The serpin antithrombin is a slow thrombin inhibitor that requires heparin to enhance its reaction rate. In contrast, α1-proteinase inhibitor (α1PI) Pittsburgh (P1 Met → Arg natural variant) inhibits thrombin 17 times faster than pentasaccharide heparin-activated antithrombin. We present here x-ray structures of free and S195A trypsin-bound α1PI Pittsburgh, which show that the reactive center loop (RCL) possesses a canonical conformation in the free serpin that does not change upon binding to S195A trypsin and that contacts the proteinase only between P2 and P2’. By inference from the structure of heparin cofactor II bound to S195A thrombin, this RCL conformation is also appropriate for binding to thrombin. Reaction rates of trypsin and thrombin with α1PI Pittsburgh and antithrombin and their P2 variants show that the low antithrombin-thrombin reaction rate results from the antithrombin RCL sequence at P2 and implies that, in solution, the antithrombin RCL must be in a similar canonical conformation to that found here for α1PI Pittsburgh, even in the nonheparin-activated state. This suggests a general, limited, canonical-like interaction between serpins and proteinases in their Michaelis complexes.

Although antithrombin is the principal inhibitor of the blood coagulation proteinases thrombin and factor Xa, its rate of reaction with each of these proteinases is slow in the absence of heparin (1, 2). In marked contrast, a variant of the serpin α1-proteinase inhibitor that contains a P1 mutation of methionine to arginine, named α1PT Pittsburgh, was found to be a very effective inhibitor of thrombin, with a second order rate constant for thrombin inhibition 17-fold higher than that of antithrombin that had been conformationally activated by pentasaccharide heparin (3, 4). In the individual who carried the Pittsburgh mutation, the potent thrombin inhibitory capability of this variant serpin resulted in hemorrhage and ultimately death.

In antithrombin, heparin is a multifactorial enhancer that can not only act as a bridging cofactor to bring serpin and proteinase together but can also induce a conformational change in antithrombin that results in expulsion of the two distal residues of the RCL from β-sheet A (5, 6). It has been proposed that this loop expulsion permits alteration of the RCL conformation in the vicinity of the scissile bond to a more optimal one for interaction with proteinase and so contributes significantly to the heparin-induced acceleration of proteinase inhibition. In keeping with this proposal, the RCL conformation of antithrombin in published x-ray structures has the P1 arginine residue turned inwards toward the body of the protein, where it forms a salt bridge to Glu255 (7, 8). However, these x-ray structures are all of antithrombin dimers that contain one molecule of functionally active antithrombin and one in an inactive, latent conformation. Importantly, the RCL of the functional molecule forms the interface with the latent molecule, as an extraneous strand of β-sheet C of that molecule. This raises the question of whether the conformation of the active molecule has been artificially altered by heterodimer formation and thus of whether it represents the conformation of antithrombin in solution.

In an attempt to understand the structural basis for α1PI Pittsburgh being able to inhibit thrombin at a high rate, and thus also to provide insight into the low basal reactivity of antithrombin, we have determined crystal structures of free and S195A trypsin-bound forms of the Pittsburgh variant of the serpin α1-proteinase inhibitor and correlated these with rate constants for inhibition of thrombin and trypsin by α1PI Pittsburgh, by antithrombin, and by P2 variants of each of these serpins.

MATERIALS AND METHODS

Protein Expression and Purification—α1PI Pittsburgh, containing seven stabilizing mutations that hinder polymerization (9) and a C328S mutation that prevents intermolecular disulfide bond formation, was expressed and refolded as previously described (10). The P2 Pro → Gly variant was made by site-directed mutagenesis and expressed and purified as for α1PI Pittsburgh. S195A bovine trypsinogen was expressed in Escherichia coli as inclusion bodies and refolded, purified, and activated as previously described (11). The noncovalent complex between S195A trypsin and α1PI Pittsburgh was formed at pH 7.5 in 25 mM Tris-HCl, 50 mM NaCl, and the complex was purified on Superdex 200 (Amersham Biosciences), dialyzed, and concentrated to 15 mg ml⁻¹ in 10 mM MOPS, pH 6.0, containing 25 mM NaCl.

Crystallography, Data Collection, Structure Determination, and Refinement—α1PI Pittsburgh crystals were obtained at 20 °C using the hanging drop method, by mixing equal volumes of protein (25 mg/ml) and well solutions (0.2 M potassium citrate, 20% polyethylene glycol).
**Table I**

| Crystal | Data collection and refinement statistics for α_{PI} Pittsburgh and α_{PI} Pittsburgh-S195A trypsin noncovalent complex |
|---------|---------------------------------------------------------------------------------------------------|
| α_{PI} Pittsburgh | α_{PI} Pittsburgh-S195A trypsin |
| **Crystal** | | |
| Space group | P2₁,2,2₁ | C2 |
| Cell dimensions | | |
| a = 39.31 Å | a = 132.2 Å |
| b = 52.16 Å | b = 62.3 Å |
| c = 208.68 Å | c = 98.9 Å |
| β = 110.3° | | |
| No. of crystals | 1 | 2 |
| **Data collection** | | |
| Temperature | 298K | 100K |
| Resolution limit (Å) | 100-2.65 | 100-2.3 |
| Wavelength (Å) | 1.541 | 1.00 |
| Reflections (observed/unique) | 103,240/12,840 | 628,540/33,539 |
| Completeness (overall/last shell) (%) | 96.7/93.6 | 99.5/99.7 |
| Rmerge (overall/last shell) (%) | 10.5/56.4 | 9.6/24.5 |
| Rdiff (overall/last shell) | 15.9/92.7 | 17.6/4.1 |
| **Refinement** | | |
| No. of molecules (complexes) in asymmetric unit | 1 | 1 |
| Resolution range (Å) | 40.00-2.65 | 40.00-2.30 |
| Average B-factor (Å²) | 50.6 | 51.5 |
| No. of reflections (work/test) | 11,125(1)/1,256 | 29,499(3)/277 |
| No. of protein atoms (amino acids) | 2,786 (371) | 4,542 (598) |
| No. of solvent molecules | 123 | 377 |
| Rcryst (%) | 18.8 | 19.3 |
| Rfree (%) | 18.9 | 22.8 |
| Root mean square deviations from ideality | | |
| Bond lengths (Å) | 0.006 | 0.006 |
| Bond angles (degrees) | 1.3 | 1.3 |
| Ramachandran statistics | | |
| Most favored | 277 (83.7%) | 524 (87.6%) |
| Additionally allowed | 51 (15.4%) | 73 (12.2%) |

3350, pH 6.30. An irregular rod-shaped crystal (0.9 × 0.25 × 0.1 mm) grew within 1 month. Diffraction data were collected at room temperature from a single crystal on a Rigaku RAXIS II detector, with a Rigaku RU-H2R rotating anode x-ray generator. Data were indexed and scaled using SCALEPACK. Initial phases were determined by molecular replacement using the structure of wild type α_{PI} (1QLP). Molecular replacement calculations were performed with CNS. Electron density maps were interpreted using Quanta. The model was progressively refined using simulated annealing at constant temperature, followed by energy minimization and manual inspection and rebuilding. 22 N-terminal amino acids and the histidine tag were not visible in the final electron density maps. Crystallographic data and statistics are shown in Table I.

S195A trypsin-α_{PI} Pittsburgh complex crystals (0.15 × 0.20 × 0.20 mm) were obtained within 10 days at 18 °C from hanging drops in 0.2 M potassium citrate buffer, pH 8.3–8.5, containing 17.5% (w/v) polyethylene glycol 3350 and 2% glycerol. Diffraction data were collected from two single, flash frozen, crystals, cryoprotected in oil, using synchrotron radiation at the SERCAT beamline (Advanced Photon Source, Argonne National Laboratory). Data were indexed and processed as described above. The structure of the complex was solved by molecular replacement using CNS programs with the structures of human α_{PI} (1QLP) and bovine trypsin (1EJM) as search models. The final refined model of the complex was obtained as described for α_{PI} Pittsburgh. Statistics are given in Table I.

**Kinetic Measurements**—Inhibition of trypsin by α_{PI} Pittsburgh was followed at 25 °C by progress curve analysis using a continuous assay. 1 nm trypsin was mixed with 10–50 nm α_{PI} Pittsburgh in 10 mM sodium phosphate, pH 7.40, 2 mM EDTA, 0.1 mM NaCl, 0.1% polyethylene glycol 8000, containing 250 mM S-2222 chromogenic substrate. Product release was followed by change in absorbance at 405 nm. Curves obtained at different α_{PI} concentrations were fitted by a single exponential function with Sigma-Plot software, and values for k₉ were plotted against [I]₀/1 + [S]₀/Kᵢ, where [I]₀ and [S]₀ are the initial concentrations of α_{PI} and S-2222, respectively. The second-order rate constant was obtained from the slope.

The trypsin-P2 Pro → Gly α_{PI} Pittsburgh complex had very low stability. The rate of complex formation was therefore determined from SDS-PAGE analysis of the reaction products obtained under conditions where dissociation of the covalent complex was negligible (12). The α_{PI} Pittsburgh P2 Gly variant (0.2 μg) was mixed with trypsin (0.05 μg) at room temperature (22 °C) in 10 μl of assay buffer. Samples were removed at intervals from 3 to 80 s, the reaction was quenched by mixing immediately with 10 μl of preheated reduced SDS-loading buffer and heating for 2 min, and reaction products were immediately analyzed by 10–20% gradient Tris/glycine SDS-PAGE. Band intensities were determined by densitometry and converted to relative concentrations. The second-order rate constant was determined from the slope of a plot of time against log([I]₀/[I]ₑ), where I represents serpin and E is proteinase. The rate of thrombin inhibition by the P2G variant was determined by discontinuous assay. 25 nM thrombin was mixed with 250 nM P2G α_{PI} Pittsburgh, and the residual proteinase activity was determined at intervals by diluting the reaction mixture into assay buffer containing 75 μM S-2238 chromogenic substrate. Residual proteinase activity was plotted against time and analyzed by nonlinear least-squares fitting in Sigma-Plot software using the following equation: [E]₀ = [E]ₑe⁻k₉t. The second order rate constant, k₉, was determined by dividing k₉ by the initial α_{PI} concentration.

**Stoichiometries of Inhibition**—The stoichiometry of inhibition of α_{PI} Pittsburgh P2G variant for thrombin was determined by plotting residual proteinase activity against the serpin/proteinase ratio, after incubating the serpin (200–400 nM) with 200 nM thrombin in assay buffer at 25 °C and allowing the reactions to go to completion. Residual thrombin activity was determined by diluting the reaction mixture 100-fold with assay buffer containing 100 μM S-2238 and measuring the rate of substrate hydrolysis at 405 nm. The stoichiometry of inhibition of α_{PI} Pittsburgh for trypsin was determined analogously, except that residual activity of trypsin was measured by diluting the reaction mixture 500-fold into assay buffer containing 250 μM S-2222.

**RESULTS**

**Structure of the α_{PI}-Pittsburgh-S195A Trypsin Noncovalent Complex**—Although the serpin-proteinase reaction cannot readily be halted at the stage of the initial noncovalent Michaelis complex, this complex is thought to be closely approximated by the noncovalent complex formed between the dehydroalanine or S195A form of the proteinase and the serpin. We have determined the structure of such a complex formed between S195A trypsin and the serpin α_{PI} Pittsburgh (Fig. 1b).

The α_{PI} Pittsburgh RCL in the complex had a canonical conformation (Fig. 1b) similar to that found previously for noncomplexed wild-type serpin (13, 14). As expected, the active site of the proteinase interacted with the serpin RCL, centered on the P1–P1' scissile bond. No contacts were present between the serpin body and the proteinase, unlike the
noncovalent complex of another serpin, heparin cofactor II, with S195A thrombin. Surprisingly, considering the high stability of the $K_d$ of the $\alpha_1$PI Pittsburgh-S195A trypsin complex ($5 \text{ nM}$) (15), contacts between the serpin RCL and the proteinase are quite limited (Fig. 1c). Only the P1 arginine side chain has extensive interaction with the proteinase, being buried in the S1 specificity pocket. The remaining contacts involve hydrogen bonds between the backbone of serpin residues P2 to P2' with backbone atoms of the proteinase, in a manner almost identical to that of classical canonical serine proteinase inhibitors (16). This differs from an earlier structure of S195A rat trypsin with $Manduca sexta$ serpin 1B A353K variant, which showed some additional contacts between more distal parts of the RCL and surface loops of trypsin that were suggested to be important for binding (17). We do not, however, find such a difference for our complex, in which the interaction of the serpin with S195A trypsin is equivalent to that of BPTI, and consequently the serpin has an RCL conformation superimposable on that of the interaction loop of BPTI.

**Structure of Free $\alpha_1$PI Pittsburgh**—We also determined the x-ray crystal structure of native $\alpha_1$PI Pittsburgh and found minimal differences between the structure of the free serpin and the serpin in noncovalent complex with S195A trypsin. The serpin body in the two species overlaid with root mean square deviation of only 0.6 Å for 240 non-RCL Cα atoms. This lack of conformational change for both the serpin body and the trypsin moiety upon forming complex is what had been found previously from NMR measurements for the same complex (10, 11). Even within the RCL, the only significant differences were in the region where the loop joins the serpin body, distally from the scissile bond. Within P5 to P5' (Glu-Ala-Ile-Pro-Arg-Ser-Ile-Pro-Pro-Glu), however, which encompasses the whole of the region that interacts with S195A trypsin, there were almost identical backbone conformations in free and complexed states (Fig. 2). This RCL conformation was also very similar to that of
wild-type α1PI, which has previously been pointed out to have a canonical conformation (13, 14).

Comparison of RCL Conformations—Whether this same conformation of the RCL of α1PI Pittsburgh could also fit into the active site of thrombin without need for structural change was evaluated from a comparison of the present structure with the recent x-ray structure of the equivalent noncovalent complex of S195A thrombin with the thrombin-specific serpin heparin cofactor II (18). The corresponding P5–P5′ sequences of heparin cofactor II and (for later comparison) of antithrombin are Gly-Phe-Met-Pro-Leu-Ser-Thr-Gln-Val-Arg and Val-Ile-Ala-Gly-Arg-Ser-Leu-Asn-Pro-Asn, respectively. Although the P1 residue of heparin cofactor II is a leucine residue rather than arginine, it is nevertheless a good serpin for comparison with α1PI Pittsburgh, since, in addition to both serpins being thrombin inhibitors, both have P2 proline residues and P1′ serine residues, and the P1 leucine of heparin cofactor II occupies the S1 pocket of S195A thrombin in the same way that arginine in α1PI Pittsburgh does in our noncovalent complex with S195A trypsin. Superpositioning of the RCLs of α1PI Pittsburgh and heparin cofactor II, both from their noncovalent complexes with S195A proteinase, shows that the backbone conformations are almost identical in the region flanking P1, whereas for cleavage of thrombin, there is very much higher specificity, particularly at the P2 position (20). Thus, change of the P2 residue from Pro to Gly results in almost no change in rate for cleavage by trypsin but a 20-fold reduction in rate for cleavage by thrombin. To determine whether the same pattern of specificity is shown in the more constrained RCL of α1PI Pittsburgh, we created a P2 Pro → Gly variant of α1PI Pittsburgh and compared the rates of reaction of the P2 Pro and P2 Gly forms of the serpin with both trypsin and thrombin.

For reaction with trypsin, both α1PI variants gave similar rates of reaction, as was seen with peptide substrates (Table II). In addition, the absolute values of the rate constants were less than 1 order of magnitude lower than for the peptides. For reaction with thrombin, a similar relationship was observed between rates of peptide substrate cleavage and serpin reaction for these two α1PI variants. Thus, α1PI Pittsburgh (P2 Pro) reacted about 30-fold faster than the P2 Gly variant, which was similar to the equivalent peptide substrates (Table II). In addition, the absolute values of the rate constants showed only a slightly larger decrease between free peptide and serpin RCL reactions than did the reactions with trypsin.

Compared with the α1PI Pittsburgh analog, pentasaccharide-activated wild-type antithrombin (P2 Gly) reacts about as fast or faster with either trypsin or thrombin (Table II). The antithrombin RCL conformation in this heparin-bound form is therefore likely to require no more change in conformation to be able to bind to either of these proteinases than does α1PI Pittsburgh. Similarly, for the P2 Pro variant of antithrombin bound to heparin pentasaccharide, the rate of reaction with thrombin is almost the same as for the reaction of α1PI Pittsburgh, again implying equivalent minimal change in conformation upon binding. Whereas it is true that the rate of reaction of antithrombin with thrombin is lower in the absence of heparin pentasaccharide than in its presence, the reduction is only 1.6-fold for the wild-type and a little more (6-fold) for the P2 Pro variant (Table II).
DISCUSSION

Inhibition of serine proteinases by serpins involves initial noncovalent binding of the proteinase to the RCL of the serpin, followed by attack of the active site serine on the P1–P1' peptide bond of the serpin. The rate-limiting step for inhibition is typically formation of the initial Michaelis-like complex that involves docking of the proteinase active site with the serpin RCL (21). Based on the x-ray structures of α1-PI Pittsburgh, alone and in noncovalent complex with S195A trypsin presented here, and on previous structures of wild-type α1-PI, we can conclude that the RCLs of these serpins are already in a preferred, canonical conformation to interact with trypsin or elastase, respectively, and form the initial, rate-determining Michaelis-like complex. This may account both for the high affinity of the noncovalent complexes and the very high rates of reaction with active trypsin and elastase, for the Pittsburgh and wild-type proteins, respectively.

Although we did not determine the structure of the noncovalent complex of α1-PI Pittsburgh with S195A trypsin, the availability of such a structure for another serpin, heparin cofactor II, allowed us to compare the RCL conformations of heparin cofactor II in complex with S195A thrombin with that of α1-PI Pittsburgh in complex with S195A trypsin. Based on a near identity of conformation of the two RCLs, it is expected that α1-PI Pittsburgh could also form an optimal, canonical noncovalent complex with thrombin without need for change in conformation of the RCL compared with that in the free state. In contrast, the conformation of the antithrombin RCL from published x-ray structures is noncanonical and consequently would not fit into the active site of thrombin without major distortion. This would presumably require significant energy and consequently result in a major rate reduction for formation of this complex compared with its formation by α1-PI Pittsburgh. That this is not the case, either for P2 Gly or P2 Pro forms of antithrombin compared with the equivalent α1-PI Pittsburgh species, strongly suggests that the solution conformation of the antithrombin RCL around the scissile bond is not like that in the crystal structure but is already like that of α1-PI Pittsburgh and hence requires little further conformational change to bind to thrombin.

Our kinetic results also suggest that, for α1-PI Pittsburgh, the 30-fold reduction in rate upon replacement of proline at P2 by glycine is not a result of conformational differences, since the free peptides show a similar rate reduction. Since the rate of reaction of α1-PI Pittsburgh with thrombin is only 6-fold lower than with trypsin, it is likely that the RCL conformations are the same as those found for the complexes of HCII with S195A thrombin and α1-PI Pittsburgh with S195A trypsin and that the
modest rate reduction results from the need to bring about the same kind of displacement of the 60 and 150 loops of thrombin as was seen in the HCII-S195A thrombin noncovalent complex. Taken together, these rate correlations imply that the RCL of \( \alpha \)-PI Pittsburgh, with either P2 Pro or P2 Gly, has the same canonical conformation in complexes with S195A trypsin and S195A thrombin and that differences in rates of reaction result from changes in P2 side chain contacts in thrombin and from the need to displace the 60 and 150 loops of thrombin to accommodate the serpin RCL.

A similar conclusion can be drawn with regard to pentasaccharide-activated antithrombin, thus providing an answer to the question of why \( \alpha \)-PI Pittsburgh is a faster inhibitor of thrombin than pentasaccharide-activated antithrombin. Since the 15-fold increase in the rate of inhibition of thrombin by pentasaccharide-activated antithrombin primarily because it has a proline at P2 rather than the glycine that is present in antithrombin.

For antithrombin in the absence of activating heparin, the small reductions in the rate of reaction with thrombin are unlikely to result from major conformational differences between pentasaccharide-activated and native antithrombin in the vicinity of the scissile bond but may result from the greater restraint placed on the RCL in the native state by the anchoring of two RCL residues, P14 and P15, in the \( \beta \)-sheet A. Indeed, a fluorescence lifetime study of a \( N-(2\text{-acetoxy})\text{ethyl-N-methyl} \)amino-7-nitrobenz-2-oxa-1,3-diazole fluorophore covalently bound to cysteine introduced at P1 as a replacement for arginine showed that the fluorophore experiences a similarly solvent-exposed environment in heparin-free and heparin-bound forms of antithrombin (22). The fluorescence enhancement that the \( N-(2\text{-acetoxy})\text{ethyl-N-methyl} \)amino-7-nitrobenz-2-oxa-1,3-diazole fluorophore undergoes upon heparin binding results from a dequenching of about 50% of the \( N-(2\text{-acetoxy})\)
ethyl-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole, probably as a result of movement of the RCL outward from the serpin body, but without need for a change in backbone conformation at and near to the P1-P1’ bond.

Significance of Canonical Binding of Serpins—Our finding that α1P1 Pittsburgh interacts with S195A trypsin analogously to true canonical inhibitors, as had been predicted by Elliott and colleagues (13, 14), and the further demonstration that this mode of interaction is also used in two other noncanonical serpin-proteinase complexes, those of heparin cofactor II with S195A thrombin and M. sexta serpin 1B with S195A rat trypsin, raises the question of what makes serpins different from canonical inhibitors. The present findings show that the difference is not due to a fundamental difference in the conformation of the RCLs presented to proteinase. In addition, since proteolytic cleavage of the RCL of canonical inhibitors can take place, cleavage versus noncleavage is not per se a fundamental difference between these types of inhibitor. Instead, the difference appears to be in the position of the equilibrium between the cleaved and intact forms of the inhibitor. With classical canonical inhibitors, the equilibrium constant between intact and RCL-cleaved forms is close to 1 at physiological pH, demonstrating that there is little if any energy difference between these two states as a result of the constraints placed on the RCL. In serpins, however, cleavage of the RCL at the acyl enzyme intermediate stage removes the physical constraint that maintains the RCL as a fully exposed loop at the “top” of the molecule. Since the serpin conformation in which the cleaved RCL has inserted into β-sheet A is very much more stable than that in which it remains exposed, there is an enormous thermodynamic push toward the loop-inserted state, once the acyl intermediate has been formed. That some of this energy is used to “crush” the proteinase and hence trap it kinetically is a consequence of the evolution of the complete serpin mechanism. However, it should not detract from the realization that serpins, in their initial interaction with the proteinase, even up to acyl enzyme formation, are behaving as canonical inhibitors.

This conclusion has relevance to the question of how the serpin inhibitory mechanism may have evolved, since the mechanism imposes severe requirements on the serpin for the mechanism to work efficiently. First, the serpin must fold into a metastable state, such that the loop insertion that follows reactive center loop cleavage is highly favorable energetically. Such metastable folding of proteins is extremely rare. Second, the rate at which loop insertion occurs must be fast enough that translocation of the proteinase and subsequent “crushing” of the proteinase must occur fast enough to compete with the alternative of simple hydrolysis of the peptide bond. Inappropriate residues within the hinge of the RCL that might slow down insertion or too slow an opening of β-sheet A to accommodate the inserting RCL would be sufficient to inactivate the mechanism or greatly reduce its effectiveness. It is hard to imagine that all of the structural requirements needed by a serpin to make it work properly came about simultaneously. Instead, serpins more likely evolved by a progressive incorporation of features of this mechanism. It is possible that a primordial serpin, which may have originally had an alternative function, had an exposed loop that could interact with a protease but was not constrained enough to inhibit it efficiently. Mutations both within the hinge of the loop and within and under β-sheet A may have promoted partial insertion of the loop, which would have reduced the conformational freedom of the loop and perhaps made it into a better canonical inhibitor (indeed, there is precedent for such partial insertion in antithrombin). Subsequent changes within the serpin body may have allowed further insertion upon cleavage of the scissile bond and therefore the start of movement of the proteinase, perhaps resulting in a partial compromise of activity. Gradually, full insertion might have evolved to more effectively compromise the proteinase. If all serpins thus started out more like canonical inhibitors with little or no ability to loop-insert into β-sheet A, it may be that some serpins whose RCLs are still not able to insert may be like these primordial serpins and actually be inhibitors of the canonical type. This may be especially true if residues within the RCL restrict conformational freedom. A possible example is pigment epithelium-derived factor, which appears incapable of facile loop insertion upon RCL cleavage but has a well exposed RCL that contains rigidifying prolines at positions P10, P8, P4, and P3’ (23).

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