Formation of a Noncovalent Serpin-Proteinase Complex Involves No Conformational Change in the Serpin. Use of ^{1}H-^{15}N HSQC NMR as a Sensitive Nonperturbing Monitor of Conformation†

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ABSTRACT: A structural understanding of the nature and scope of serpin inhibition mechanisms has been limited by the inability so far to crystallize any serpin−proteinase complex. We describe here the application of [^{1}H-^{15}N]-HSQC NMR on uniformly and residue-selectively ^{15}N-labeled serpin α_1-proteinase inhibitor (Pittsburgh variant with stabilizing mutations) to provide a nonperturbing and exquisitely sensitive means of probing the conformation of the serpin alone and in a noncovalent complex with inactive, serine 195-modified, bovine trypsin. The latter should be a good model both for the few examples of reversible serpin−proteinase complexes and for the initial Michaelis-like complex formed en route to irreversible covalent inhibition. Cleavage of the reactive center loop, with subsequent insertion into β-sheet A, caused dramatic perturbation of most of the NMR cross-peaks. This was true for both the uniformly labeled and alanine-specifically labeled samples. The spectra of uniformly or leucine- or alanine-specifically labeled α_1-proteinase inhibitor in noncovalent complex with unlabeled inactive trypsin gave almost no detectable chemical shift changes of cross-peaks, but some general increase in line width. Residue-specific assignments of the four alanines in the reactive center loop, at P12, P11, P9, and P4, allowed specific examination of the behavior of the reactive center loop. All four alanines showed higher mobility than the body of the serpin, consistent with a flexible reactive center loop, which remained flexible even in the noncovalent complex with proteinase. The three alanines near the hinge point for insertion showed almost no chemical shift perturbation upon noncovalent complex formation, while the alanine at P4 was perturbed, presumably by interaction with the active site of bound trypsin. Reporters from both the body of the serpin and the reactive center loop therefore indicate that noncovalent complex formation involves no conformational change in the body of the serpin and only minor perturbation of the reactive center loop in the region which contacts proteinase. Thus, despite the large size of serpin and serpin−proteinase complex, 45 and 69 kDa respectively, NMR provides a very sensitive means of probing serpin conformation and mobility, which should be applicable both to noncovalent and to covalent complexes with a range of different proteinases, and probably to other serpins.

Serpins are members of a large and growing superfamily of structurally related proteins, most of which are proteinase inhibitors (*3*). An indication of their abundance is the identification of 35 serpins, mostly new, in the recently published *Drosophila* genome (*4*), which is less than 1/20th the size of the human genome. Members of this superfamily are widely distributed throughout nature and are found in eukaryotic organisms, plants, parasites, and viruses (*3*). In humans, serpins are found both extra- and intracellularly and are responsible for the regulation of essential proteolytic cascades which control coagulation, fibrinolysis, inflammation, and the immune response and are involved in apoptosis. Disruptions of normal serpin function within humans result in disease pathologies which include thrombosis, bleeding disorders, emphysema, and liver disease.

Serpins primarily function as inhibitors of serine proteinases, though some can also inhibit cysteine proteinases (*5*). Based largely on crystal structures of complexes, the mechanism of serine proteinase inhibition by members of the Kunitz, Kazal, and Bowman-Birk families of inhibitors is well understood as a rigid-body docking of the proteinase with the reactive center loop of the inhibitor (*6*). In contrast, the inhibitory mechanism of serpins remains controversial. Although there is much evidence to support an irreversible suicide-substrate-based covalent mechanism of inhibition of proteinases by serpins (*7*−*9*), there is also compelling evidence, at least in some specific instances such as scuPA/PAI-3 (*10*) or α_2-antiplasmin/chymotrypsin (*11*), of noncovalent reversible inhibition. Furthermore, the irreversible inhibition mechanism is thought to involve major conformational rearrangement of the reactive center loop and β-sheet A of the serpin (*12*, *13*), whereas reversible inhibition has been proposed to involve either a modest insertion of the uncleaved reactive center loop or no conformational change at all (*14*−*16*). Since irreversible inhibition involves

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a normal proteolytic attack by the proteinase on the scissile bond and therefore proceeds through an initial noncovalent Michaelis-like complex, the difference between reversible and irreversible inhibition by serpins may be only at which point in the proteolytic attack the complex is stabilized. What is needed to address the structural basis for inhibition by serpins, in both specific and general instances, is a nonperturbing, highly sensitive, and widely reporting means of monitoring the conformation of the serpin.

The solution methods used to dissect the serpin inhibitory mechanism have been constrained in their usefulness by limitations in one or more of these criteria. Studies thus far have focused on structural changes within the reactive center loop and/or β-sheet A using fluorescence and other spectroscopic techniques to monitor conformational changes (*13, 17*−*19*). Results from these studies can only elucidate structural changes within the vicinity of the probe and not provide insight into the global structural changes occurring within the serpin. Additionally, the use of extrinsic fluorophores to monitor local structural changes is potentially perturbing to the serpin mechanism, complicating the interpretation of experimental results. NMR spectroscopy, however, is a nonperturbing structurally sensitive technique appropriate to the challenge of characterizing the global conformational changes involved in the serpin inhibitory mechanism. NMR has previously been used primarily on nonisotopically labeled serpins to provide limited information on such questions as the effects of heparin binding to antithrombin (*20, 21*), mobility within the reactive center loop (*22*), or changes associated with reactive center loop insertion (*23, 24*). A single, heroic effort that incorporated ^{13}C label into methionine α-carbonyls was used to examine the nature of the serpin−proteinase bond in covalent complex (*25*). The usefulness of these NMR studies was severely limited by the large size of the serpin−proteinase complexes ($\sim$70 kDa) and by restriction to one-dimensional methods, with consequently poor resolution of individual resonances.

We report here the successful application of a 2D NMR technique, [^{1}H-^{15}N]-HSQC,[1] on the ^{15}N-labeled serpin α_1-proteinase inhibitor (Pittsburgh variant with seven stabilizing mutations and C232S change) (multi-9 α_1PI) to specifically determine whether conformational change occurs in the serpin upon formation of a noncovalent complex with bovine trypsin, rendered inactive by conversion of the active site serine either by chemical means to dehydroalanine or by mutagenesis to alanine. Since this technique is nonperturbing and correlates directly bonded ^{1}H and ^{15}N nuclei, we could monitor changes in the environment of NH groups in the protein backbone at sites of labeling. The exquisite sensitivity of this technique in the case of this serpin allowed for the characterization of the global conformational changes associated with the formation of the Michaelis-like noncovalent complex. Both uniform and residue-specific ^{15}N-labeling was used to highlight both global conformation changes and conformational changes within the reactive center loop when the Michaelis-like noncovalent complex was formed. These methods should be more broadly applicable to covalent complexes of this serpin with a range of proteinases and probably to other bacterially expressible serpins.

[1] Abbreviations: HSQC, heteronuclear single quantum correlation; α_1PI, α_1-proteinase inhibitor; multi-9 α_1PI, α_1PI in which the seven stabilizing mutations reported by Kwon et al. (*1*) have been incorporated as well as change of C232 to serine and the P1 residue from methionine to arginine (Pittsburgh mutation); P1, P4, etc., designation of residues in the reactive center loop, using the nomenclature of Schechter and Berger (*2*) 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.

MATERIALS AND METHODS

Construction of the Multi-9 α_1PI/pQE30 Construct. The pET plasmid DNA coding for the multi-7 variant of α_1PI (*26*) was a kind gift from Dr. Myeong-Hee Yu of the Korea Research Institute of Biosciences and Biotechnology. The polymerase chain reaction was used to amplify the multi-7 coding region from amino acid 4 to 394 of the mature sequence. Primers were designed to add a 5′ *Bam*HI and a 3′ *Hin*dIII restriction endonuclease site to facilitate subcloning into the pQE30 vector (Qiagen) to add an amino-terminal 6× histidine tag. Amplification products were purified and digested with *Bam*HI and *Hin*dIII and ligated into a similarly digested pQE30. The M358R (Pittsburgh variant) and C232S mutations were introduced into the multi-7 α_1PI/pQE30 construct using the Quick Change kit (Stratagene), yielding the multi-9 α_1PI/pQE30 construct. The coding region of the multi-9 α_1PI/pQE30 construct was confirmed by DNA sequencing.

Expression and Purification of Isotopically Labeled Multi-9 α_1PI. The multi-9 α_1PI/pQE30 construct was transformed into SG13009 cells (Qiagen) and selected by antibiotic resistance. A 1 L culture of M9 minimal medium supplemented with [^{15}N]ammonium chloride was grown to an OD_{600} of 0.5 and induced with 1 mM isopropyl-β-thiogalactopyranoside. Incubation was continued for 4.5 h, and the cells were harvested by centrifugation. Cells were resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 300 mM sodium chloride and lysed by sonication. The cellular lystate was separated from inclusion bodies by centrifugation and stored at −80 °C until purification. Inclusion bodies were refolded by the method of Kwon et al. (*27*) and dialyzed into 50 mM sodium phosphate buffer, pH 7.4, 300 mM sodium chloride. The cellular lysate and refolded protein were loaded onto a Ni^{2+} affinity column in 50 mM sodium phosphate, pH 7.4, 300 mM sodium chloride, and 10 mM imidazole. The protein was eluted using a step gradient with rests at 50, 100, and 200 mM imidazole. The desired protein eluted at 100 mM imidazole. Fractions were pooled and dialyzed into 20 mM sodium phosphate, pH 6.0, containing 150 mM sodium chloride.

Specific labeling with either [^{15}N]leucine or [^{15}N]alanine was accomplished using a modified M9 minimal medium. M9 minimal medium containing natural-abundance ammonium chloride supplemented with 100 mg/L of each amino acid except for that corresponding to the desired ^{15}N amino acid label. A 50 mL overnight culture was used to inoculate a 1 L culture additionally supplemented with the desired ^{15}N-labeled amino acid just prior to inoculation. Expression and purification were carried out as described above.

Mutagenesis of Multi-9 α_1PI. Residue-specific assignments of alanines within the reactive center loop were carried out using variants in which either P12/P11, P11/P9, or P4 residues were replaced with serine using the Quick Change Kit (Stratagene). This series of three variants allowed for

the assignment of all four reactive center loop alanines in both native and reactive center loop cleaved forms. The N-terminal alanines for residue positions 7 and 8 of mature multi-9 α_1PI were assigned as an undifferentiated pair using an A7S/A8S variant.

Preparation of Anhydrotrypsin and S195A Trypsin. Anhydrotrypsin was prepared from crystallized bovine trypsin (Worthington Biochem) by β-elimination of the PMSF adduct according to the method of Ako et al. (*28*). Following the β-elimination reaction, TLCK was added to a final concentration of 100 μM to inhibit any remaining trypsin activity. β-Anhydrotrypsin was then purified using a soybean trypsin inhibitor agarose matrix (*29*). The absence of proteolytic activity (<0.01%) was confirmed spectrophotometrically using the chromogenic substrate S-2222 (Pharmacia Hepar, Franklin, OH). S195A bovine trypsin was prepared from recombinant S195A bovine trypsinogen by reaction with enterokinase. Recombinant trypsinogen was expressed in high yield in *E. coli* and purified and refolded from inclusion bodies (Peterson, Gordon, and Gettins, submitted for publication). The ability of the S195A to substitute for β-anhydrotrypsin was confirmed independently of the NMR experiments, which themselves show direct evidence for complex formation with α_1PI, by the appearance of a band corresponding to a noncovalent complex on nondenaturing PAGE (not shown).

Preparation of Reactive Center Loop-Cleaved Multi-9 α_1PI. Reactive center loop-cleaved multi-9 α_1PI species were prepared by dissociation of the covalent complex formed between ^{15}N-labeled multi-9 α_1PIs and β-trypsin, exploiting the extremely high thermal stability of cleaved serpins compared with the native state (*23*, *30*). Multi-9 α_1PI in 10 mM sodium phosphate, pH 7.4, was reacted with β-trypsin at a 1.5:1 molar ratio at room temperature for 10−15 min before TLCK (100 μM) was added to inhibit any remaining β-trypsin activity. The mixture of multi-9 α_1PI−trypsin complex, unreacted multi-9 α_1PI, and any residue of unreacted trypsin was then incubated with 50 mM dithiothreitol and heated to 75 °C for 10 min. This caused dissociation of the covalent complex, through destabilization of the trypsin moiety and precipitation of all protein components except the cleaved multi-9 α_1PI that represented the serpin moiety of the dissociated covalent complex. The sample was then clarified by centrifugation and transferred to NMR buffer, as for other samples. This gave quantitative production of cleaved multi-9 α_1PI, and so was the method of choice for preparing cleaved α_1PI species. The alternative of using an arginine-specific proteinase with SI >1 was considered inferior, since it gave a mixture of cleaved serpin and covalent complex that needed to be separated and never gave a high percentage of the desired cleaved serpin.

[^{1}H−^{15}N]-HSQC NMR. All NMR experiments were carried out on a Bruker DRX600 spectrometer equipped with a 5 mm (^{1}H/^{15}N/^{13}C) triple resonance probe and pulse field gradients. All NMR spectra were collected at 318 K in 90% H_2O/10% D_2O, 20 mM sodium phosphate, pH 6.0, and 150 mM sodium chloride. Sample concentrations ranged between 0.5 and 1.2 mM. Noncovalent complex samples were prepared by mixing ^{15}N-labeled multi-9 α_1PI with a 15% molar excess of unlabeled β-anhydrotrypsin (or recombinant S195A trypsin) followed by concentration. Two-dimensional [^{1}H−^{15}N]-HSQC spectra were collected for 3 or 6 h for uncomplexed and noncovalent complex samples, respectively. NMR data were processed using Tripos 6.3 software (Tripos, Inc., St. Louis, MO).

RESULTS AND DISCUSSION

Optimization of α_1PI Variant for NMR Studies. The ultimate selection of the "multi-9" variant of α_1PI (seven core stabilizing mutations, plus methionine 358 changed to arginine and cysteine 232 changed to serine) for all of our NMR studies was the result of several refinements to optimize the stability and complex-forming ability of the serpin. The choice of the Pittsburgh variant, i.e., P1 changed from methionine to arginine, was based upon our previous finding that this variant forms an extremely tight noncovalent complex with anhydrotrypsin (K_d = 5 nM) (*17*), whereas the wild-type methionine-containing protein gives only a weak complex with either elastase or trypsin (multi-micromolar K_d). Thus, our ability to examine a stable noncovalent complex was facilitated by using this variant. In addition, the change in P1 residue to arginine holds out the future prospect of making covalent complexes with a wide range of P1 arginine-specific proteinases of blood coagulation, fibrinolysis, and complement activation, for study by NMR. Since the Pittsburgh variant was originally identified in a boy with a bleeding disorder in which his mutated α_1PI was an effective inhibitor of thrombin (*31*), such studies would be of functional as well as mechanistic significance. The choice of a "multi-7" background was based on a desire to have enhanced stability against loop−sheet polymerization and to enable higher temperatures to be used for NMR data collection, if needed. This background was identified by the Korean group of Yu and colleagues (*1*), who sought mutations that enhanced the stability of α_1PI, without affecting inhibitory function. The multi-7 represented a cumulative enhancement of stability such that the unfolding temperature was raised from approximately 59 to 80 °C (*1*). However, since the stoichiometry of inhibition of proteinase remains close to 1, despite the greatly enhanced stability to denaturation, the ability of the reactive center loop to insert in a facile manner has not been compromised. Accordingly, this variant is unlikely to result in atypical and therefore misleading results with regard to proteinase-induced loop insertion. A further advantage of the multi-7 background was that the mutations reduced the tendency to polymerize at lower temperatures. Finally, the replacement of the single free cysteine at position 232 with serine was necessary to prevent unwanted disulfide cross-linking. Our initial studies on a "multi-8" variant that still contained C232 showed a tendency of the protein to precipitate at high concentrations and at elevated temperatures.

[^{1}H−^{15}N]-HSQC NMR of Uniformly ^{15}N-Labeled Multi-9 α_1PI, in Native, Cleaved, and Noncovalently Complexed States. The [^{1}H-^{15}N]-HSQC spectrum of the 45 kDa serpin multi-9 α_1PI, recorded at 318 K and pH 6.0, showed not only a wide spread of chemical shifts in the ^{1}H and ^{15}N dimensions but also very good resolution of individual cross-peaks outside of the crowded central region (Figure 1A). The wide spread of chemical shifts was expected from the known globular nature of α_1PI, with an extensive hydrophobic core and the presence of extensive secondary structure, including three major β-sheets (Figure 2A). The good resolution of peripheral peaks was, however, not

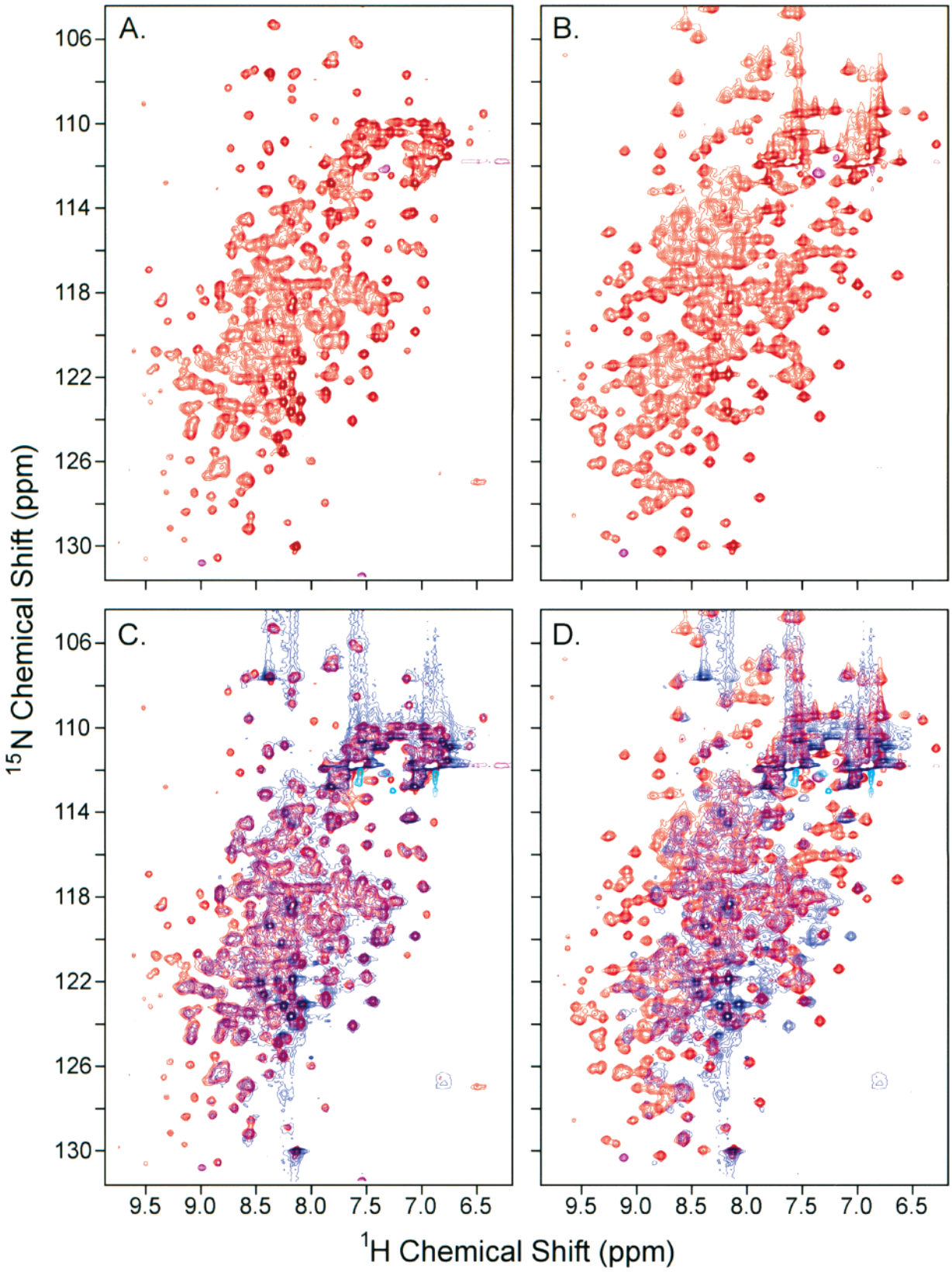

FIGURE 1: [^{1}H-^{15}N]-HSQC spectra of uniformly ^{15}N-labeled multi-9 α_1PI recorded at 318 K and pH 6.0. Panel A, native multi-9 α_1PI; panel B, reactive center loop-cleaved multi-9 α_1PI; panel C, complex of ^{15}N-labeled multi-9 α_1PI with unlabeled β-anhydrotrypsin (blue spectrum) overlaid by the spectrum of native multi-9 α_1PI (red, spectrum same as in panel A); panel D, complex of ^{15}N-labeled multi-9 α_1PI with unlabeled β-anhydrotrypsin (blue spectrum) overlaid by the spectrum of reactive center loop-cleaved multi-9 α_1PI (red, spectrum same as in panel B). The multi-9 α_1PI concentration was 900 μM for the spectra of native protein and noncovalent complex and 520 μM for cleaved multi-9 α_1PI. Intensities are normalized to constant α_1PI concentration.

necessarily expected given the large size of the protein, but was very encouraging for our proposed comparative studies.

A reactive center loop-cleaved form of the same multi-9 α_1PI, with cleavage at the P1−P1′ bond, gave a comparably

well-dispersed and resolved [^{1}H-^{15}N]-HSQC spectrum relative to that of the native protein (Figure 1B), but with striking change in chemical shift of many of the peaks. This is most easily seen in the peripheral regions. From the crystal structure of cleaved α_1PI, it is known that the structural change that has occurred following cleavage of the reactive center loop at P1−P1′ is insertion of the loop into β-sheet A as a central strand in antiparallel orientation (*32*). While we expected that residues associated with the strands of β-sheet A that end up flanking the newly inserted reactive center loop would have their chemical shifts perturbed, we were surprised by the extensive nature of the chemical shift perturbations of what appeared to be a majority of the resonances. This presumably reflects the concerted changes that need to occur to allow such loop insertion to take place, involving not only opening of β-sheet A and insertion of the reactive center loop, but also movement of this sheet over underlying α-helices and consequent perturbation of these structural elements (see Figure 2A). This exquisite sensitivity of the HSQC spectrum to loop insertion was, however, ideal for subsequently examining the structure of the noncovalent complex with anhydrotrypsin.

A [^{1}H-^{15}N]-HSQC spectrum of a noncovalent complex of uniformly labeled multi-9 α_1PI and unlabeled anhydrotrypsin gave reduced intensity for most resonances and some broadening, consistent with alteration in the rotational properties of the resulting serpin−proteinase complex from an ∼50% increase in size compared to the isolated serpin (Figure 1C,D). We attempted to obtain better resolved spectra of both free and complexed multi-9 α_1PI by using the TROSY method (*33, 34*) to obtain the ^{1}H-^{15}N correlations, but found that the loss of signal intensity, particularly for the complex, negatively offset any increase in resolution (spectra not shown), making the spectrum of the complex much poorer for the purposes of comparison with the spectra of native and cleaved α_1PI. Despite the broadening and reduced intensity of some resonances in the HSQC spectrum of the noncovalent complex, overlays of this spectrum with those of native and cleaved multi-9 α_1PI provide a persuasive demonstration that the serpin in the noncovalent complex has an almost identical conformation to that of native multi-9 α_1PI, from the coincidence of almost all peaks present in the complex spectrum with ones in the native spectrum (Figure 1C) (though note that, because of lowered intensity in the complex, some of the cross-peaks present in the spectrum of native multi-9 α_1PI are missing from the spectrum of complex at this contour level in Figure 1C). In addition, there can be no degree of loop insertion, judged from the fundamental mismatch between peaks in the spectrum of complex and peaks in the spectrum of cleaved multi-9 α_1PI (Figure 1D).

Specific Labeling of Multi-9 α_1PI with ^{15}N-Labeled Leucine. Although the spectra presented above of uniformly labeled multi-9 α_1PI strongly support the conclusion that the conformation of the serpin moiety in the noncovalent complex is nearly identical to that of native multi-9 α_1PI, but not of cleaved multi-9 α_1PI, the considerable spectral overlap in the central region of the spectrum might obscure differences that could be present. To eliminate such spectral overlap, while still using nonperturbing and widely reporting methods to examine the system, we next chose to simplify the spectrum of native multi-9 α_1PI by selectively labeling

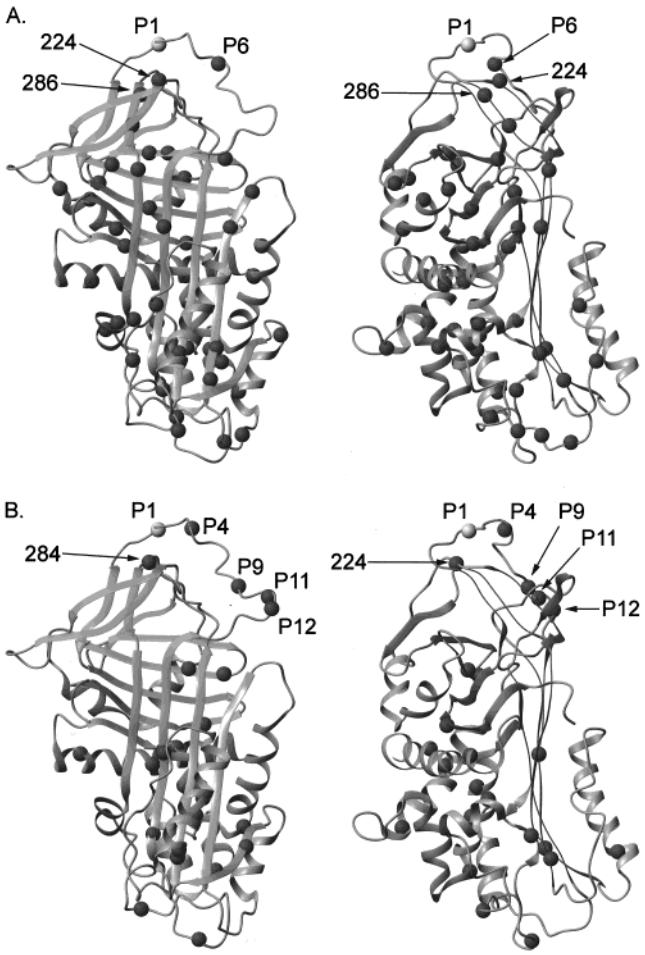

FIGURE 2: Location of leucine and alanine residues within α_1PI, based on the crystal structure of multi-7 variant α_1PI (pbd file 1atu) (*39*). The right panels are an orthogonal view from the left panels. Top, distribution of leucine residues (spheres), with the P1 residue also indicated, and leucines close to the reactive center loop numbered; bottom, distribution of alanine residues (spheres) with reactive center loop alanines P4, P9, P11, and P12 indicated, and the alanine closest to the P1 residue numbered.

only the leucine residues with ^{15}N (see Materials and Methods). Leucine was chosen because there are 46 of these residues in the protein that are well distributed throughout the molecule, including several in β-sheet A and even one in the reactive center loop (Figure 2A).

The [^{1}H-^{15}N]-HSQC spectrum of [^{15}N]leucine-labeled multi-9 α_1PI was very well resolved, with 44 of the expected 46 peaks discernible (Figure 3A). Furthermore, the large spread of chemical shifts was consistent with the large range of environments for leucines in multi-9 α_1PI. However, given that random coil leucine has ^{1}H and ^{15}N chemical shifts of about 8.2 and 122 ppm, respectively (*35*), the peaks in the bottom left and upper right corners, being most shifted from random coil positions, are likely to be from regions with defined secondary structure associated with the core of the protein. These peaks are therefore likely to be sensitive to even small conformational changes in the core of the serpin.

The spectrum of the complex of [^{15}N]leucine-labeled multi-9 α_1PI and unlabeled anhydrotrypsin (Figure 3B) showed almost as good resolution as that of the uncomplexed, native protein, though with more overlap in the central region of the spectrum resulting from some line broadening of the higher molecular weight complex. Neverthless, superposi-

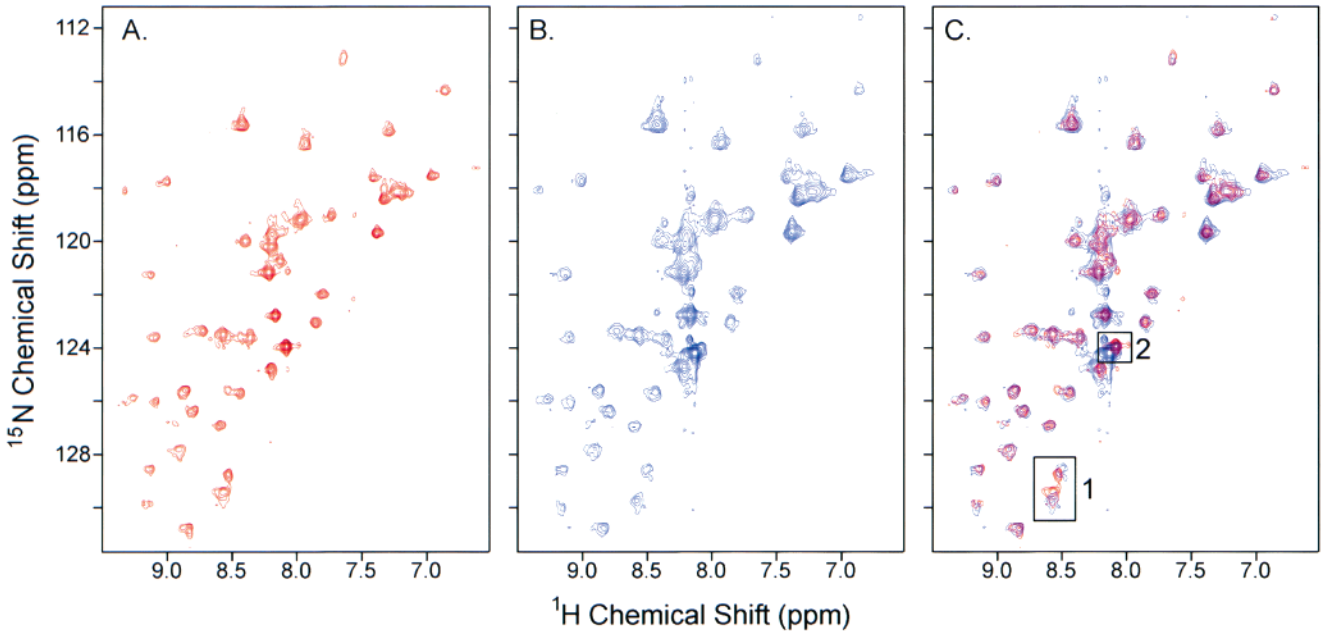

FIGURE 3: [^{1}H-^{15}N]-HSQC spectra of [^{15}N]leucine-labeled multi-9 α_1PI recorded at 318 K and pH 6.0. Panel A, native multi-9 α_1PI; panel B, complex of native multi-9 α_1PI with β-anhydrotrypsin; panel C, overlay of spectra of native multi-9 α_1PI (red) and complex with β-anhydrotrypsin (blue). The two boxed regions in panel C represent the only significant differences between the two spectra. The numbers of the boxed regions are those used in the text.

tioning of the two spectra (Figure 3C) showed them to be almost identical in position of the resonances. Only two regions of difference were seen, labeled 1 and 2. Inspection of the structure of α_1PI and identification of leucines relative to the expected site of docking with the proteinase at the P1 position suggest that the leucine in the reactive center loop at P6 and leucines 224 and 286 may be close enough to the proteinase to be directly perturbed by contact, even without a conformational change in the body of the serpin. The very different chemical shifts of resonances in regions 1 and 2 are also consistent with the different environments of these leucines, one solvent-exposed and therefore expected to be close to random coil (region 2) and one strongly shifted in both ^{1}H and ^{15}N dimensions, consistent with being part of a β-sheet (region 1). However, such assignments are not essential for the most important conclusion that, given the near-identity of the remainder of the spectra, the conformation of the body of the serpin must be the same in the complex as in the native state.

Behavior of Multi-9 α_1PI Labeled Selectively with [^{15}N]-Alanine. The successful specific incorporation of ^{15}N-labeled leucine into multi-9 α_1PI encouraged us to extend this strategy to production and examination of an [^{15}N]alanine-labeled sample, since, while there are fewer alanines than leucines in α_1PI, and hence fewer potential reporters of global conformational change, there are 4 alanines (out of 26) that are within the reactive center loop itself, which might thereby provide direct information on the conformational and flexibility changes of the loop upon complex formation.

The [^{1}H-^{15}N]-HSQC spectrum of native [^{15}N]alanine-labeled multi-9 α_1PI was of excellent quality and resolution with 25 of the 26 expected resonances distinguishable (Figure 4A). As with the effect of reactive center loop cleavage on the spectrum of uniformly labeled multi-9 α_1PI, cleavage of the alanine-labeled sample resulted in dramatic alteration in the chemical shift of most of the resonances in the [^{1}H-^{15}N]-HSQC spectrum (Figure 4B), showing high sensitivity to loop insertion-induced conformational change. In this spectrum, all 26 of the alanines gave resolved resonances.

The spectrum of the noncovalent complex of multi-9 α_1-PI with S195A trypsin (a recombinant equivalent of chemically produced anhydrotrypsin that we had available to us toward the end of this study) was of good quality and superimposed very well with the spectrum of native multi-9 α_1PI (Figure 4C), but not with the spectrum of cleaved multi-9 α_1PI (Figure 4D). This is the same result as was found for both the uniformly ^{15}N-labeled and [^{15}N]leucine-labeled samples (though note that comparison of leucine-labeled complex was not made with the cleaved serpin) and again supports the conclusion that the conformation of the serpin in noncovalent complex closely resembles that in native multi-9 α_1PI. The only differences that are seen are discussed in more detail below, since they become specifically interpretable as a result of selective assignment of resonances from the reactive center loop alanines.

Assignment of the Reactive Center Loop Alanines. The reactive center loop of multi-9 α_1PI contains four alanines, which are located at positions P12, P11, P9, and P4, i.e., close to the hinge point for insertion in the case of the P12 and close to the expected contact point with the proteinase in the case of the P4, with the two others in between. To permit fuller interpretation of the HSQC spectra of alanine-labeled multi-9 α_1PI in terms of specific changes within the reactive center loop, a series of alanine-to-serine variants was made, which enabled assignment of the reactive center loop alanines and two N-terminal alanines at positions 7 and 8 of the mature α_1PI sequence to be made (spectra not shown). Resonances corresponding to the reactive center loop alanines are identified in Figure 4A while the two N-terminal alanines are marked with asterisks. Both the ^{15}N and ^{1}H chemical shifts of these resonances are consistent with all of these alanines being in random coil conformations (*35*), suggesting

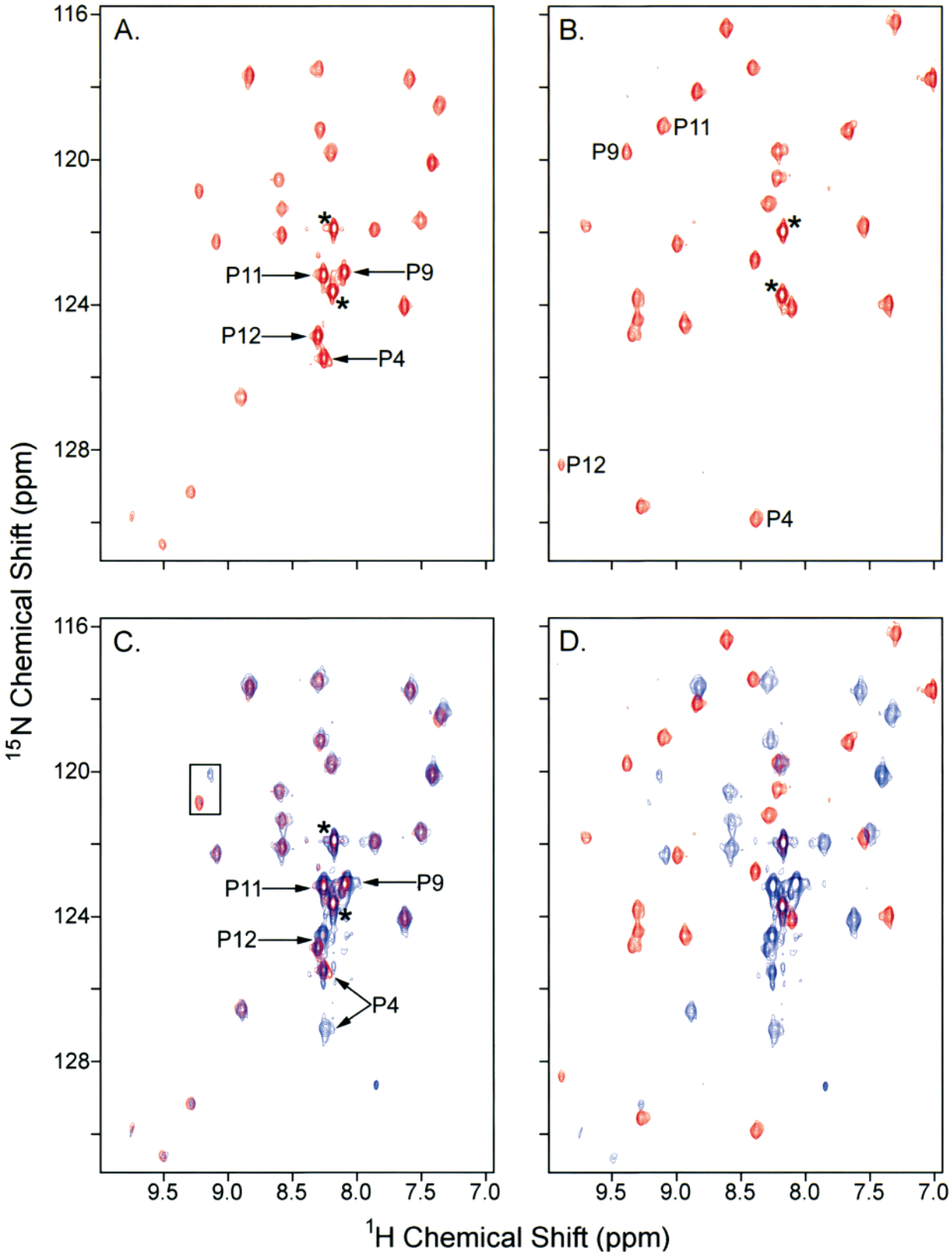

FIGURE 4: [^{1}H-^{15}N]-HSQC spectra of [^{15}N]alanine-labeled multi-9 α_1PI recorded at 318 K and pH 6.0. Panel A, native multi-9 α_1PI; panel B, reactive center loop-cleaved multi-9 α_1PI; panel C, complex of [^{15}N]alanine-labeled multi-9 α_1PI with unlabeled S195A trypsin (blue spectrum) overlaid by the spectrum of native multi-9 α_1PI (red, spectrum same as in panel A); panel D, complex of [^{15}N]alanine-labeled multi-9 α_1PI with unlabeled S195A trypsin (blue spectrum) overlaid by the spectrum of reactive center loop-cleaved multi-9 α_1PI (red, spectrum same as in panel B). Whereas almost all peaks are shifted between spectrum B and that of the complex, only one difference outside of the central region is apparent in panel C (boxed). The specific assignments to reactive center loop alanines (P4, P9, P11, and P12) and the two N-terminal alanines that are located in highly mobile, unstructured regions (marked with asterisks) are indicated for the spectra of native and cleaved multi-9 α_1PI.

that the reactive center loop is unstructured. Such a conclusion is further supported when the signal intensity for each assigned alanine is compared with the remaining resonances. In all instances, the assigned alanines have intensities which are ~2−10-fold greater than the other resonances.

We also made assignments of the four reactive center loop resonances in the spectrum of cleaved alanine-labeled multi-9 α_1PI (Figure 4B), which shows that each of the four reactive center loop alanines undergoes a large change in both ^{1}H and ^{15}N chemical shifts. In contrast, the two alanines from the N-terminus remain at the same positions and with comparable high signal intensity, and thus serve as an internal control. The large changes in chemical shift and intensity for the four reactive center loop alanines in the cleaved protein are fully expected from the known drastic change in structure and environment of the reactive center loop upon cleavage and insertion into β-sheet A (*35*). Thus, the whole loop changes conformation from unstructured, solvent-exposed, and flexible to being part of a β-sheet, buried and rigid.

Superpositioning of the spectra of alanine-labeled multi-9 α_1PI in native and noncovalently complexed states (Figure 4C) shows that, with the exception of residue P4, there are only very minor changes in chemical shift of the reactive center loop alanines upon complex formation. This contrasts with the very large chemical shift changes for each of these upon loop insertion (Figure 4B) and clearly indicates that none of the three residues P9, P11, or P12 can be inserted into β-sheet A in the noncovalent complex. The larger chemical shift changes for P4 (~1.6 ppm for ^{15}N) may well be due to direct contact with the proteinase, which must at the very least be bound tightly to the P1 arginine and hence be close to residues in the reactive center loop immediately adjacent to this position.

The assignment of the four reactive center loop alanines in complex with S195A trypsin leaves only one other, non-reactive center loop alanine that is significantly perturbed by complex formation (boxed in Figure 4C). This is an alanine with non-random coil chemical shifts for both ^{1}H and ^{15}N and therefore probably in a structured region of the protein. Inspection of Figure 2B shows that alanine 284 lies at the end of a strand of β-sheet C and only 6 Å from the P1 residue (N-to-N separation). It is therefore likely that this residue is perturbed by simple proximity to the proteinase.

Nature of the Noncovalent Complex of Multi-9 α_1PI with Anhydrotrypsin. From the above comparisons of spectra of native, cleaved, and noncovalently complexed multi-9 α_1-PI, a consistent conclusion can be drawn that the serpin moiety, outside of the reactive center loop, remains unperturbed, except possibly for a few residues that are close enough to the docking site of the proteinase to be perturbed directly by proximity of the proteinase. The large changes in chemical shift seen throughout the serpin upon reactive center loop cleavage for the alanine residues, including large changes for the reactive center loop alanines, make these perturbations sensitive markers for loop insertion. The absence of any such effects for the noncovalent complex reinforces the conclusion that there can be no insertion of the reactive center loop into β-sheet A in the complex. This is opposite to the recent conclusion of Mellet and Bieth that formation of a Michaelis-like noncovalent complex between α_1PI and anhydroelastase induces insertion of the reactive center loop into β-sheet A (*16*). We do not think that this is a result of different behavior of different serpin−proteinase pairs. Instead it seems more likely that the results of the fluorescence study are artifactual and accordingly misinterpreted. In that study, the identification of a "loop inserted" but noncleaved serpin conformer depends on correct interpretation of a small slow fluorescence change as resulting from proteinase translocation in a noncovalent complex. No data are presented to show the expected concentration dependence of each of the steps, and no evidence of actual complex formation is presented for what is likely to be a quite weak interaction.

Structure and Flexibility of the Reactive Center Loop in Different States of α_1PI. Even using only the four definitive assignments of alanine residues within the reactive center loop, we are in a good position to make some useful conclusions with regard to the structure and flexibility of this region in the native state and of the interactions and structural changes that occur in the loop upon noncovalent complex formation. First, the random coil ^{1}H and ^{15}N chemical shifts of all four of the alanines, which report on a significant fraction of the whole reactive center loop, are consistent with the crystal structure of α_1PI, which shows an extended conformation for this region, with no significant contacts between the backbone and the underlying body of the serpin (*36*). What the crystal structure did not show was whether this region is highly mobile. The presence of an extended, β-strand-like structure in the crystal structure implied a degree of immobilization. Our finding that the intensities of only the resonances from these four alanines, and two in the extreme, unconstrained N-terminus, are 2−10 times higher than all other resonances in the spectrum of alanine-labeled multi-9 α_1PI, strongly supports a much higher degree of motional freedom for the reactive center loop compared with the body of the serpin. This is contrary to the conclusion of our earlier 1D NMR study of the reactive center loop of α_1PI (*22*), and highlights the advantage of the present 2D NMR approach to allow unambiguous identification of individual resonances even in a 45 kDa protein. Such high flexibility is also consistent with the most common role of the reactive center loops of serpins being as proteinase substrates and hence being able to adopt an optimal conformation for attack by the proteinase catalytic serine. Precise measurements of relaxation parameters for these and other resonances will be necessary to put a more quantitative value on the relative motions. What was unexpected was that in the noncovalent complex with S195A trypsin, at least three of these reactive center loop alanines (P12, P11, and P9) retained high intensity, both in absolute terms and relative to the remainder of the alanine resonances. This retention of mobility demonstrates that complex formation does not constrain the entire reactive center loop to adopt a rigid "canonical"-like structure, such as is found in noncovalent complexes of proteinases with Kazal, Kunitz, and Bowman-Birk inhibitors. In addition, the perturbation of chemical shift for the P4 alanine and broadening suggest that interactions between the proteinase and the reactive center loop may extend from P1 to P4, consistent with the very high affinity for this noncovalent complex.

Utility of the NMR Method Applied to Serpins. While some aspects of the present system were optimized for NMR studies, such as choice of a more stable variant of α_1PI, there

are some common serpin characteristics that should make the present approach generally applicable to other serpins. Thus, the excellent spectral dispersion is a reflection of the common type of secondary structure present in all serpins. In addition, insertion of the reactive center loop is known to cause similar structural changes in those serpins for which there are crystal structures of both native and cleaved states (*32*, *37*, *38*). Therefore, the exquisite sensitivity of resonance chemical shift seen here for both uniformly labeled and alanine-specifically labeled multi-9 α_1PI should also be true in other serpins. The successful incorporation of specific amino acids, without detectable dilution of the label through catabolic processes, should also be possible for other serpins that can be bacterially expressed. The incorporation of alanine is particularly felicitous, since many serpin reactive center loops contain large numbers of alanines, thought to be necessary for facile loop insertion close to the hinge region (*3*). Finally, the use of ^{15}N label in only the serpin will enable complexes of a given serpin with different proteinases to be compared, with focus only on the serpin part of the complex. This should enable dissection of those aspects of inhibition that are proteinase-specific and those that are common to a given serpin.

A particularly exciting extension of the present NMR studies would be to the scuPA/PAI-3 complex, which behaves as a reversible, noncovalent complex analogous to what we have examined here. It has been shown that dissociation of this noncovalent complex in the presence of the plasminogen activator receptor yielded active PAI-3 and scu-PA. The dissociated PAI-3 could then reversibly inhibit another scuPA molecule or irreversibly inhibit the activated two-chain u-PA through the formation of a covalent inhibitory complex, suggesting two modes of inhibition by a single serpin, one noncovalent and the other covalent (*10*). The NMR methods described here should be able to provide direct structural insight into what the nature of each of these types of complex of PAI-3 is with each form of the plasminogen activator.

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