ΔG_{H_2O} (Pace, 1990)] of the unfolded state. This is done by extrapolating the transition curve to 0 M urea as described by Goldenberg (1989).

TUG gels of native, reactive site cleaved, and (where appropriate) proteinase-complexed serpins are shown in Figure 5. Examination of the native forms of the six serpins revealed that each had a unique TUG gel signature, with all except ovalbumin having distinct unfolding transitions. With the

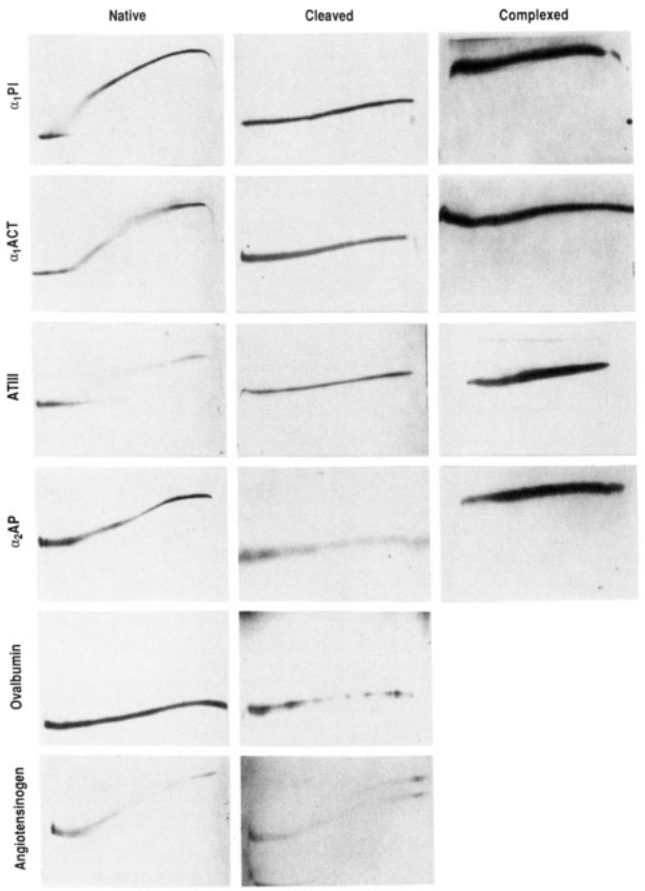

FIGURE 5: TUG–polyacrylamide gel electrophoresis of serpin forms. First column, unreacted serpins; second column, reactive site cleaved (proteolytically modified) serpins; third column, serpin–proteinase complexes. Each panel represents a single gel, with each row of gels aligned to show the relative migration distance of the forms. Gels in the first and second columns were stained with Coomassie blue whereas gels in the third column represent autoradiograms. For each panel, the direction of migration is from top to bottom, and the urea gradient (0–8 M) is from left to right. The downward curve on the right end of several panels is an edge effect due to current leakage down the side of the gels. The cleaved ovalbumin panel is spotty due to uneven sample loading, and the cleaved α_2AP is diffuse due to the high sample volume loaded.

exception of ovalbumin, which showed no detectable transition, and ATIII, which displayed a discontinuous transition, we were able to estimate conformational stabilities for the native serpins in the range of 5–15 kJ mol^{-1}. In contrast to the unique TUG gel signatures of the native serpins, the modified serpins, with the exception of modified angiotensinogen, appeared as similar bands without unfolding transitions (Figure 5). Modified angiotensinogen displayed an unfolding transition identical with the native protein. The band in the cleaved angiotensinogen TUG gel, absent from the native angiotensinogen TUG gel, is from *S. aureus* V8 proteinase (Figure 5). Modified α_1ACT had the same TUG signature with or without the N-terminal cleavage (data not shown). The bands of the modified serpins migrated with a slight slope because of the decreased pore size in the gel matrix caused by the high urea concentration on one side of the gel.

The high isoelectric point of the proteinases used prevented uncomplexed proteinases from entering the gel under the buffer conditions used (data not shown for all proteinases; see Figure 6 for HNE data). Therefore, the radioactive band in the autoradiograms of the serpin–proteinase complexes represents radioiodinated proteinase bound to an unlabeled serpin, allowing specific visualization of the rate of migration of each complex. Most serpin–proteinase complexes migrated as

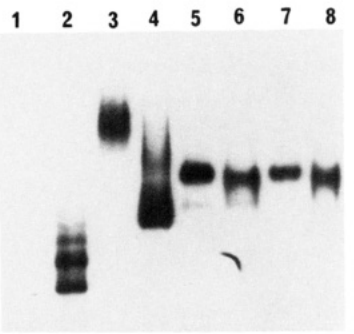

FIGURE 6: Autoradiogram of nondenaturing polyacrylamide gel electrophoresis for selected proteins before (odd-numbered lanes) and after (even-numbered lanes) 2-h incubation in 8 M urea. Electrophoresis was performed under conditions identical with those used for the TUG gels. Only the proteinases are radiolabeled. (1 and 2) HNE (no band is visible in lane 1); (3 and 4) α_1PI–HNE complex; (5 and 6) α_1PI–PPE complex; (7 and 8) α_1PI–trypsin complex.

continuous bands without unfolding transitions (Figure 5), similar to modified serpins. The one exception was the α_1PI–HNE complex which had an unfolding transition at approximately 2.5 M urea. This transition differed from that seen with the native serpins in that the complex migrated further into the gel at higher urea concentrations (data not shown). When sections of this band at 0, 3, and 8 M urea were excised from the gel and electrophoresed in SDS gels, all three sections migrated as the proteinase complex, indicating that the transition was not due to dissociation of the complex (data not shown). Moreover, it appears that HNE migrates into the TUG gels only after unfolding in urea, as indicated in Figure 6, and we conclude that the transition is due to the unfolding of complexed HNE. Of the several serpin–proteinase complexes analyzed, HNE was the only proteinase to exhibit this behavior (Figure 6).

Discussion

Previous studies from this laboratory have demonstrated that serpin–proteinase complexes are rapidly removed from the circulation. Since the clearance of any given serpin complex is independent of the proteinase used to form the complex, it has been concluded that a change in the structure of the serpin is the signal for recognition (Gonias et al., 1982; Pizzo et al., 1988). Two distinct pathways for the plasma elimination of serpin complexes have been identified; a receptor designated serpin receptor 1 (SR1) recognizes complexes of proteinases with α_1PI, α_1ACT, ATIII, and another serpin, heparin cofactor II (Pizzo et al., 1988). Similarly, SR2 recognizes and eliminates complexes of proteinases with α_2AP (Gonias et al., 1982; Pizzo et al., 1988). These receptors, present mainly on hepatocytes, are probably responsible for bulk removal of proteinase activity during episodes of inflammation, coagulation, and fibrinolysis. In the present study, we show that proteolytically modified serpins are not recognized by SR1 or SR2, extending a recent report that modified ATIII is cleared very slowly from the circulation of rabbits (Jordan et al., 1989).

These data contrast with the reaction of certain antibodies with the serpin conformations; some antibody preparations have been found which recognize both modified and proteinase-complexed ATIII (Wallgren et al., 1981) and C1 inhibitor (Agostini et al., 1985), but not the native counterparts, suggesting that the conformation of proteinase-complexed serpins is close to that of the modified forms. Conversely, lack of recognition of modified serpins by SR1 and SR2 raises the possibility that they differ from the conformation of proteinase-complexed forms. To address this possibility, we inves-

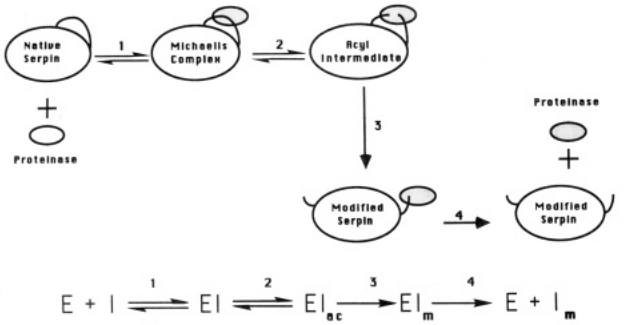

FIGURE 7: Scheme for the reaction of proteinases with serpins. The reaction pathway is equivalent to steps in the hydrolysis of a substrate by a proteinase, with certain of the intermediates identified. In this case, the substrate is the reactive site loop of a serpin, which may be hydrolyzed to yield the final product of a modified serpin. The equation at the bottom of the figure mirrors the steps and intermediates of the diagram, where the ac and m subscripts denote the acyl intermediate and modified serpin, respectively. For those proteinases that catalytically inactivate the loop, all steps are relatively fast. When a proteinase is inhibited, steps 2 and 3, which govern the turnover rate of a substrate, are relatively slow, and stabilization is at the level of the Michaelis complex, acyl intermediate, or some stage in between. Under physiological conditions, the complex has probably not attained the relaxed conformation; when it does, the proteinase should be rapidly released by hydrolysis, resulting in an inactive modified serpin. Consequently, the recognition of a serpin, by a serine proteinase, as a substrate or an inhibitor depends on the forward rates of steps 2 and 3, which in turn probably depend on how well the conformation of the reactive site loop fits the substrate binding sites of the proteinase. The key divergence between the proposed pathway for serpins and the standard mechanism of proteinase inhibition (Laskowski & Kato, 1980) concerns step 3, drawn here as an irreversible step to emphasize the low probability of resynthesis of the reactive site bond in relaxed serpins.

tigated the thermodynamic stabilities of native, proteinase-complexed, and proteolytically modified serpins.

Conformational Stabilities of Serpins. The estimated conformational stability values of 5–15 kJ mol^{-1} for native serpins are significantly lower than those of most globular proteins, which range from 21 to 63 kJ mol^{-1} (Pace, 1990). This means that the native serpins we have examined, with the exception of ovalbumin, have a greater tendency to unfold than most proteins. The striking difference between the migration of the native and modified forms of the inhibitory serpins on the TUG gels is direct visual evidence of the well-documented fact that most serpins become more thermodynamically stable after cleavage within the reactive site loop. This has been referred to as the "stressed" to "relaxed" transition by Carrell and Boswell (1986), the proposal being that a native serpin is stressed as a metastable intermediate in a folding pathway to the lower energy, relaxed, form. Proteolysis in the reactive site loop is required to allow the transition (see Figure 7). Since we observed no unfolding transitions in modified serpins, with the exception of angiotensinogen, we were unable to estimate the stabilities of the modified conformations, though these are certainly greater than the native counterparts.

The noninhibitory serpins angiotensinogen and ovalbumin were clearly distinct from the inhibitory ones since they failed to undergo any detectable change in conformation following modification. This is consistent with previous reports obtained via different methodologies (Bruch et al., 1988; Gettins, 1989; Stein et al., 1989). Reactive site modified angiotensinogen maintained the relatively unstable conformation of its native counterpart, yet ovalbumin exhibited high stability independent of reactive site cleavage. The variation in conformational stabilities of serpins may be explained with reference to the three currently available serpin molecular structures, based on X-ray crystallography: modified α_1PI (Loebermann, et al., 1984), native ovalbumin (Stein et al., 1990), and modified ovalbumin (Wright et al., 1990). The dominant structural feature of modified α_1PI is the "A" β-sheet, whose central strand consists of much of the reactive site loop; to create the native structure by rejoining the P_1 and P_1' residues, it is neccessary to pull this strand out of the plane of the sheet (Loebermann et al., 1984). This would disrupt the favorable interactions of the modified form and is probably responsible for the conformational instability of the native serpin. In modified ovalbumin, the strand is absent; the cleaved reactive site loop has not incorporated into the A β-sheet but instead points away from it. This β-sheet in native ovalbumin has the same structure as its modified counterpart. Consequently, the following proposals, discussed in part by Loebermann et al. (1984), Huber and Carrell (1989), Wright et al. (1990), and Stein et al. (1990), may explain the differences in conformational stabilities between native and modified serpins revealed by the TUG gels. Native α_1PI, α_1ACT, ATIII, α_2AP, and angiotensinogen are unstable since they lack the central strand of the A β-sheet. Upon cleavage of a bond in the reactive site loop, the loop becomes free to incorporate into the sheet, increasing its stability by allowing new favorable interactions. For modified angiotensinogen, the loop does not incorporate or, if it does, fails to increase the stability of the A sheet. In the case of ovalbumin, the sheet is stabilized independent of the reactive site loop, suggesting that the interactions within the A sheet of ovalbumin may be stronger than those of other native serpins.

Conformation of the Serpin–Proteinase Complexes. The relaxed conformation distinguishes serpins from other known macromolecular inhibitors of serine proteinases, since cleavage of the reactive site of non-serpin inhibitors does not lead to inactivation or any large-scale conformational change of the inhibitor (Laskowski & Kato, 1980). This has led some investigators to suggest that the mechanism of inhibition by serpins is significantly different from other inhibitors and that the serpin in the complex is in the relaxed conformation (Wallgren et al., 1981; Agostini et al., 1988; Haris et al., 1990; Perlmutter et al., 1990). This would require that the target proteinase had hydrolyzed the reactive site peptide bond of the inhibitory loop resulting in the separation of P_1 and P_1' residues to the modified structure reported by Lobermann et al. (1984). According to this reasoning, complex stabilization, and therefore inhibition, would result from esterification of the catalytic serine by attachment to the P_1 carbonyl moiety which sits, in the case of relaxed α_1PI, at one pole of the serpin.

It is hard to reconcile this proposition with known mechanisms of proteinase inhibition (Read & James, 1986), or with current knowledge of serpin–proteinase interactions. Here, we have demonstrated that the circulatory removal of modified serpins is not mediated by the hepatic receptors which recognize serpin–proteinase complexes. This suggests a significant structural difference between these two serpin conformations. Other workers have also obtained results which are difficult to explain if the serpin–proteinase complex is in the relaxed conformation. Schechter et al. (1989) characterized two pathways for the reaction of human skin chymase with α_1ACT, only one of which resulted in proteinase inhibition. The second pathway resulted in inactivation of α_1ACT, primarily by cleavage of the P_1–P_1' reactive site bond, presumably resulting in modification of the serpin. A similar phenomenon was reported for the reaction of thrombin, factor IXa, and factor Xa with ATIII (Bjork et al., 1982; Bjork & Fish, 1982) and heparin cofactor II with cathepsin G (Pratt et al., 1990). Since

inactivation of a serpin can be caused by a target proteinase cleaving the reactive site peptide bond, it is unlikely that this could be a mechanism of inhibition. Sheih et al. (1989) have shown that the complex of trypsin or chymotrypsin with α_2AP is reversible and that native α_2AP can be quantitatively recovered from the complex. This would almost certainly not be possible if the P_1 and P_1' residues were separated in the complex as they are in modified α_1PI. Finally, serpins are capable of forming complexes with anhydroproteinases (Tomono & Sawada, 1986; Moroi & Aoki, 1977), although the strengths of these interactions are unknown. These proteinases have their active site serine chemically modified to dehydroalanine, thereby inactivating the hydrolytic activity of the proteinase. This modification prevents cleavage of the serpin reactive center, implying this is not necessary for complex formation.

These data suggest that the serpin–proteinase complex is stabilized in a conformation that is close to the native one. In the serpin complex, the interaction between O^γ of the catalytic serine of the proteinase and the carbonyl carbon of the reactive site of the inhibitor may be closer to a fully formed bond than in other proteinase inhibitor families (Read & James, 1986). Indeed, some data suggest that the complex may even exist in a stage equivalent to the acyl intermediate of substrate hydrolysis (Travis & Salvesen, 1983), implying that the reactive site bond is hydrolyzed. However, it is extremely unlikely that "relaxation" of a serpin is involved in complex stabilization. When this does occur, the serpin is inactivated, and proteinase is released from the complex (Johnson & Travis, 1978; Bjork et al., 1982; Travis & Salvesen, 1983; Laine et al., 1985) (see Figure 7). The frequent finding of proteinase covalently associated with serpin following SDS–polyacrylamide gel electrophoresis of complexes may be akin to the trapping of covalent intermediates present in very low amounts under nondenaturing conditions, but which are stabilized and accumulate under denaturing conditions, a possibility raised previously by Travis and Salvesen (1983).

Since the serpin is present as a stable complex with a proteinase, denaturing conditions force the covalent intermediate very efficiency. From this perspective, it is surprising that other families of protein inhibitors do not form covalent complexes under denaturing conditions (Laskowski & Kato, 1980). However, it has recently been confirmed that protease nexin 2 forms SDS-stable complexes with some serine proteinases (Van Nostrand et al., 1990). Since this inhibitor is related to aprotinin, an archetypal standard mechanism inhibitor (Tanzi et al., 1988), it is likely that covalent complexes of non-serpins with proteinases may occur more frequently than previously suspected (Travis & Salvesen, 1983). If serpin–proteinase complexes exist, under native conditions, in a form in which the P_1 and P_1' residues are not separated, how do we explain their apparent stability revealed by TUG gel electrophoresis? With the exception of HNE, the proteinase component of each serpin complex is stabilized with respect to unfolding (see Figure 5). Indeed, this stabilization is also seen in the ATIII–thrombin complex as an increased resistance of thrombin to thermal unfolding (Atha et al., 1984). Since the proteinase in a serpin complex can resist unfolding, the complexed serpin may also be resistant. The lack of unfolding transitions of the serpin–proteinase complexes may be due to a stabilization of both partners, thereby mimicking the stability of the relaxed conformation. Conversely, it is possible that, just as with SDS, denaturants such as urea may force covalent intermediates and reactive site cleavage. In this case, the result would be a true relaxed serpin–proteinase complex, but one that had been forced to occur under denaturing conditions. The resolution of these questions will probably require less invasive methods of investigating protein conformation. However, our results indicate that structural elucidations of proteinases in complex with serpins must consider the possibility that experimental conditions may force the complex to adopt a conformation not found under physiological conditions.

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