Active Site Distortion Is Sufficient for Proteinase Inhibition by Serpins

STRUCTURE OF THE COVALENT COMPLEX OF \( \alpha_1 \)-PROTEASE INHIBITOR WITH PORCINE PANCREATIC ELASTASE

Received for publication, September 27, 2005, and in revised form, November 28, 2005 Published, JBC Papers in Press, December 1, 2005, DOI 10.1074/jbc.M510564200

Alexey Dementiev, József Dobó, and Peter G. W. Gettins

From the Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60607

We report here the x-ray structure of a covalent serpin-proteinase complex, \( \alpha_1 \)-proteinase inhibitor (\( \alpha_1 \)-PI) with porcine pancreatic elastase (PPE), which differs from the only other x-ray structure of such a complex, that of \( \alpha_1 \)-PI with trypsin, in showing nearly complete definition of the proteinase. \( \alpha_1 \)-PI complexes with trypsin, PPE, and human neutrophil elastase (HNE) showed similar rates of deacylation and enhanced susceptibility to proteolysis by exogenous proteinases in solution. The differences between the two x-ray structures therefore cannot arise from intrinsic differences in the inhibition mechanism. However, self-proteolysis of purified complex resulted in rapid cleavage of the trypsin complex, slower cleavage of the PPE complex, and only minimal cleavage of the HNE complex. This suggests that the earlier \( \alpha_1 \)-PI-trypsin complex may have been proteolysed and that the present structure is more likely to be representative of serpin-proteinase complexes. The present structure shows that active site distortion alone is sufficient for inhibition and suggests that enhanced proteolysis is not necessarily exploited in vivo.

Over the last 10 years, many of the details of the remarkable conformational change-based serpin inhibition mechanism have been worked out (1). The proteinase initially recognizes the serpin as a potential substrate by using residues in the reactive center loop (RCL), and, in some cases, additional exosite interactions. This initial Michaelis-like complex forms without major conformational change in either serpin or proteinase. This is borne out by five x-ray structures of such complexes formed using inactivated proteinase to prevent further reaction (2–6). When functional proteinases are used, however, cleavage of the scissile bond of the serpin occurs with cleavage of the RCL and formation of an acyl enzyme intermediate (7). This cleavage removes the constraints that hold the RCL out from the serpin body and allows the cleaved RCL to insert into \( \beta \)-sheet A of the serpin, dragging the proteinase with it and moving it over 70 Å to the distal end of the serpin (8). The length of the RCL available to insert into the \( \beta \)-sheet is tightly linked to the length of the RCL, such that full insertion results in steric clashes between serpin and proteinase at the interface, which appear to be resolved by extraction of the P1 side chain from the S1 pocket of the proteinase (9) and consequent displacement of the covalently attached active site Ser-195 by ~6 Å from its catalytic partner His-57 (10). This separation of the two key catalytic residues provides a very plausible explanation for the loss of catalytic function in the covalent complex and, hence, an explanation for the mechanism whereby serpins inhibit serine proteinases by kinetically trapping the acyl intermediate of the substrate cleavage reaction.

Although there are fluorescence (8, 11–13) and NMR (14, 15) data for several covalent serpin-proteinase complexes that all indicate that proteinase translocation is a common aspect of the mechanism, there is only a single x-ray structure of such a complex that provides the details of the molecular structure and rearrangement, that is, of the \( \alpha_1 \)-PI-trypsin complex (10). The structure confirms the expectations of active site distortion and steric compression between serpin and proteinase and provides the quantitation of the movements of the catalytic residues and the serpin substrate noted above. What was quite unexpected was that 37% of the proteinase was crystallographically disordered. This was interpreted as the structural manifestation of the altered biochemical properties of serpin-complexed proteinases that have been known for many years. These alterations include increased susceptibility of complexed proteinase to proteolysis and a reduction in overall proteinase stability (16–22). It was therefore suggested that such apparent "partial denaturation" of the proteinase in the x-ray structure is an integral part of the inhibition process and might provide a means for degradation and clearance in vivo (10).

Given the remarkable and unexpected loss of ordered structure for a major part of the proteinase in the crystal structure of the \( \alpha_1 \)-PI-trypsin complex and the suggestion that this is part of the inhibition process, it is essential to test whether it is indeed a common aspect of the mechanism and therefore to determine the x-ray structure of a second such complex for comparison. We report here such a structure, formed between the same serpin, \( \alpha_1 \)-PI, and PPE, a proteinase closer than trypsin in specificity to the natural target HNE. We find no evidence for partial denaturation of the elastase, even though the biochemical properties of the proteinase are altered in equivalent ways to trypsin or HNE in complex with \( \alpha_1 \)-PI. Our structural and biochemical findings, taken together, suggest that the present structure of \( \alpha_1 \)-PI with PPE is more likely to be representative of the serpin-proteinase complex than that published previously of \( \alpha_1 \)-PI with trypsin.

MATERIALS AND METHODS

Protein Isolation and Purification—The His-tagged multi-8 variant of \( \alpha_1 \)-PI, containing seven stabilizing mutations (23), C232S (24), and an R101H mutation, which randomly appeared during cloning, was expressed and purified as described previously (15). The His tag was not
removed but has been found not to alter the inhibitory properties of the serpin. PPE and HNE were from Calbiochem. Bovine pancreatic trypsin was from Sigma. The covalent complex of α₁PI and PPE was made and purified by chromatofocusing as previously reported (15) with some modifications, namely inclusion of 5 μM methoxy-succinyl-Ala-Ala-Pro-Val-chloro-methyl-ketone (MS-AAPV-CMK, Sigma) in all chromatography buffers to minimize complex degradation and a subsequent gel filtration step to remove residual polybuffer following the chromatofocusing column. The complex was concentrated to 2 ml, applied to a 24-ml Superdex-75 column (Amersham Biosciences), equilibrated with 100 mM NaCl, 10 mM HEPES, 0.5 mM EDTA, pH 8.0, 5 μM MS-AAPV-CMK, and eluted with the same buffer. Fractions containing pure complex were combined, 400 μM final concentration MS-AAPV-CMK and 1% (by weight) α₁PI were added to prevent any degradation by liberated proteinase, and the protein was concentrated to 12 mg/ml final concentration and stored at −80 °C in aliquots.

**Crystallization and Data Collection**—α₁PI-PPE covalent complex was crystallized from hanging drops containing 2 μl of protein (12.6 mg/ml) and 2 μl of reservoir solution (0.2 M bicine buffer, pH 8.1, 60 mM sodium citrate, 16–18% polyethylene glycol-3350). Small crystals appeared within 4–5 days at 18 °C. Several cycles of microseeding gave better quality crystals with an average size of 50 × 50 × 200 μm. Diffraction data were collected at 100 K from a single flash-frozen crystal cryoprotected in oil on a Mar CCD-300 detector at the SER-CAT beamline (Advanced Proton Source, Argonne National Laboratory). Data were indexed and processed with the HKL 2000 software suite (25). The crystal diffracted up to 3.3 Å and contained one molecule of complex/asymmetric unit (Table 1). The structure was solved by molecular replacement using CNS (26). Initial phases were determined by Patterson search, with the structures of native PPE (Protein Data Bank (PDB) entry 1H9L) and cleaved α₁PI from the covalent complex with trypsin (PDB entry 1EZX) as search models. The final model of the complex was obtained after multiple cycles of refinement consisting of a manual model building on an SGI Silicon Graphics work station with Quanta crystallography NMR software. Crystallographic statistics are given in Table 1. Coordinates have been deposited with the Protein Data Bank with accession number 2D26.

**Determination of Dissociation Rates**—All covalent complexes were made and purified analogously to α₁PI-PPE, except that no inhibitors were added and the gel filtration step was omitted. The purified complexes were concentrated to 0.5–3 mg/ml in ultrafiltration cells fitted with 30-kDa cutoff membranes and stored at −80 °C prior to use. The assay was performed in 100 mM HEPES buffer, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% polyethylene glycol-8000 at 37 °C at a final protein concentration of 10–200 nM and substrate concentration of 200 μM. Succinyl-Ala-Ala-Ala-pNA (Calbiochem) was used as a substrate in the assay for α₁PI-PPE and the proteinase loop containing the active site Ser-195 of elastase and Met-358 (P1) of α₁PI are shown in ball-and-stick representation.

**RESULTS**

**Crystallization and Structure of the α₁PI-Elastase Complex**—α₁PI-PPE complex was purified and crystallized as described under "Materials and Methods." Crystals grown for different periods of time under the same conditions were dissolved in sample buffer and examined by SDS-PAGE for evidence of complex degradation. After 11 days, a small amount of complex cleavage could be seen (~5%), whereas after 16 days, as much as 20% appeared to have been cleaved. Data collection was therefore restricted to crystals grown for no more than 9 days to minimize heterogeneity in the crystal resulting from traces of cleaved complex.

With this limitation, it was possible to obtain diffraction data to 3.3 Å that were 97.8% complete and to determine a structure for the complex using molecular replacement. To avoid model bias, the starting model for the cycles of rebuilding and refinement had 43 residues of the proteinase and 81 residues of the serpin removed, including loops on the serpin and proteinase that were likely to be in contact with one another as well as the serpin RCL and the proteinase loop containing the active site Ser-195. After 25 cycles of refinement, a final structure was obtained (Fig. 1) with Rwork of 0.25 and Rfree of 0.31 (Table 1). In overall organization, the structure resembles that determined previously for α₁PI-
trypsin by both fluorescence resonance energy transfer (8) and x-ray crystallography (10), with the RCL fully inserted into β-sheet A and the attached proteinase translocated to the distal end of the serpin, where it is held tightly against the serpin body by the RCL tether. The serpin moiety is almost identical to that of cleaved α1PI, with root mean square deviation for all Cα of only 0.85 Å. The location of the proteinase is very similar, although not identical, to that of trypsin in the α1PI-trypsin complex, with the elastase rotated ~40° clockwise about an axis running along the inserted RCL and viewed from the serpin toward the proteinase. In the elastase-active site, there is clear density for Ser-195 and His-57 and clear evidence that the acyl group of the cleaved RCL is in an ester linkage with the γ-O of Ser-195, that the P1 methionine has been removed from the elastase S1 pocket, and that the Ser γ-O is ~6 Å away from the ring Ne-2 nitrogen of His-57 (Fig. 2). The oxyanion hole, formed by backbone amides of residues 193 and 195, is disrupted, with the nitrogens moved 3.4 and 2.8 Å, respectively, compared with free elastase and with the Asp-194 Cα displaced by 2.6 Å. These findings are as expected from the inhibition mechanism in that they show the same type of active site distortion as in the α1PI-trypsin structure and hence provide a structural basis for kinetic trapping of the complex.

The major difference between the present complex and that of α1PI-trypsin is that almost all of the proteinase is ordered and gives rise to well defined electron density. Of the secondary structural elements that make up 45% of the native elastase structure, all are visible in elastase complex. Of the remaining turns and loops, only residues 142–149, 186–190, and the N-terminal 4 residues cannot be defined. The 142–149 loop is likely to be involved in the interface with α1PI, whereas the stretch from 186 to 190 is much more buried and is close to the active site, suggesting some very limited disorder induced by complex formation. An overlay of the structures of free and complexed elastase (Fig. 3A) also clearly demonstrates the absence of major conformational change upon complex formation for almost the whole protein, with root mean square deviation for all defined Cα of 1.0 Å. The only exception is the surface loop 218–225, which is slightly displaced relative to free elastase, with the Cα differences ranging from 1.4 to 3.5 Å. This may result from distortions in the active site being propagated to this loop via the linking disulfide between Cys-191, adjacent to oxyanion hole-forming Gly-193, and Cys-220 in the surface loop.

In contrast to the well defined structure of complexed elastase, the structure of trypsin co-crystallized with α1PI is missing not only surface loops but about one-third of the secondary structure. Because both elastase and trypsin have the same chymotrypsin fold, an overlay of the structures of complexed elastase and trypsin serves well to visualize the differences between them (Fig. 3B). That there is no fundamental difference in the degree of ordering for that part of elastase that is unde

### TABLE 1

| Statistics on data collection and refinement |
|---------------------------------------------|
| **Space group** | C2 |
| **Cell constant (a, b, c in Å; α, β in °)** | 109.83, 85.18, 76.26; 121.01 |
| **Wavelength (Å)** | 1.0 |
| **No. of reflections** | 31,619 |
| **No. of unique reflections** | 9,161 |
| **Resolution range (Å)** | 50.0–3.3 (3.45–3.30)* |
| **Completeness (%)** | 97.8 (96.4) |
| **Rmerge** | 0.078 (0.275) |
| **Average intensity ([Fobs]/[σ(Fobs)])** | 9.5 (5.7) |
| **Average redundancy** | 3.6 (3.4) |
| **No. of reflection used (test set)** | 8,959 (821) |
| **Crystallographic Rwork (Rfree)** | 0.25 (0.31) |
| **No. of protein atoms/water molecules** | 4,118/75 |
| **Average B-factors (Å²)** | 64 |

* Values in parentheses are for the highest resolution shell.

### FIGURE 2.

Electron density map for the catalytic residues of the complexed elastase and the α1PI P1 methionine (stereo). Continuous density links the backbone of the methionine 358 to the γO of Ser-195, indicating the presence of a covalent ester linkage between the serpin and the proteinase.

### FIGURE 3.

Comparison of complexed PPE with free PPE and with complexed trypsin. **A**, overlay (stereo) of complexed PPE (cyan) with free PPE (PDB entry 1H9L). The free elastase is colored yellow for those regions that are visible in the complexed PPE, purple for the missing contact loop (141–152), red for the missing internal loop (186–190), and blue for the missing four N-terminal residues. There are minimal differences between the two structures. **B**, overlay (stereo) of α1PI-complexed PPE (cyan) with α1PI-complexed trypsin (PDB entry 1EZX) (brown). Note that extensive regions of the complexed PPE structure are absent from complexed trypsin and that these include regions of secondary structure as well as surface loops.

### FIGURE 4.

Deacylation Rates of α1PI-Protease Complexes—Rates of deacylation were determined under physiological conditions for complexed of
α₁PI with trypsin, PPE, and HNE as described under “Materials and Methods.” The rates of deacylation were all very low, as expected for complexes that are sufficiently long-lived to be crystallized, and were similar to those reported previously for the α₁PI-trypsin complex (27). Although there was some variation in deacylation rate for the three complexes (Table 2), this represented a difference of less than a factor of two between α₁PI-PPE and either α₁PI-trypsin or α₁PI-HNE.

Proteolytic Susceptibility of α₁PI-Proteinase Complexes—We next examined whether there were differences in the enhancements in proteolytic susceptibility of the three complexes in complex with α₁PI. Each of the three purified serpin-proteinase complexes was incubated separately with proteinases of different specificity (trypsin, subtilisin Carlsberg, and V8 proteinase). Compared with the susceptibility to proteolysis of the uncomplexed proteinases, each of the three complexed proteinases showed similar greatly enhanced reactivity. Trypsin cleavage was the most extensive (Fig. 5), followed by subtilisin cleavage, and then relatively little cleavage by V8 proteinase (data not shown). Importantly, each of the complexed proteinases behaved similarly toward each of the three proteinase probes. Complex formation thus appears to have qualitatively similar effects on the structures of each proteinase that renders some regions more reactive to exogenously added proteinase.

Self-proteolysis of α₁PI-Proteinase Complexes—A separate question that was addressed was whether the proteinase released by deacylation of a given complex would cause degradation. Because each complex would give rise to a different proteinase (trypsin, PPE, or HNE), this is a different question from that addressed above but one with important practical consequences for crystallization studies, where maintenance of complex homogeneity through prevention of self-proteolysis is critical.

Each complex was incubated at room temperature, with samples removed after different times and examined by SDS-PAGE for evidence of complex degradation. The three complexes showed dramatic differences. The trypsin complex showed nearly complete cleavage of the complexed proteinase after even 1 day (Fig. 6). The PPE complex also showed cleavage of the complexed proteinase, although this was much less than for the trypsin complex, with only 50% of the complex cleaved after 1 day (Fig. 6). In contrast, the HNE complex showed minimal degradation over the 2-day period. Given that each complex undergoes deacylation at similar rates (Table 2), the amount of proteinase released and available for cleavage of complex should have been a similarly small amount in each case. Thus, the differences for the three complexes must represent a combination of different proteinase specificities for trypsin, PPE, and HNE and different available sequences within the regions of enhanced proteolytic susceptibility. The presence of cleaved complex for PPE was in keeping with what we found above in examining PPE-α₁PI crystals grown for different periods of time. There was, however, a time period during which crystals were of sufficient size to use for data collection without being so old that major cleavage of the complex had occurred. In contrast, the greater rate of degradation for the trypsin complex suggests that it might be very difficult to obtain crystals of the trypsin-α₁PI complex without incurring significant cleavage, even though the pH used in the earlier study (7.4) was a little lower than that used here for crystal growth (8.1).

**TABLE 2**  
Rate constants for dissociation of α₁PI-proteinase complexes

| Complex       | $k_{diss}$  | $t_{50}$ |
|---------------|-------------|----------|
| α₁PI-PPE     | $3.8 \pm 0.3 \times 10^{-7}$ | 21       |
| α₁PI-HNE     | $2.9 \pm 0.3 \times 10^{-7}$ | 28       |
| α₁PI-trypsin | $5.3 \pm 0.6 \times 10^{-7}$ | 15       |

Values are the average and spread of duplicate measurements.

**DISCUSSION**

The structure presented here of the covalent complex of α₁PI with PPE is only the second of a serpin with a target proteinase. It shows many features in common with the first structure of α₁PI with bovine trypsin, including full insertion of the cleaved RCL and concomitant translocation of the proteinase to the distal end of the serpin, a tight interface between serpin and proteinase, and a distortion of residues in the active site that can alone account for the kinetic trapping of the acyl-enzyme reaction intermediate. There is, however, one major difference between the two structures. Although 37% of the polypeptide of the trypsin moiety in complex was crystallographically disordered (10), this was not the case for PPE in the present complex. For the trypsin complex, residues both within surface loops as well as some strands of β-sheet were untraceable. In the present structure, only 17/243 residues from elastase cannot be traced. These are restricted to one loop in direct...
contact with the serpin body, where the nonspecific nature of the compression between serpin and proteinase may give rise to multiple conformations for these loops, an internal loop adjacent to the active site, and the extreme N terminus, which interacts with Asp-194 in the active site of free elastase. In addition, the $C_{\alpha}$ root mean square deviation for the 93% of the PPE structure that can be seen in the complex shows only 1.0 Å root mean square deviation from the structure of free PPE, implying that the forces imposed on the proteinase by RCL insertion and compression against the serpin body result in changes that are restricted to the active site and the surrounding surface loops. No major conformational change occurs within the stable secondary structure of the proteinase.

Given this difference between the two complexes, we sought to understand the basis for it by comparing the solution properties of the two complexes with respect to deacylation rates and susceptibility to proteolysis. It has been proposed that the final conformational state of the serpin-proteinase complex is in equilibrium with one in which the RCL has been completely or almost completely inserted into β-sheet A but in which the catalytic function of the proteinase has not been fully compromised (28). Biochemical evidence supports such a two-state model in which a very small fraction retains some catalytic function, whereas the dominant species is catalytically inactive. It has been shown for trypsin, under physiological conditions, that the small fraction with reduced catalytic activity accounts for almost all of the observed deacylation of the acyl intermediate (27). Deacylation rates therefore are a direct measure of the fractional population of this conformation. Because the equilibrium populations of these conformers are predicted to be influenced by how much of the energy derived from RCL insertion is expended on structurally modifying the proteinase to change it to the final catalytically compromised conformation, it would be expected that a complex in which there was less structural modification would have a smaller population in the catalytically active conformation and therefore deacylate at a rate reduced in proportion to the reduction in conformer population. Although the comparison is not perfect, it has been shown that there is a difference in stability between free and complexed proteinase for PPE and $\alpha_{1}$PI, using denaturants to follow unfolding of the proteinase moiety (16). That study gave a substantial difference in stability (6.3 kcal mol$^{-1}$) between these two proteinase conformations, suggesting that there is a large enough expenditure of energy needed to alter the proteinase structure that differences in such structural alteration for different proteinases might result in large shifts in the equilibrium between the two conformations. Accordingly, if the apparently greater structural alteration in complexed trypsin compared with complexed elastase results from more of the energy derived from RCL insertion being used to alter the trypsin conformation, there should be less of a free energy difference between the two states in the trypsin complex than in the PPE complex, a higher fractional population of partially active trypsin in complex than PPE in complex, and a correspondingly higher rate of catalyzed deacylation for the trypsin complex than the PPE complex. This is not what was found. Instead, the trypsin and PPE complexes deacylated at very similar rates, which were also similar to that for the complex with the true $\alpha_{1}$PI target, HNE. This suggests that, for each complex, the fraction of complex in the partially active conformation is similar and in turn implies similar $\Delta G^\circ$ values for the conformational equilibrium for each complex. Because the serpin is the same in all three cases, the energy available from RCL insertion should be very similar for each complex. This suggests that the energy expended in each case to produce the final conformation of the proteinase is similar and therefore that each is likely to be altered in structure in a similar way. Finally, it should be noted that, although the stabilized multi-8 variant of $\alpha_{1}$PI was used in the present study, the rate of $\alpha_{1}$PI-trypsin complex dissociation is very similar to that reported elsewhere for the complex of wild-type $\alpha_{1}$PI with trypsin (27).

A separate measure of possible differences in conformational alteration of the proteinase in the trypsin and PPE complexes was provided by their susceptibility to proteolysis by a range of proteinases of different specificities. Such enhanced proteolytic susceptibility of the proteinase in complex has been shown for a number of serpin-proteinase complexes and has been taken as evidence for the conformational alteration of the complexed proteinase (17–22). Using subtilisin, V8 proteinase, and trypsin as probes, we found that both the PPE and trypsin complexes showed similar enhanced susceptibility to proteolysis and even that HNE was affected in the same way. Although the three proteinases thus appear to have undergone similar structural alteration upon complex formation, it is not possible from such studies to deduce what the exact nature of these changes is, which may be subtle. However, the absence of defined electron density for the extreme N terminus, which would normally interact with Asp-194, suggests a linkage between disruption of the active site caused by the “tugging” of the acyl intermediate from its normal site and structural and/or dynamic changes elsewhere in the proteinase. It was also noted above that movement of Cys-191 near the active site might explain the perturbation of the disulfide-linked loop 218–225.

Similar deacylation rates and increases in proteolytic susceptibility support the conclusion that the structural alterations that occur in PPE upon complex formation with $\alpha_{1}$PI are the same as those for trypsin. Furthermore, this similarity extends to HNE. Why then is there such a large difference in the x-ray structures of the trypsin and PPE complexes? A possible answer lies in the results obtained above on self-proteolysis within the complexes. The studies on enhanced proteolysis used three proteinases, including trypsin. Trypsin resulted in the greatest degree of proteolysis for each of the three complexes. This implies that, although both trypsin and PPE complexes would be expected to release a similar amount of proteinase during a comparable period of crystal growth, the released trypsin might be more potent at cleaving the remaining complex than a similar amount of released PPE. The results obtained above bear this out. After an incubation period of only 1 day at room temperature, all of the $\alpha_{1}$PI-complexed trypsin was cleaved at one or more sites, resulting in bands of complex that migrated on SDS-PAGE much faster than intact complex. Although there was also degradation for the complexed PPE, this was significantly less. Even more striking was the near absence of degradation for complexed HNE, suggesting that, for similar structural changes within the complexed proteinase, the degree of cleavage by released proteinase is a combined function of the proteinase, its activity and specificity, and the availability of appropriate cleavage sites in the regions of the trapped proteinase that are made generally more reactive to proteolysis.

There is thus the possibility that the structure determination of the trypsin-$\alpha_{1}$PI complex was of partially degraded complex and that the apparent crystallographic disorder of 37% of the proteinase resulted from self-proteolysis. This is supported by two pieces of evidence. The first is from a detailed study of the sites of enhanced proteolysis in uPA complexed with plasminogen activator inhibitor 1 (22). That study shows that only a subset of potential cleavage sites undergoes increased susceptibility to proteolysis upon complex formation with plasminogen activator inhibitor 1. These are confined to loop regions, including the activation loop. If the regions undergoing such changes in proteolytic susceptibility are common to other serpin-proteinase complexes, it would be expected that the missing regions in the trypsin-$\alpha_{1}$PI complex would map exclusively to these sites, whether increased flexibility or
proteolysis was the cause of the loss of electron density. However, this was not the case. The loop connecting h1C and s3C of uPA was cleaved by both V8 proteinase and trypsin in the uPA-plasminogen activator inhibitor 1 complex. In the trypsin-α1PI complex, however, the equivalent region of trypsin (residues 173PGQITSN) is well defined. The significance is that this region in uPA contains a potential trypsin cleavage site, whereas trypsin itself does not, suggesting that altered flexibility per se is not the determinant for observing electron density in the α1PI-trypsin structure, but it is whether there is also a trypsin cleavage site that has then been used to give cleaved complex. The second piece of evidence is the SDS gel of the crystal of complex used for the trypsin structure, but it is whether there is also a trypsin cleavage site that has been used to give cleaved complex. The dissolution crystal of the complex showed high heterogeneity, with three bands rather than one and with the lowest mobility band (likely to correspond to uncleaved complex) representing no more than 25% of the total complex. In the case of the present study with PPE, we carefully monitored complex degradation as a function of time and used crystals grown for a short enough time that such degradation was not a major concern.

We conclude that the present structure of a serpin-proteinase complex carried out on crystals in which minimal complex cleavage had occurred is a better representation of the true nature of the structural changes that occur within the proteinase upon complex formation than is the earlier structure of α1PI-trypsin. The failure to observe electron density for 37% of the trypsin in regions that are well defined in the PPE complex, despite similar structural changes detected in solution for the two complexes, suggests that this may have resulted from trypsin self-proteolysis and consequent heterogeneity rather than simply from the much higher mobility of an intact polypeptide. The limited active site distortion seen in both complexes is therefore sufficient to account for kinetic trapping of the acyl intermediate. Furthermore, there is no support for the idea that proteolysis of complexed proteinase is exploited in vivo. Indeed, neither HNE in the present study nor uPA in an earlier study (22) gives rise to self-proteolysis of serpin-complexed proteinase.

Acknowledgment—We thank Dr. Steven Olson for helpful comments on the manuscript.

REFERENCES

1. Gettins, P. G. W. (2002) Chem. Rev. 102, 4751–4804
2. Ye, S., Crech, A. L., Belmares, R., Bergstrom, R. C., Tong, Y., Corey, D. R., Kanost, M., and Goldsmith, E. (2001) Nat. Struct. Biol. 8, 979–983
3. Baglin, T. P., Carrell, R. W., Church, F. C., Esmon, C. T., and Huntington, J. A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11079–11084
4. Dementiev, A., Simonovic, M., Volz, K., and Gettins, P. G. W. (2003) J. Biol. Chem. 278, 37881–37887
5. Dementiev, A., Petitou, M., Herbert, J. M., and Gettins, P. G. W. (2004) Nat. Struct. Mol. Biol. 11, 863–867
6. Li, W., Johnson, D. J., Esmon, C. T., and Huntington, J. A. (2004) Nat. Struct. Mol. Biol. 11, 857–862
7. Lawrence, D. A., Ginsburg, D., Day, D. E., Beekenpas, M. B., Verhamme, I. M., Kvassman, J.-O., and Shore, J. D. (1995) J. Biol. Chem. 270, 25309–25312
8. Stratikos, E., and Gettins, P. G. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4808–4813
9. Futamura, A., Stratikos, E., Olson, S. T., and Gettins, P. G. W. (1998) Biochemistry 37, 13110–13119
10. Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) Nature 407, 923–926
11. Stratikos, E., and Gettins, P. G. W. (1998) J. Biol. Chem. 273, 15582–15589
12. Fa, M., Bergstrom, F., Hagglof, P., Wilczynska, M., Johansson, L., and Ny, T. (2000) Arch. Biochem. Biophys. 380, 102, 1182–1191
13. Backovic, M., Stratikos, E., Lawrence, D. A., and Gettins, P. G. W. (2002) Protein Sci. 11, 1182–1191
14. Peterson, F. C., and Gettins, P. G. W. (2001) Biochemistry 40, 6284–6292
15. Dobo´, J., and Gettins, P. G. W. (2004) J. Biol. Chem. 279, 9264–9269
16. Herve´, M., and Ghelis, C. (1991) Arch. Biochem. Biophys. 285, 142–146
17. Fish, W. W., Orre, K., and Bjork, I. (1979) Eur. J. Biochem. 101, 39–44
18. Cooperman, B. S., Stavridi, E., Nickbarg, E., Rescorla, E., Scheckter, N. M., and Rubin, H. (1993) J. Biol. Chem. 268, 23616–23625
19. Kaslik, G., Patthy, A., B´alint, M., and Gr´af, L. (1995) FEBS Lett. 370, 179–183
20. Kaslik, G., Kardos, J., Szabo´, L., Za´vodszky, P., Westler, W. M., Markley, J. L., and Gra´f, L. (1997) Biochemistry 36, 5455–5474
21. Stavridi, E. S., O’Malley, K., Lukacs, C. M., Moore, W. T., Lambris, J. D., Christianson, D. W., Rubin, H., and Cooperman, B. S. (1996) Biochemistry 35, 10608–10615
22. Egeldun, R., Petersen, T. E., and Andreassen, P. A. (2001) Eur. J. Biochem. 268, 673–685
23. Lee, K. N., Park, S. D., and Yu, M. H. (1996) Nat. Struct. Biol. 3, 497–500
24. Peterson, F. C., Gordon, N. C., and Gettins, P. G. W. (2000) Biochemistry 39, 11884–11892
25. Osivonovski, Z., and Minor, W. (1997) Methods Enzymol. 276, 461–472
26. Brünger, A. T. (1998) Acta Crystallogr. Sect. D 54, 905–921
27. Calugaru, S. V., Swanson, R., and Olson, S. T. (2001) J. Biol. Chem. 276, 32446–32455
28. Gettins, P. G. W. (2002) FEBS Lett. 523, 2–6