Insight into the Mechanism of Serpin−Proteinase Inhibition from 2D [^{1}H-^{15}N] NMR Studies of the 69 kDa α_1-Proteinase Inhibitor Pittsburgh−Trypsin Covalent Complex†

Francis C. Peterson and Peter G. W. Gettins*

Department of Biochemistry and Molecular Biology, M/C 536, College of Medicine, University of Illinois at Chicago, 1853 West Polk Street, Chicago, Illinois 60612

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ABSTRACT: We have used [^{1}H-^{15}N]-HSQC NMR to investigate the structural changes that occur in both serpin and proteinase in forming the kinetically trapped covalent protein−protein complex that is the basis for serpin inhibition of serine proteinases. By alternately using ^{15}N-alanine specifically-labeled α_1-proteinase inhibitor (α_1PI) Pittsburgh (serpin) and bovine trypsin (proteinase), we were able to selectively monitor structural changes in each component of the 69 kDa complex. Residue-specific assignments of four alanines in the reactive center loop and seven other alanines aided interpretation of the spectral changes in the serpin. We found that the majority of the alanine resonances, including those from reactive center loop residues P12, P11, and P9, were at identical positions in covalent complex and in cleaved α_1PI. Five alanines that are close to the contact region with proteinase showed some chemical shift perturbation compared with cleaved α_1PI, indicating some degree of structural deformation. With ^{15}N label in the proteinase, an HSQC spectrum was obtained that more closely resembled that of a molten globule, suggesting that the structure of the proteinase had been significantly altered as a result of complex formation. Large increases in line width for all α_1PI resonances in the covalent complex, with the sole exception of two residues in the flexible N-terminal tail, indicate that, unlike the noncovalent α_1PI−anhydroproteinase complex, the covalent complex is a rigid body of effectively increased molecular weight. We conclude that the mutual perturbations of serpin and proteinase result from steric compression and distortion, rather than simple contact effects. This distortion provides a structural basis for the greatly reduced catalytic efficiency of the proteinase in the complex and hence kinetic trapping of the covalent reaction intermediate.

Serpins are a most unusual family of protein proteinase inhibitors that employ a unique mechanism to inhibit their serine proteinase targets. Each contains an exposed reactive center loop consisting of approximately 20 residues within which is an appropriate substrate recognition site for the target proteinase. Rather than forming a thermodynamically stabilized noncovalent complex with the serpin, however, the proteinase attacks the P1−P1′ [1] bond within the reactive center loop (*2*). Upon formation of the acyl enzyme intermediate, and hence cleavage of the peptide bond, a major conformational change occurs within the serpin that involves insertion of the reactive center loop into β-sheet A of the serpin and translocation of the covalently bound proteinase toward the distal end of β-sheet A (*3*, *4*). The resulting complex thus chemically represents an intermediate on the normal substrate cleavage pathway, but one that, as a result of the conformational changes, deacylates ~6−8 orders of magnitude slower than in a normal substrate reaction (*5*). The proteinase has thus been *kinetically* trapped. The critical question for understanding the mechanism of proteinase inhibition is therefore how this dramatic reduction in catalytic efficiency of the proteinase has been effected.

We have previously used fluorescence, including fluorescence resonance energy transfer, to localize the proteinase trypsin in covalent complex with the serpin α_1-proteinase inhibitor (α_1PI) Pittsburgh [a natural variant in which the P1 residue has been changed from methionine to arginine (*6*)], and shown that it is at the "bottom" of the serpin with respect to the location of the reactive center loop ("top") in the native serpin (*7*). A similar placement of proteinase has more recently been demonstrated by fluorescence energy transfer for other serpin−proteinase pairs (*8*), suggesting that this aspect might be a common feature of kinetically trapped serpin−proteinase complexes. The limitation of fluorescence, however, for providing a detailed structural explanation for the kinetic trap is that it uses a single reporter group with consequent limited ability to report on other than local structural changes. X-ray crystallography or NMR spectros-

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* Correspondence should be addressed to this author. Phone: 312 996 5534; FAX: 312 413 0364; E-mail: pgettins@tigger.uic.edu.

[1] Abbreviations: HSQC, heteronuclear single quantum correlation; BPTI, bovine pancreatic trypsin inhibitor; SBTI, soybean trypsin inhibitor; α_1PI, α_1-proteinase inhibitor; P1, P4, etc., designation of residues in the reactive center loop, using the nomenclature of Schechter and Berger (*1*) in which the scissile bond is between residues P1 and P1′; residues N-terminal to this are designated P2, P3, etc. and those C-terminal P2′, P3′, etc.

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copy represent the only real ways of obtaining the necessary structural details for the whole complex. The former approach had previously been handicapped by the difficulty of obtaining crystals of the complex, but was recently successful with a nearly identical serpin−proteinase pair to that examined here (*9*). We chose NMR spectroscopy as a sensitive and information-rich means of looking at serpin−proteinase complexes in solution that might be fairly generally applicable to a range of serpin−proteinase pairs. Since it is a solution method, it has the capability of examining conformational heterogeneity and dynamics as well as more straightforward structural changes. We have recently demonstrated the utility of this approach on a noncovalent α_1-PI−anhydrotrypsin complex, showing that HSQC NMR can readily distinguish between loop-inserted and nativelike structures for the serpin in the complex and also provide information both on conformational change within the proteinase and on motional freedom of the component proteins in the complex (*10*). We report here the application of 2D [^{15}N-^{1}H] NMR to a structural examination of ^{15}N-alanine-labeled α_1-proteinase inhibitor (Pittsburgh variant)−bovine trypsin complexes, where the ^{15}N label was incorporated either into the serpin or into the proteinase, thereby providing information on each component. The results are discussed in light of the recent X-ray structure of an almost identical complex (*9*).

MATERIALS AND METHODS

Protein Expression. Recombinant bovine trypsinogen was expressed in high yield in *E. coli* as inclusion bodies and refolded after guanidine hydrochloride resolubilization, as described in the preceding paper (*18*). A D189S background was used rather than wild-type, since this had been reported to have lower substrate reactivity (*11*) and was found, in our hands, to give a covalent complex with α_1PI Pittsburgh that deacylated at least 10 times slower than that formed with wild-type trypsin, but was otherwise normal. This was advantageous for long data acquisitions used for the covalent complexes to minimize decomposition of the complex through deacylation. For production of ^{15}N-alanine-labeled trypsinogen, 100 mg/L ^{15}N-labeled alanine was added to the growth medium, which consisted of M9 minimal medium supplemented with 100 mg/L of all other amino acids. Trypsin was prepared from recombinant trypsinogen by activation with highly purified porcine enterokinase (Sigma).

Recombinant α_1PI Pittsburgh was also expressed in *E. coli* as inclusion bodies and purified and refolded as previously described (*10*). The background was the multi 9 variant that we have previously used for NMR studies, which comprises seven mutations that have been found to stabilize the native conformation with respect to polymerization, but not otherwise to alter the inhibitory rate or efficiency of the serpin (*12*), as well as the change of P1 from methionine to arginine to permit more efficient complex formation with trypsin, and of the single cysteine at position 232 to serine to prevent disulfide-mediated aggregation. The last two changes have also been shown not to alter the fundamental serpin inhibition mechanism of formation of SDS-stable covalent complexes with target proteinase (*4*). In addition, a reversion of one of the seven stabilizing mutations was made to reintroduce a reporter alanine at position 70. Variants used for assignment of reactive center loop alanines have been described previously (*10*) and involved single and double changes of alanine to serine and comparison of the resulting HSQC spectrum with that of the normal protein.

Sample Preparation. Cleaved α_1PI samples were prepared by dissociation of the covalent complex formed between ^{15}N-labeled α_1PI Pittsburgh and β-trypsin through raising the temperature to 75 °C for 10 min. This exploits the extremely high thermal stability of the cleaved form of serpins (T_m > 120 °C) (*13*), so that the trypsin moiety, once dissociated, can be removed by thermal denaturation and precipitation. Covalent complexes of α_1PI Pittsburgh and recombinant D189S trypsin were generated by addition of a small excess of serpin (20% molar excess) over proteinase zymogen, in situ activation of the zymogen with enterokinase, and subsequent purification of the covalent complex by HPLC using a chromatofocusing column. The reactions were carried out in 25 mM Bis-Tris at pH 6.25; 5 units of enterokinase (Sigma) was added to the mixture of α_1PI and trypsinogen and reacted for 4 h at room temperature. The mixture was run over a monoP column at a flow rate of 1 mL/min. Native and cleaved α_1PI bound to the column, whereas the covalent complex passed through. The sample was dialyzed into NMR buffer and concentrated. For covalent complexes where the ^{15}N label was in the trypsin, a 15% excess of unlabeled α_1PI was added to labeled trypsinogen in NMR buffer and the proteinase activated in situ by addition of 5 units of enterokinase. The reaction was allowed to proceed at room temperature for 4 h. The sample was then concentrated.

[^{1}H-^{15}N]-HSQC NMR. All NMR experiments were performed on a Bruker DRX600 spectrometer equipped with a 5 mm (^{1}H/^{15}N/^{13}C) triple resonance probe and pulse field gradient capability. All NMR spectra were collected at 310 K, unless otherwise noted, in 90% H_2O/10% D_2O, 20 mM sodium phosphate, pH 6.0, and 150 mM sodium chloride. Protein concentrations and data accumulation times are as indicated in the figure legends. NMR data were processed using Tripos 6.3 software (Tripos, Inc., St. Louis, MO).

RESULTS

Assignments of Resonances from Alanine Residues in Native and Cleaved α_1PI. We have previously made residue-specific assignments for the four reactive center loop alanines of α_1PI (P12, P11, P9, and P4; residues 347, 348, 350, and 355, respectively) as well as the two alanines close to the N-terminus (residues 7 and 8), in both the native and reactive center loop-cleaved forms of α_1PI (*10*). This showed that, whereas the two N-terminal alanines, which have high mobility, are insensitive to the major conformational change that occurs when the reactive center loop is cleaved and inserts into β-sheet A as an additional, central, antiparallel strand, the four reactive center loop alanines all experience large changes in both ^{1}H and ^{15}N chemical shifts, consistent with a marked change in the exposure and hydrogen bonding of their backbones (Table 1) (Figure 1). In our examination of HSQC spectra of the covalent complex of α_1PI with trypsin (see below), we found four other, nonreactive center loop alanine resonances that were significantly perturbed compared to the spectrum of cleaved α_1PI. To permit more definitive interpretation of these perturbations in terms of structural changes to the serpin moiety in the covalent complex, we made additional single site mutants of α_1PI,

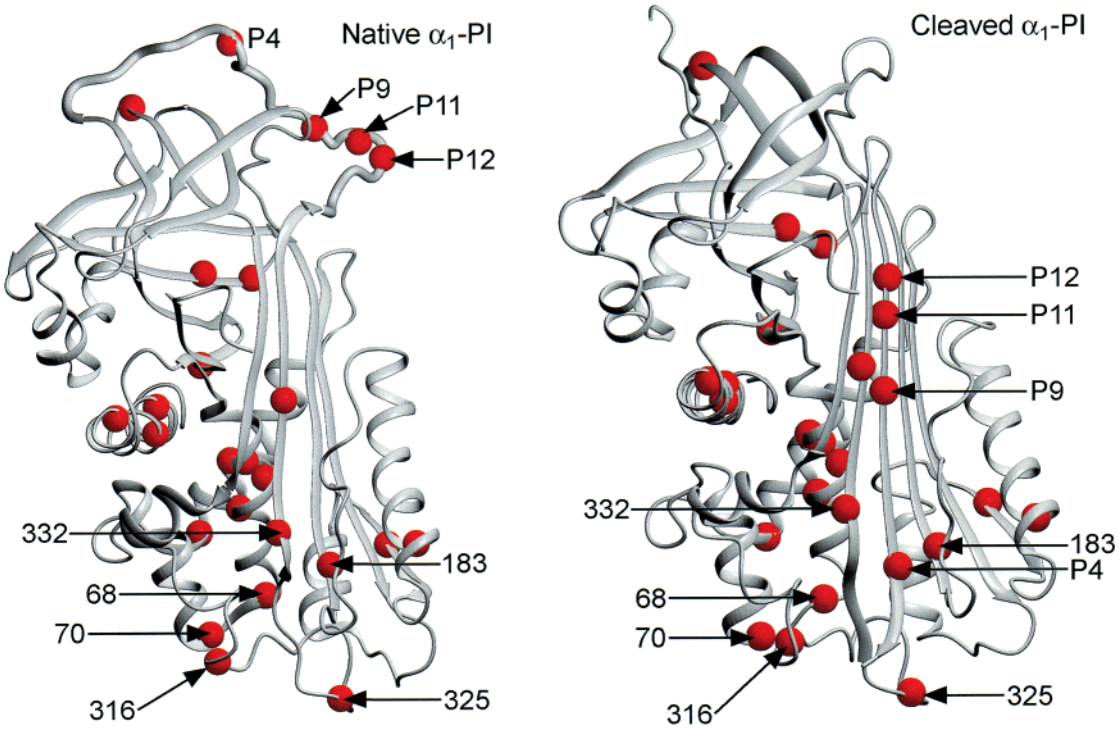

FIGURE 1: Ribbon representations of α_1PI in native and cleaved states with alanine backbone amides shown as spheres. Left panel, native α_1PI (pdb file 1atu); right panel, cleaved α_1PI (pdb file 7api). The alanines whose NMR resonances were assigned by mutagenesis are numbered (with the exception of residues 7 and 8, which do not give visible electron density). Note the dramatic change in location and exposure of the four reactive center loop alanines, P12, P11, P9, and P4, between the two structures. The use of the designations "top" and "bottom" in the text relates to the orientation of α_1PI depicted here.

Table 1: ^{1}H and ^{15}N Chemical Shifts of Assigned Alanines in α_1PI Pittsburgh

	^{15}N chemical shift (ppm)				^{1}H chemical shift (ppm)			
residue	native	NCC	cleaved	CC	native	NCC	cleaved	CC
7 & 8[a]	122.0	122.0	122.0	121.9	8.12	8.17	8.13	8.13
7 & 8[a]	123.7	123.7	123.7	123.7	8.13	8.18	8.13	8.14
P4 (355)	125.7	127.2	129.7	127.7	8.23	8.27	8.27	8.16
P9 (350)	123.2	123.2	119.6	119.6	8.04	8.08	9.31	9.32
P11 (348)	123.4	123.2	118.9	118.8	8.21	8.26	9.03	9.04
P12 (347)	125.0	124.6	128.4	128.4	8.25	8.26	9.84	9.86
68	121.5	121.7	121.7	121.8	7.41	7.50	7.45	7.44
70	123.0	nm[b]	123.1	123.1	8.77	nm	8.82	8.82
183	nv[c]	nv	121.5	122.3	nv	nv	9.64	9.55
316	124.1	124.1	124.0	123.4	7.53	7.62	7.26	7.00
325	120.2	120.1	117.7	116.0	7.34	7.41	6.92	6.86
332	129.9	130.1	124.3	123.4	9.69	9.76	9.25	9.08

[a] Alanines 7 and 8 were assigned as an undifferentiated pair. Their high mobility makes them insensitive to structural or dynamic changes in the rest of the serpin. [b] nm: not measured (the G70→A mutation was only made for examination of the covalent complex). [c] nv: not visible.

with change of individual alanines to glycine (residues 183 and 332) or serine (residues 316 and 325), and recorded HSQC NMR spectra of their native and cleaved forms to give additional residue-specific assignments.

To minimize the number of variants that had to be made and expressed, we selected alanines to replace based on our earlier model of the serpin−proteinase complex derived from fluorescence measurements (*7*). Accordingly, alanines 183, 316, 325, and 332 were mutated, based on their location in the bottom part of β-sheet A or in a loop at the bottom of the serpin, and therefore possibly sensitive to structural changes caused by the proteinase in the body of the serpin. Alanines at 68 and 70 (the latter was introduced as a glycine-to-alanine mutation) were additionally chosen as being surface residues that might be close to the interface with trypsin in the covalent complex, if our earlier positioning of the proteinase were slightly in error (Figure 1).

Consistent with their surface location, alanines 68 and 70 were almost insensitive to loop insertion, giving both ^{1}H and ^{15}N chemical shifts that were unchanged within experimental error between native and cleaved states (Table 1, Figure 2A,B). In contrast, alanines 316, 325, and 332 showed mostly large changes in both ^{1}H and ^{15}N chemical shift between native and cleaved states (Table 1, Figure 2A,B). The largest changes were for residue 332, for which there was a difference of 0.44 ppm in ^{1}H chemical shift and 5.6 ppm in ^{15}N chemical shift. This residue is in strand 5 of β-sheet A and so experiences a major change in environment upon insertion of the cleaved reactive center loop adjacent to it. The smaller ^{15}N chemical shift change for alanine 316 is consistent with its loop location at the bottom of the serpin. Alanine 183 was only visible in the spectrum of cleaved α_1PI, indicating possible exchange broadening in the native protein, but also thus showing sensitivity to cleavage and loop insertion of the reactive center loop.

Structural Changes in α_1PI upon Covalent Complex Formation. A 1:1 covalent serpin−proteinase complex was made between ^{15}N-alanine-labeled α_1PI Pittsburgh and unlabeled recombinant bovine trypsin, and its HSQC NMR spectrum was recorded. All 27 alanines present gave observable, relatively broad resonances with good overall dispersion in both the ^{15}N and ^{1}H dimensions. Comparison with the spectrum of the same serpin in the uncomplexed native state (Figure 2C) shows an almost complete mismatch between the resonance positions in the two spectra. Even with only

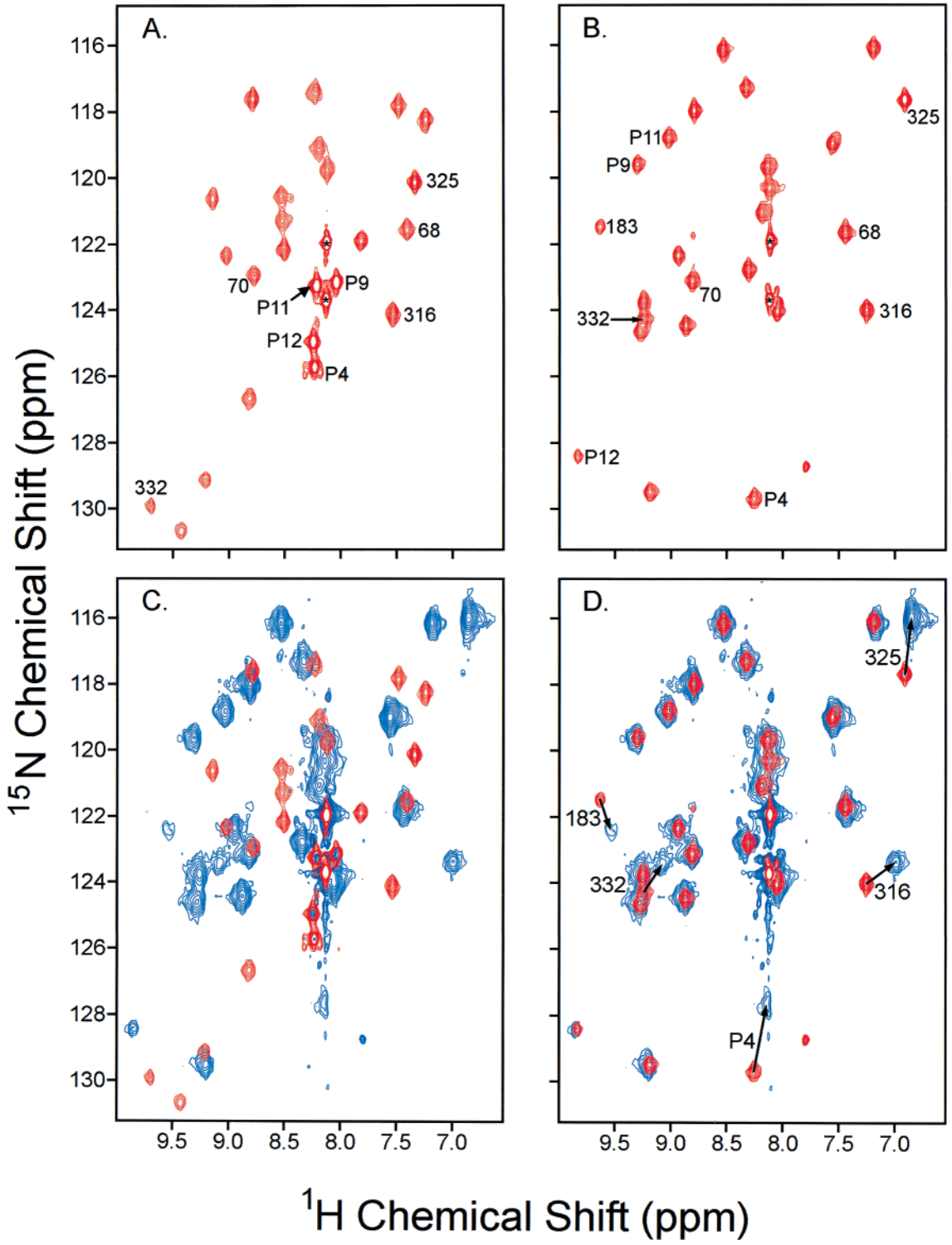

FIGURE 2: [^{15}N-^{1}H]-HSQC NMR spectra of native, cleaved, and covalently complexed α_1PI Pittsburgh, with ^{15}N-alanine labeling only of the α_1PI. (A) Native α_1PI (1 mM). (B) P1-cleaved α_1PI (1 mM). (C) Covalent complex of α_1PI with recombinant bovine trypsin (1 mM) (blue spectrum) overlaid by the spectrum of native α_1PI (same as panel A). (D) Covalent complex of α_1PI with recombinant bovine trypsin (1 mM) (blue spectrum) overlaid by the spectrum of cleaved α_1PI (same as panel B). In panels A and B, all assigned alanines are marked. Those from the reactive center loop are given their Schechter and Berger (*1*) numberings, P12, P11, P9, and P4, while the remaining resonances are given their number in the primary structure. Alanines 7 and 8 were assigned as an undifferentiated pair and are each indicated by an asterisk. In panel D, the most likely assignments of the five alanines that shift between spectra of cleaved and complexed α_1PI are indicated, together with arrows linking their position in each spectrum. Spectra were recorded at 310 K in pH 6.0 phosphate buffer.

limited assignments for the nonreactive center loop resonances, the fundamental difference in appearance of the two spectra indicates that the conformation of the serpin in the covalent complex is very different from that in the native state.

In contrast to the mismatch between spectra of covalent complex and native α_1PI, there is very close coincidence in resonance positions between spectra of covalent complex and cleaved α_1PI (Figure 2D). For the nonreactive center loop resonances, 19 out of 23 are in identical positions, whereas

Table 2: Line Widths of Assigned Alanine Residues

residue	native (Hz)[a]	cleaved (Hz)[a]	covalent complex (Hz)[a]
7 & 8	18	18	18
7 & 8	18	18	24
68	36	30	48
70	36	24	54
183	nv[b]	24	60
316	30	24	54
325	30	24	42
332	36	30	48
P4	24	36	48
P9	18	30	48
P11	18	36	54
P12	18	30	48

[a] Digital resolution limited accuracy of line width measurements to ±3 Hz. [b] nv: resonance not visible in the spectrum of the native protein.

for the 4 reactive center loop alanines, those at positions P12, P11, and P9 gave resonances at identical chemical shifts in the spectra of complexed and cleaved α_1PI, with only the resonance from P4 not being coincident in the 2 spectra. Reasonably confident assignments can be made for the five alanines in the covalent complex that are not at identical chemical shifts to cleaved α_1PI, based on the relatively small chemical shift differences between each of these resonances and a resonance in cleaved α_1PI (Figure 2D). Any alternative set of assignments would require much larger shift perturbations for at least two of the alanines, and so is considered much less likely.

As had been hoped, the five resonances that were at slightly different chemical shifts in the spectra of cleaved and covalently complexed α_1PI were all from alanines that we had assigned by mutagenesis, and which are located at the bottom of the serpin (Figure 1B). The largest difference in chemical shift was for the P4 alanine, with an ^{15}N $\Delta\sigma$ of 2 ppm. The other four alanines had differences in ^{15}N chemical shift between 0.6 and 1.7 ppm. Differences in ^{1}H chemical shift were between 0.06 and 0.26 ppm (Table 1). Significantly, none of the other three reactive center loop alanines (P12, P11, and P9) showed any differences in chemical shift between cleaved and complexed states, despite each of these alanines having chemical shifts that are very sensitive to loop insertion itself (see above). The surface alanines 68 and 70 also showed no difference between cleaved and complexed states. Although these residues are not sensitive to loop insertion, they should have been perturbed by contact with proteinase if they were located within the α_1PI−trypsin interface.

Mobility Changes in α_1PI upon Cleavage and Covalent Complex Formation. Although we did not carry out detailed relaxation time measurements of alanine resonances from α_1PI in native, cleaved, and complexed states, we can nevertheless make useful conclusions based on very large changes in line width and/or peak intensity seen for some of the resonances. In the native state, all resonances have similar intensity and/or line width with the exception of the four reactive center loop alanines and residues 7 and 8, consistent with the body of the protein behaving as a rigid entity. Values are given in Table 2 for the assigned resonances, though it can be seen from Figure 2A that the line widths for other nonassigned residues appear to be similar. In contrast, both the N-terminal alanine pair 7 and 8 and the four alanines from the reactive center loop (P12, P11, P9, and P4) all have very high intensity and narrow line width, consistent with a much higher degree of motional freedom. While this is expected for residues 7 and 8, which are likely to be in a mobile region of the protein, since the region up to residue 22 is not visible in the crystal structures of either native or cleaved forms (*14*, *15*), it is not necessarily expected for the reactive center loop, which the X-ray structure shows to be in extended β-conformation, with a potentially strong salt bridge between the P5 glutamic acid (residue 354) and an arginine in the body of the serpin (residue 196) that anchors the reactive center loop to the serpin core. Our data in solution suggest, however, a very mobile, untethered reactive center loop in native α_1PI.

Upon formation of the cleaved, loop-inserted form of the serpin, the nonreactive center loop alanines showed no significant change in line width (Figure 2B), within experimental error, consistent with their location in the body of the serpin, or in the mobile N-terminal tail, and the expected similar mobility of these regions compared with native α_1PI. In contrast, all four of the reactive center loop alanines changed line width and intensity from those characteristic of more mobile residues to those of the remainder of the serpin body. This is entirely consistent with the known changes in secondary structure of inhibitory serpins upon cleavage and loop insertion.

Formation of the covalent complex with trypsin resulted in the most widespread changes in line width. With the exception only of the alanines in the N-terminus, 7 and 8, all other resonances showed large increases in line width (Figure 2D), consistent with the covalent complex being a rigid body, with larger mass and size than the serpin alone, and with a corresponding increase of approximately 50% in line width (Table 2). This suggests that the proteinase in the complex must be held tightly against the serpin.

Changes in Trypsin upon Covalent Complex Formation. As described in the preceding paper (*18*), we have established a high-yield expression system for bovine trypsin that has allowed us to specifically incorporate ^{15}N-labeled alanine into trypsin and to obtain good-quality, well-resolved, and dispersed spectra of S195A trypsin, both alone and in noncovalent complexes with proteinase inhibitors, including α_1PI. An [^{15}N-^{1}H]-HSQC NMR spectrum of D189S/S195A trypsin (Figure 3B) showed the expected number of alanine resonances (*15*) while that of trypsinogen gave 13 of the 15 (Figure 3A). Although there are significant differences in chemical shift for a number of the alanines between the zymogen and activated forms of the proteinase, there is nevertheless good chemical shift dispersion in both ^{15}N and ^{1}H dimensions for both spectra. The original motivation for developing this system was to enable us to examine the proteinase in covalent complex with trypsin. Accordingly, we recorded an HSQC spectrum of covalent D189S trypsin−α_1PI complex, in which there was ^{15}N-labeling of alanines only in the trypsin moiety. Given the well-behaved nature of spectra of both trypsin and trypsinogen, as well as for the noncovalent complex of S195A with α_1PI shown in the preceding paper, the appearance of the spectrum of covalent complex was altogether unexpected (Figure 3C). With the exception of resonances at 119/7.2 ppm and 123/7.5 ppm, which are still clearly resolved, albeit with significantly increased line width, there is poor correspondence between the spectrum of the covalent complex and the spectrum of

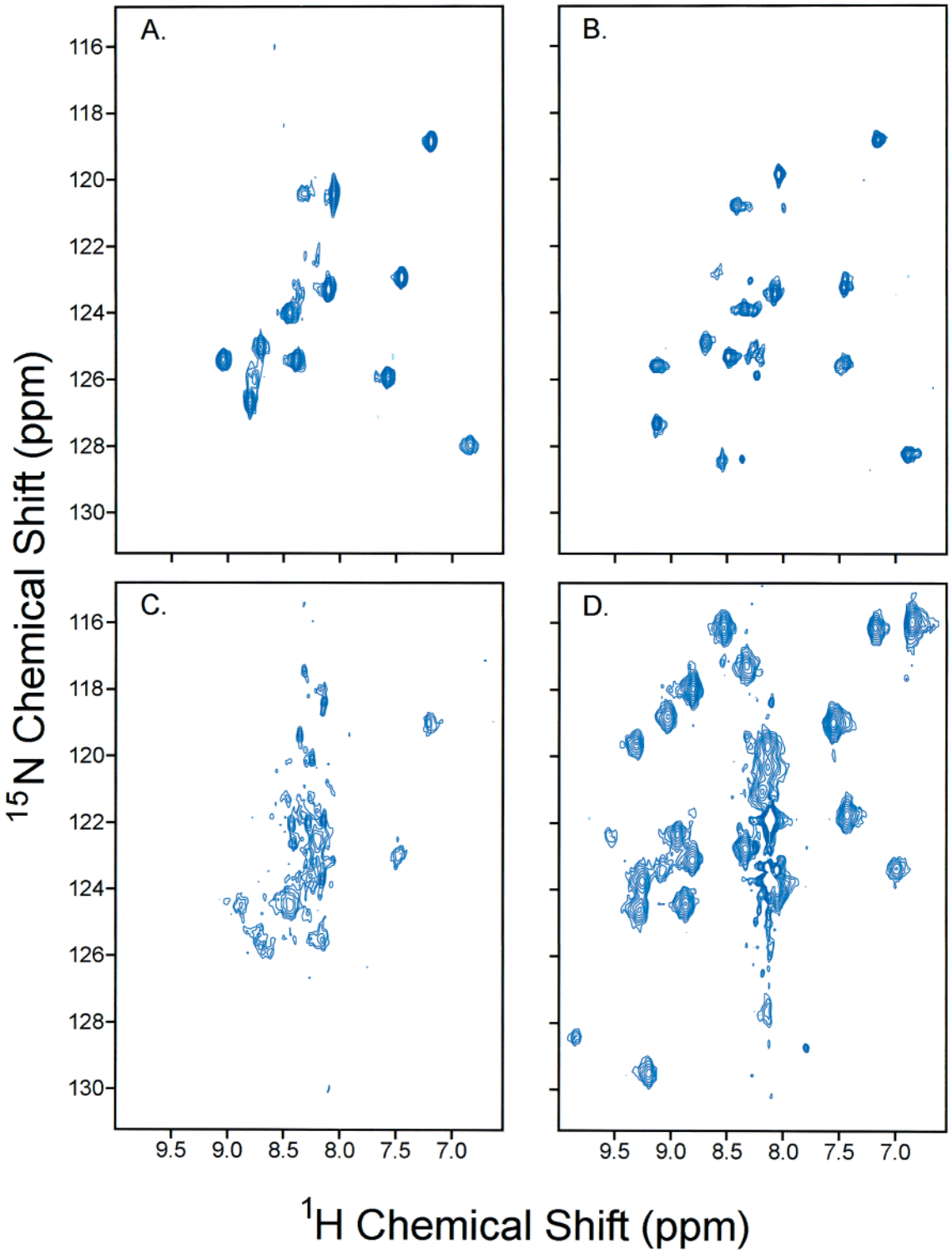

FIGURE 3: [^{15}N-^{1}H]-HSQC NMR spectra of trypsinogen, trypsin, and trypsin covalently complexed with α_1PI Pittsburgh, with ^{15}N-alanine labeling only of trypsin or trypsinogen (panels A−C) and, for comparison, spectrum of the same covalent complex as in panel C, but with ^{15}N-alanine only in the α_1PI (panel D). All samples were at 1 mM for the labeled species. Panel A, D189S/S195A trypsinogen; panel B, D189S/S195A trypsin; panel C, covalent complex of D189S trypsin with α_1PI Pittsburgh. All spectra were recorded at 37 °C and pH 6.0. The spectra in panels C and D were collected under similar conditions. Note the much lower intensity in panel C compared with panel D, and poorer spectral dispersion in panel C compared with panels A and B.

trypsin (Figure 3B), as well as a loss of expected signal intensity. Comparison to the spectrum of trypsinogen (Figure 3A) shows a closer resemblance, particularly the group of four peaks around 125/8.5 ppm, though even here there are clear absences of some of the trypsinogen resonances. To emphasize the difference in behavior of the serpin and proteinase components of the complex, the spectrum of the equivalent covalent trypsin−α_1PI complex with ^{15}N-alanine in α_1PI, and collected under the same experimental conditions, is shown in Figure 3D.

Because of the unusual appearance of the spectrum, we sought to determine if it arose from conformational interconversion on an intermediate NMR time-scale, by recording the spectrum at both higher and lower temperature. Spectra

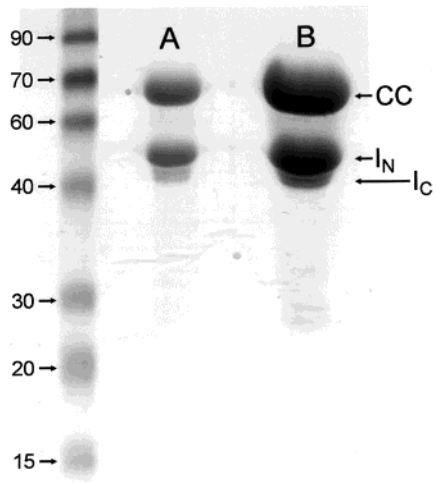

FIGURE 4: SDS−polyacrylamide gel of the covalent complex of α_1PI Pittsburgh with recombinant ^{15}N-alanine-labeled bovine trypsin. Lane 1, complex frozen immediately after generation and just preceding recording of the NMR spectrum in Figure 3C; lane 2, same sample >14 days after collection of the NMR spectrum, showing no change in the relative amounts of native, cleaved, and complexed α_1PI. The NMR sample was generated with a molar excess of α_1PI to ensure that there was no free trypsin present in the sample. Since ^{15}N-alanine is only present in the trypsin moiety, the presence of some native and cleaved α_1PI does not affect the NMR spectrum, since these species are essentially invisible at the natural abundance of ^{15}N in the serpin.

were also recorded at 4, 12, and 20 °C (data not shown). There was a further reduction in signal intensity due to slower molecular motion as the solution was cooled, but no discernible increase in dispersion at the expense of intensity from resonances in the central region of the spectrum, such as might have been expected if any exchange process had been slowed from intermediate to slow exchange conditions. To confirm that the trypsin in the complex was otherwise intact, i.e., that it had not been adventitiously cleaved by free trypsin, we ran an SDS gel of the complex after collection of the NMR spectrum and compared this with the same sample immediately after it had been formed. The NMR sample complex gave a single, well-defined band at the position expected for a covalent complex of mass equal to the sum of the masses of the proteinase and the serpin (Figure 4), confirming that the trypsin was unaltered, other than being covalently linked through its active site serine to the serpin. Although there are additional bands from cleaved α_1PI (very minor) and native α_1PI, these resulted from addition of excess α_1PI in the initial formation of the complex to ensure that there would be no free trypsin. Importantly, these do not show a time-dependent change such as would occur if the complex were being degraded over time (note that the presence of some native and cleaved α_1PI has no effect on the appearance of the NMR spectrum, since visible ^{15}N-alanine is only present in the trypsin, all of which is in covalent complex).

DISCUSSION

We have used ^{15}N NMR on residue-specific ^{15}N-labeled protein components to provide insight into the structure and dynamics of the covalent serpin−proteinase complex formed between α_1PI Pittsburgh and bovine trypsin and hence into the inhibition mechanism of the serpin. Although we have previously shown by fluorescence resonance energy transfer for the same α_1PI Pittsburgh−trypsin covalent complex that the trypsin is located at the bottom of the serpin, and consequently involves full insertion of the reactive center loop into β-sheet A, as shown in Figure 5, we were not able to use such fluorescence approaches to provide direct evidence for loop insertion, for the structure of the remainder of the serpin moiety, for the conformation of the proteinase, or for the rigidity of the complex. The NMR spectra described above have been able to address each of these issues and thereby illuminate the mechanism of proteinase inhibition by serpins. Consistent with the global placement of the proteinase at the bottom of the serpin by FRET, our NMR data have shown that the reactive center loop of the serpin is fully inserted into β-sheet A and that most of the remainder of the serpin has a conformation identical to that of cleaved α_1PI. From previous modeling of trypsin and cleaved α_1PI (*7*), we had found that the structure in Figure 5 involved steric clashes between surface loops of the trypsin and the bottom of the serpin. The present findings that five alanines in α_1PI in complex are at chemical shifts close to, but not quite coincident with, those of cleaved α_1PI is most easily explained as resulting from small distortions of the serpin moiety, restricted just to the bottom part of the serpin, but including residues of β-sheet A including P4 of the reactive center loop and alanines in the immediately flanking strand 3 (alanine 183) and strand 5 (alanine 332), to accommodate the proteinase. For the proteinase, the consequence of this "tight fit" is comparable distortion of its conformation that results in a spectrum that is more similar to that of either inactive zymogen or molten globule forms of trypsin than of active trypsin. In keeping with the expected rigidity of such a firmly interacting pair of proteins, the line widths of resonances in the complex (viewed from the serpin moiety) are uniformly and greatly increased over those for the free protein components. This contrasts with our findings for a noncovalent complex between α_1PI Pittsburgh and anhydrotrypsin in which no conformational change occurred in either serpin or "proteinase" and for which there was still considerable flexibility of the proteinase domain relative to the serpin, made possible by docking of the proteinase with a relatively mobile reactive center loop that was not significantly rigidified by complex formation (*10*). Our NMR results thus provide compelling evidence for a mechanism of kinetic trapping of trypsin by α_1PI in which full translocation of the covalently bound trypsin to the distal end of the serpin, at the stage of the acyl enzyme reaction intermediate, results in global distortion of the trypsin, with consequent abrogation of its catalytic activity. Other solution studies have also indicated structural alterations in the proteinase moiety, both in the active site (*13*, *16*) and more globally from alterations in stability and proteolytic susceptibility (*13*, *17*). Such a mechanism also satisfies the known requirement for a precise length for the reactive center loop, since the distortion-based inhibition mechanism requires compression of the proteinase, which must therefore be held close enough to the body of the serpin. Similarly, since the distortion is based on resolving steric clashes rather than matching complementary binding surfaces, it explains the promiscuity of serpins in their ability to inhibit proteinases of common specificity but different surface structure.

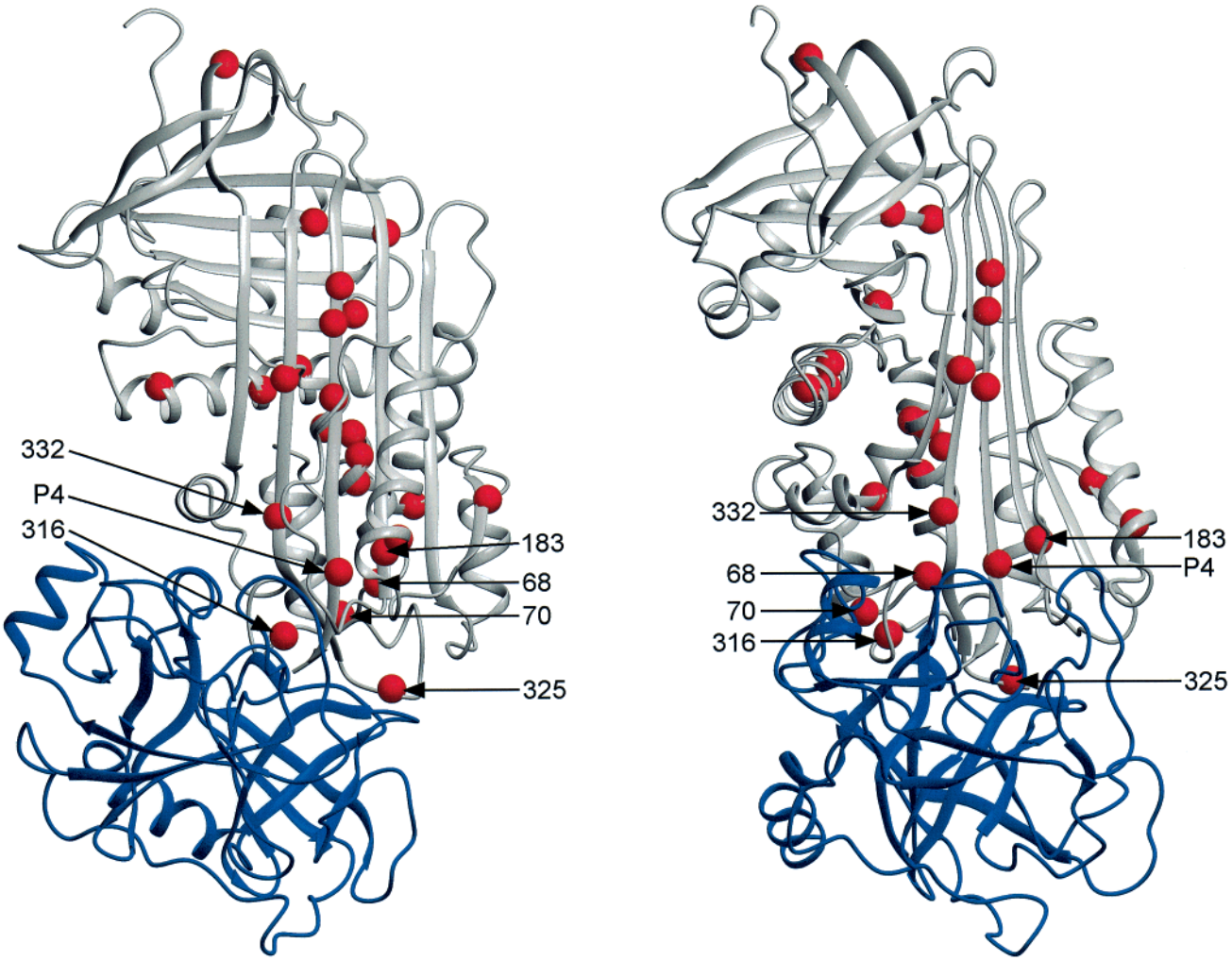

FIGURE 5: Model of the covalent complex of α_1PI and trypsin, based on our previous solution fluorescence resonance energy transfer measurements (*7*) and using the coordinates of cleaved α_1PI (7api) (*15*) and trypsin (1smf) (*19*). This model is very similar in placement of the serpin and proteinase to that found in the crystal structure of trypsin and α_1PI (pdb coordinate file 1ezx) (*9*). The latter does not include coordinates for 40% of the trypsin, which is invisible in the electron density map. The locations of assigned alanines in the bottom portion of the α_1PI are numbered.

Despite the fact that the present NMR approach is not one of complete three-dimensional structure determination, it is nevertheless a very sensitive method for addressing the specific questions of the conformation of serpin and proteinase and the location of the reactive center loop. The alanine HSQC resonances of the native protein are extremely sensitive to loop insertion, with large chemical shift changes even for alanines that are not part of the reactive center loop. The consequence of this is that for such a conformation-sensitive resonance to have identical chemical shift in the spectra of cleaved and covalently complexed serpin requires that the local environment of that alanine be essentially identical in both species. Conversely, any difference in chemical shift, such as was seen for alanines 183, 316, 325, and 332 and P4, implies that there must be some structural difference between cleaved and complexed states for these residues. What cannot be quantified is how such chemical shift perturbations correlate with specific structural differences. In this regard, it is worthwhile to compare the structures of cleaved α_1PI (*15*) and the α_1PI moiety in the recently published X-ray structure of the covalent α_1PI−trypsin complex (*9*). In the latter study, the overall rmsd of Cα atoms between the two structures was found to be 0.52 Å. However, this may not be uniformly distributed throughout the structure, and in addition any small differences, e.g., in hydrogen bond length within β-sheet A, might be too small to be detected at the resolution of the structure (2.9 Å). Our NMR findings, however, strongly suggest that, while the top two-thirds of the serpin are identical in cleaved and complexed states, the bottom portion of β-sheet A has been perturbed as a consequence of compression against the proteinase.

The strangest aspect of our results is the appearance of the spectrum of the trypsin moiety in the covalent complex. Whereas trypsin alone or in noncovalent complex with inhibitors of different sizes, up to that of α_1PI, gives well-resolved and dispersed HSQC spectra (*18*), the spectrum of covalently bound trypsin is very poorly dispersed for most resonances and appears more like that of a molten globule, hence explaining the failure of a reduction in temperature of 33 °C (from 37 to 4 °C) to improve the spectral dispersion. In the crystal structure of the complex, only 60% of the trypsin gave traceable electron density, and furthermore crystals could only be grown at 4 °C and dissolved if the temperature was raised. This suggests that the trypsin in complex may be able to sample many different conforma-

tions on a rapid time scale and for crystallization to be successful at least some of this (representing 60% of the structure) must be frozen out. This still leaves a significant fraction of the proteinase that is still plastic. While this plasticity may not be the direct cause of the loss of catalytic competence of the proteinase in the complex, it may be essential to allow suitable accommodation to occur between the interacting surfaces of the serpin and proteinase, thereby eliminating what would otherwise be steric clashes and hence allow different proteinases to be inhibited by the same serpin (e.g., thrombin and factor Xa by antithrombin).

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