High-Level Bacterial Expression and ^{15}N-Alanine-Labeling of Bovine Trypsin. Application to the Study of Trypsin−Inhibitor Complexes and Trypsinogen Activation by NMR Spectroscopy†

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ABSTRACT: We describe here the high-level expression of bovine trypsinogen in *E. coli*, its refolding and activation to β-trypsin, and the selective incorporation of ^{15}N-labeled alanine through supplementation of the growth medium. Using this procedure, we expressed ^{15}N-labeled S195A trypsinogens, both on a wild-type and on a D189S background, in amounts suitable for NMR spectroscopy. 2D [^{1}H-^{15}N]-HSQC NMR was used to follow conformational changes upon activation of trypsinogen and formation of noncovalent complexes between S195A or S195A/D189S trypsin and protein proteinase inhibitors of different structural families and different sizes, as well as to examine the effects of introduction of the D189S mutation. Spectra of good quality were obtained for both trypsins alone and in complexes of increasing size with the proteinase inhibitors BPTI (total molecular mass 31 kDa), SBTI (total molecular mass 44 kDa), and the serpin α_1-proteinase inhibitor Pittsburgh (α_1PI Pittsburgh) (total molecular mass 69 kDa). Assignments of alanines 55 and 56, close to the active site histidine, and of alanine 195, present in the S195A variant used for most of the studies, were made by mutagenesis. These three alanines, together with two others, probably close to the S1 specificity pocket, were very sensitive to complex formation. In contrast, the remaining 10 alanines were invariant in chemical shift in all 3 of the noncovalent complexes formed, reflecting the conservation of structure in complexes with BPTI and SBTI known from X-ray crystal structures, but also indicating that there is no change in backbone conformation for the noncovalent complex with α_1PI, for which there is no crystal structure. This was true both for S195A and for S195A/D189S trypsins. This high-level expression and labeling approach will be of great use for solution NMR studies on trypsin−serpin complexes, as well as for structural and mechanistic studies on trypsin variants.

Trypsin is a serine proteinase that possesses high sequence similarity and nearly superimposable polypeptide backbone to chymotrypsin. The early availability of crystal structures of each proteinase (*2, 3*), together with the ease of isolation of each protein, ensured that many mechanistic studies on serine proteinases, whether directed at understanding the role of the catalytic triad and oxyanion hole in the active site, or specificity conferred by residues in the substrate specificity pockets, were carried out on these proteinases. Trypsin is also closely related in both structure and specificity to the catalytic domains of many human plasma proteinases, such as those of the blood coagulation cascade, including thrombin (*4*) and factor Xa (*5*), and has therefore been used as a model for understanding the actions of these more complex proteinases. Our interest in trypsin arose from a structural and mechanistic interest in the family of protein proteinase inhibitors called serpins, which include the principal inhibitors in vivo of blood coagulation proteinases (*6*), and the ability of many such serpins, in vitro, to also inhibit trypsin.

The serpin inhibition mechanism, in contrast to the mechanism used by small lock-and-key type inhibitors such as pancreatic trypsin inhibitor, involves conformational change in the serpin and kinetic trapping of a normal intermediate on the proteolysis pathway. This had been proposed to result from a serpin-induced distortion of the proteinase active site (*7*). We therefore sought a means of examining conformational change in both the serpin and the proteinase that might be widely applicable to different serpin−proteinase pairs. In principle, NMR spectroscopy represents such a method, but one that is complicated by the large size of serpins (>40 kDa) and the consequent high molecular mass of any serpin−proteinase complex (>64 kDa).

Despite the large size of serpins, we have recently shown that selective introduction of ^{15}N-leucine or ^{15}N-alanine gives very well-resolved [^{1}H-^{15}N]-HSQC[1] NMR spectra of the

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[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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serpin α_1-proteinase inhibitor (α_1PI) Pittsburgh variant, both as the free protein and in a noncovalent complex with active site-inactivated trypsin (*8*) [the Pittsburgh variant involves a change of the P1 residue from methionine to arginine and so confers specificity against arginine-specific proteinases such as trypsin and thrombin (*9*)]. In the following paper, we show the application of this method to study of the covalent serpin−proteinase complex (*10*). Since we also wanted to examine structural changes that occur in the proteinase, we sought to extend our residue type-specific ^{15}N-labeling to the proteinase. Whereas natural bacterial proteinases such as subtilisin or α-lytic proteinase might most easily be so labeled (*11*), they are not effectively inhibited by serpins. In contrast, trypsin is an appropriate model proteinase for blood coagulation and fibrinolytic proteinases that can be inhibited by a wide range of plasma serpins. However, expression systems that have been reported give poor yields, and are therefore not readily adapted to cost-effective NMR label incorporation (*12*−*15*). We report here the expression of wild-type and D189S variant bovine trypsinogens at high yield in *E. coli*, the specific incorporation of ^{15}N-alanine, and the use of the activated ^{15}N-labeled trypsin to examine noncovalent complex formation with three protein proteinase inhibitors of sizes ranging from 6.5 to 45 kDa. In addition to reporting the highest level bacterial expression of any trypsinogen, this study is also the first report of expression of bovine trypsinogen, which is of significance, since many earlier mechanistic studies have been carried out on bovine trypsin and trypsinogen. Our present expression system therefore provides a means of expressing the bovine enzyme, as wild-type or variant enzyme. Through site-specific mutagenesis, we assigned resonances from alanines 55 and 56 adjacent in primary structure to the active site histidine, as well as of A195 in the S195A inactive variant used for most of the studies. Resonances of trypsin in [^{1}H-^{15}N]-HSQC spectra fell into 2 distinct classes, a class of 10 which were insensitive to complex formation and thus reflect the known structural conservation in the body of the proteinase, and a class of 5, including alanines 55 and 56 and 195, which are very sensitive both to complex formation and to the nature of the specific inhibitor. In addition, our studies provide the first direct structural information on the proteinase in a noncovalent serpin−proteinase complex as well as insight into the structural changes brought about in trypsin by the D189S mutation.

EXPERIMENTAL PROCEDURES

Cloning of Wild-Type Bovine Trypsinogen. Total RNA was isolated from a bovine pancreas using the RNAeasy Kit (Qiagen), and cDNA was prepared using oligo(dT) priming and M-MLV reverse transcriptase. A polymerase chain reaction amplified the mature coding sequence of bovine trypsinogen using the following 5′ and 3′ primers: 5′ primer, 5′-CCATGGTTGACGACGACGACAAGATCGTGGGC-3′; 3′ primer, 5′-GGAAGCTTCTAGTTGGAGGCGATGG-3′. The 5′ primer added a methionine at the start of the mature sequence, altered the first seven amino acids to bacterially preferred codons, and introduced a unique 5′ *Nco*I restriction endonuclease site. The 3′ primer introduced a unique *Hin*dIII restriction endonuclease site following the termination codon. Amplification products were purified and digested with *Nco*I and *Hin*dIII and ligated into a similarly digested pQE60 vector (Qiagen), yielding the bTrygen/pQE60 construct. The S195A, S195G, A55G, A56G, and D189S mutations were introduced into the bTrygen/pQE60 construct using the Quick Change kit (Stratagene). The coding regions of the bTrygen/pQE60 and the other constructs were confirmed by DNA sequencing.

Expression and Purification of Wild-Type and Variant Bovine Trypsinogens. The S195A, S195G, A55G, and A56G bTrypsinogen/pQE60 constructs, whether on wild-type or D189S backgrounds, were transformed into SG13009 cells and selected by antibiotic resistance. A 1 L culture of M9 minimal medium supplemented with 100 mg/L of each amino acid was grown to an OD_{600} of 0.6 and induced with 1 mM isopropyl-β-thiogalactopyranoside. Incubation was continued for 4.5 h. The cells were harvested by centrifugation and resuspended in 50 mM Tris, pH 8.0, 150 mM sodium chloride, 10 mM EDTA, and 0.5% Triton X-100 and lysed by sonication. Inclusion bodies containing bovine trypsinogen were collected by centrifugation, solubilized in 10 mL of 8 M guanidine hydrochloride, 100 mM Tris, pH 8.5, and 2 mM EDTA, and clarified by centrifugation. The solubilized trypsinogen was reduced by adding 10 mM dithiothreitol and incubating at room temperature for 1 h. The reduced protein was diluted to 100 mL in 8 M guanidine hydrochloride, 100 mM Tris, pH 8.5, and 2 mM EDTA and refolded by rapid dilution into 900 mL of 20 mM Tris, pH 8.2, 560 mM guanidine hydrochloride, 2.2 mM β-mercaptoethanol, 1.1 mM 2-hydroxyethyldisulfide, and 2 mM EDTA at 4 °C (*16*). The refolded trypsinogen was dialyzed into 12 L of 20 mM Tris, pH 8.2, at 4 °C overnight. Dialysis was continued 2 additional times in 12 L of 10 mM sodium phosphate, pH 7.4, at 4 °C. The trypsinogen was purified using high-performance SP-Sepharose (Amersham Pharmacia) in 10 mM sodium phosphate, pH 7.4, and eluted using a 0−500 mM sodium chloride gradient. The eluted protein was pooled and dialyzed into 10 mM sodium phosphate buffer, pH 7.4.

Expression and purification of the wild-type bovine trypsinogen were carried out as described above with two modifications: (i) 10 mM benzamidine was added to the refolded trypsinogen, and 1 mM benzamidine was added to the first dialysis to inhibit any proteolytic activity; (ii) the 10 mM sodium phosphate, pH 7.4, buffer was changed to 20 mM citric acid, pH 3.0, to restrict autocatalytic activation. Bovine trypsinogen was purified using high-performance SP-Sepharose in 20 mM citric acid, pH 3.0, and eluted using a 0.3−1 M sodium chloride gradient. The eluted protein was pooled and dialyzed into 5 mM hydrochloric acid.

Specific labeling with ^{15}N-alanine was accomplished using a modified M9 minimal medium. The M9 minimal medium contained natural-abundance ammonium chloride supplemented with 100 mg/L of each amino acid except alanine. A 50 mL overnight culture was used to inoculate a 1 L culture additionally supplemented with ^{15}N-alanine just prior to inoculation. Expression and purification were carried out as described above. Samples of trypsinogens with uniform ^{15}N-labeling were prepared by growth of the *E. coli* on M9 minimal medium devoid of amino acids, to which $^{15}NH_4Cl$ had been added as the source of ^{15}N.

Activation of Bovine Trypsinogens. The recombinant trypsinogens were activated using highly purified porcine

enterokinase (Sigma). Activation reactions were performed using trypsinogen at 1 mg/mL and 0.6 unit of enterokinase/mg of trypsinogen. The reactions were incubated in 10 mM sodium phosphate, pH 7.4, overnight at 4 °C with shaking. The completeness of activation was confirmed by SDS−PAGE. Completed reactions were clarified by centrifugation and dialyzed into 20 mM sodium phosphate, pH 6.0, and 150 mM sodium chloride for NMR studies.

[^{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 318 K in 90% H_2O/10% D_2O, 20 mM sodium phosphate, pH 6.0, and 150 mM sodium chloride. Samples were 1 mM in ^{15}N-alanine-labeled S195A trypsin and contained a 20% molar excess of the desired trypsin inhibitor: BPTI (Sigma), SBTI (Sigma), or α_1PI Pittsburgh variant [recombinant, nonglycosylated material expressed in this laboratory (*8*)]. Two-dimensional [^{1}H-^{15}N]-HSQC spectra of the ^{15}N-alanine-labeled samples were collected for 3 h except for that of the S195A trypsin−α_1PI Pittsburgh variant noncovalent complex, which was collected for 9 h. The spectrum of the uniformly labeled trypsin sample required 6 h data collection on a 360 μM sample at 318 K in 20 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl. NMR data were processed using Tripos 6.3 software (Tripos, Inc., St. Louis, MO).

SDS−PAGE. SDS−polyacrylamide gel electrophoresis was carried out under reducing conditions on a 12% acrylamide gel. Protein bands were visualized by staining with Coomassie brilliant blue.

RESULTS

Expression, Refolding, and Activation of Bovine Trypsinogen. Bovine trypsinogen, expressed in minimal medium supplemented with all amino acids to the level of 100 mg/L, was produced as inclusion bodies, at a level of about 40 mg per liter of *E. coli* cell culture, with no trypsinogen detectable in the supernatant. After solubilization and refolding, as described under Experimental Procedures, a yield of about 20 mg/L of trypsinogen was obtained. Activation of trypsinogen (Figure 1D) with enterokinase gave trypsin (Figure 1E) with a final yield of about 12 mg/L. This compares with a previously reported yield by others of only 0.35−0.6 mg/L final yield of recombinant rat trypsin from bacterial expression. That approach involved direct harvesting of folded trypsinogen from the supernatant (*13*).

Although the catalytically inactive S195A variant was used as the basis for most of the NMR studies to prevent any autolysis during NMR experiments and to permit noncovalent complex formation with α_1PI, true wild-type trypsin was also produced to demonstrate that the refolding procedure resulted in a catalytically functional, and therefore correctly folded, proteinase. The ability of the recombinant bovine trypsin to form a high molecular weight SDS-stable covalent complex with α_1PI Pittsburgh variant (Figure 1F), that was identical to those of plasma-derived β-trypsin (Figure 1C), together with the expected solution activity against the chromogenic substrate S2222 (Chromogenix), confirmed that the trypsinogen folded correctly and was activated by enterokinase to give functional β-trypsin. It should be noted that when minimal medium alone was used for expression (to produce the uniformly ^{15}N-labeled protein), expression levels were much lower.

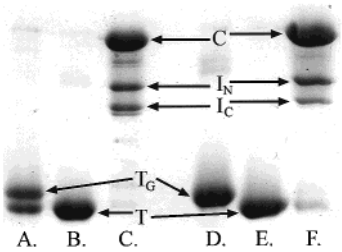

FIGURE 1: SDS−PAGE of plasma and recombinant bovine trypsin/trypsinogen. Lane A, commercial pancreatic trypsinogen (the second, lower band is contaminating trypsin that is present in the commercial material); lane B, pancreatic bovine β-trypsin; lane C, pancreatic bovine β-trypsin incubated 1:1 with recombinant α_1PI Pittsburgh to form SDS-stable high molecular weight serpin−proteinase complex; lane D, recombinant bovine trypsinogen; lane E, recombinant bovine β-trypsin generated by activation of trypsinogen with enterokinase; lane F, recombinant β-trypsin incubated 1:1 with α_1PI Pittsburgh. All lanes contained ~3 μg of trypsin or trypsinogen. The designations C, I_N, I_C, T_G, and T are covalent complex, native inhibitor, cleaved inhibitor, trypsinogen, and β-trypsin, respectively.

[^{1}H-^{15}N]-HSQC of Uniformly Labeled Bovine Trypsin. To determine how much overlap of resonances there is in the HSQC spectrum when all amides are NMR-visible, and at the same time provide additional evidence that the S195A recombinant trypsin was correctly folded, we produced uniformly ^{15}N-labeled S195A trypsin and recorded its [^{1}H-^{15}N]-HSQC spectrum. The spectrum showed good dispersion in both ^{1}H and ^{15}N dimensions (Figure 2), with approximately 200 non side chain amide resonances resolved in the 227 residue protein. Only in the central region of the spectrum was resolution of individual amides poor. However, the good line shape for those resonances that were well-dispersed, together with close to the expected number of signals being visible, strongly suggested that only a single, globular protein of the expected size was present. To our knowledge, this spectrum represents the first heteronuclear 2D spectrum of trypsin, reflecting the previous difficulty of obtaining large enough amounts of ^{15}N-labeled trypsin in a cost-effective manner. Nevertheless, both because of the extensive overlap in the central region and because of the lower yield of trypsinogen obtained from minimal medium compared with amino acid-supplemented medium, all further studies were carried out on ^{15}N-alanine-labeled trypsin.

[^{1}H-^{15}N]-HSQC of ^{15}N-Alanine-Labeled Bovine S195A and S195A/D189S Trypsins. When S195A trypsinogen was expressed with ^{15}N label only in alanine, the S195A trypsin obtained upon activation with enterokinase gave a very simple [^{1}H-^{15}N]-HSQC NMR spectrum consisting of 15 resonances (Figure 3). Whereas most resonances were of similar line width, three resonances, shown below to be sensitive to complex formation (resonances 7, 11, and 12), were broader and showed evidence of multiple conformations with slightly different chemical shifts. In addition, the resonance numbered 5 in Figure 3 had very low intensity, though it was consistently present also in spectra of complexes (see below) and is at the same position as a resonance from the D189S variant. Since the total number of resonances is as expected based on the sequence of wild-type trypsin with the S195A mutation (15 alanines), this

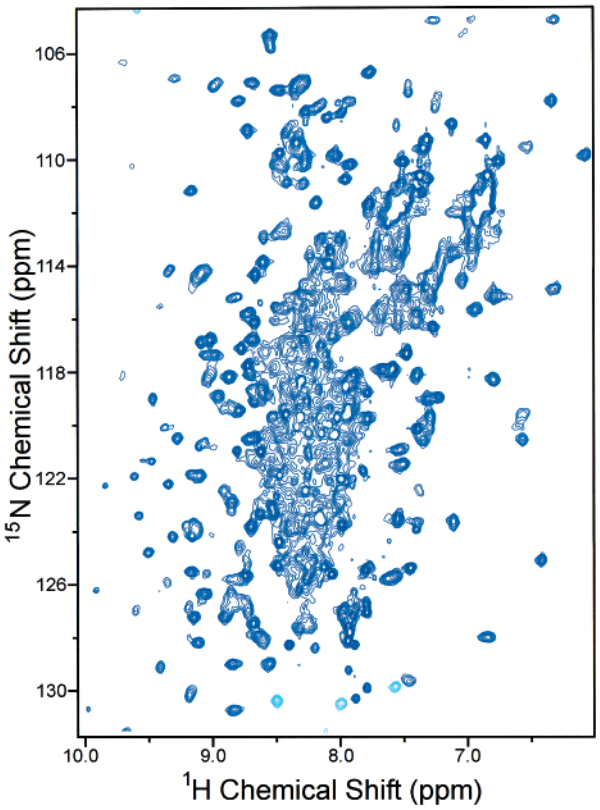

FIGURE 2: [^{1}H-^{15}N]-HSQC of uniformly ^{15}N-labeled recombinant bovine trypsin (360 μM), showing good dispersion, as expected for a folded globular protein, but with considerable spectral overlap in the central region.

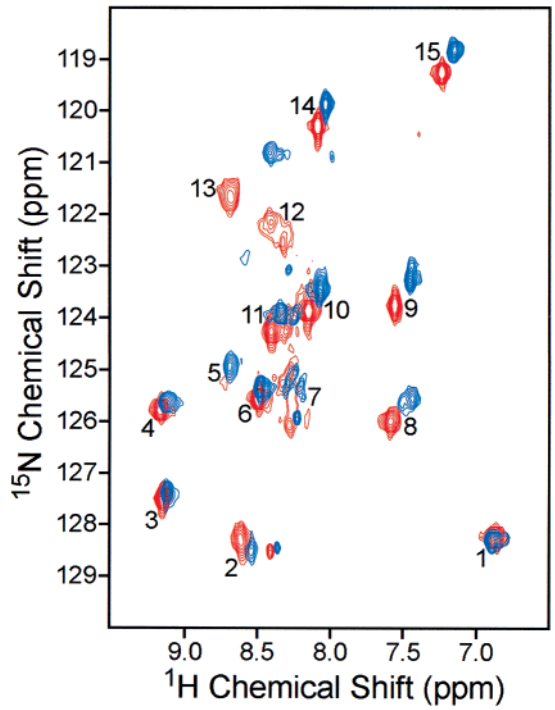

FIGURE 3: [^{1}H-^{15}N]-HSQC spectra of ^{15}N-alanine-labeled bovine β-trypsins. (Red) S195A trypsin; (blue) S195A/D189S, showing minor perturbation of many of the trypsin resonances as a result of the single mutation. The numbers are those used in the text to identify specific resonances.

suggests that there has been no significant scrambling of the label and leakage into other amino acids during bacterial growth and trypsinogen expression. The spectral dispersion was good, with a chemical shift range of 2.0 ppm for ^{1}H and of 10 ppm for ^{15}N, which reflects the diverse environments for alanine in the structure of bovine trypsin (*3*) (Figure 4).

For separate studies on covalent serpin−trypsin complexes [see following paper, (*10*)], we were also interested in preparing and examining the D189S variant of trypsin. This was first prepared by Gráf and colleagues (*18*) for the rat enzyme in an attempt to engineer chymotrypsin specificity into trypsin through removal of the negatively charged aspartate 189 from the bottom of the S1 specificity pocket. It was recently confirmed for rat anionic trypsin that the single mutation causes more extensive structural changes, particularly in the activation domain (*19*). The [^{1}H-^{15}N]-HSQC NMR spectrum of bovine S195A/D189S trypsin reflects these more extensive structural changes arising from the single mutation, through chemical shift changes for many of the resolved resonances (Figure 3). In addition, one resonance is missing, which may be due to chemical exchange. Although many of the changes are only 0.1−0.2 ppm in either the ^{1}H or the ^{15}N dimension, there is at least one resonance (number 13 in Figure 3, at 121.5 ppm/8.7 ppm) that must be perturbed by a minimum of 0.5 ppm in ^{15}N chemical shift and 0.3 ppm in ^{1}H chemical shift. Although we have not assigned this alanine specifically, we know from other assignments (see below) that it is not A55, A56, or A195 and is more likely to be one of the two alanines close to residue 189 (Figure 4).

Effect of Activation on the [^{1}H-^{15}N]-HSQC Spectrum of ^{15}N-Alanine-Labeled Bovine S195A and S195A/D189S Trypsinogens. S195A trypsinogen gave a well-resolved HSQC spectrum, with 14 of the expected 15 peaks visible (Figure 5A, green resonances). An overlay of this spectrum with that of the activated S195A trypsin (Figure 5A) shows that most of the peaks are shifted, with some perturbed by over 1 ppm in ^{15}N chemical shift. The spectrum of S195A/D189S trypsinogen was very similar to that of the S195A trypsinogen, with most peaks at nearly identical positions (Figure 5B). Upon activation, most of these peaks were perturbed similarly to activation of S195A trypsin, though the spectrum of the activated S195A/D189S trypsin differs from that of S195A trypsin in the ways noted above (Figure 3).

Effect of Formation of Noncovalent Complexes on the [^{1}H-^{15}N]-HSQC Spectra of ^{15}N-Alanine-Labeled Bovine S195A Trypsin and S195A/D189S Trypsin. We next examined the effect on the trypsin HSQC spectrum, for both S195A and S195A/D189S trypsins, of formation of noncovalent complex with three protein proteinase inhibitors of different size and family, including the serpin α_1PI Pittsburgh. Although active β-trypsin forms *noncovalent* complexes with bovine pancreatic trypsin inhibitor (BPTI) and soybean trypsin inhibitor (SBTI), without significant conformational change, it forms *covalent* complexes with the serpin α_1PI Pittsburgh variant, accompanied by major conformational rearrangement of the serpin (*20, 21*). Here we wanted to compare the *noncovalent* complexes formed between trypsin and BPTI or SBTI with the initial *noncovalent* complex that α_1PI Pittsburgh forms en route to covalent complex formation. We therefore used the inactive S195A trypsin variant or S195A/D189S variant to generate all three complexes, since these inactive trypsins can form only noncovalent complexes with serpins. S195A trypsin is essentially the same species as anhydrotrypsin in

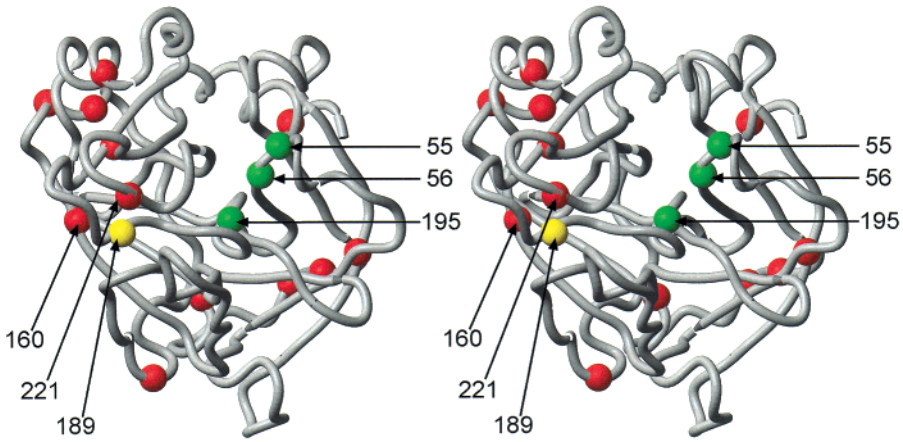

FIGURE 4: Stereoview of bovine trypsin, showing the location of the 14 alanines present in the wild-type protein, together with the positions of residue S195 (green) of the active site and D189 at the bottom of the S1 specificity pocket (yellow). Alanines 55 and 56 are also colored green and numbered. Alanines 160 and 221, which are close to D189, are indicated.

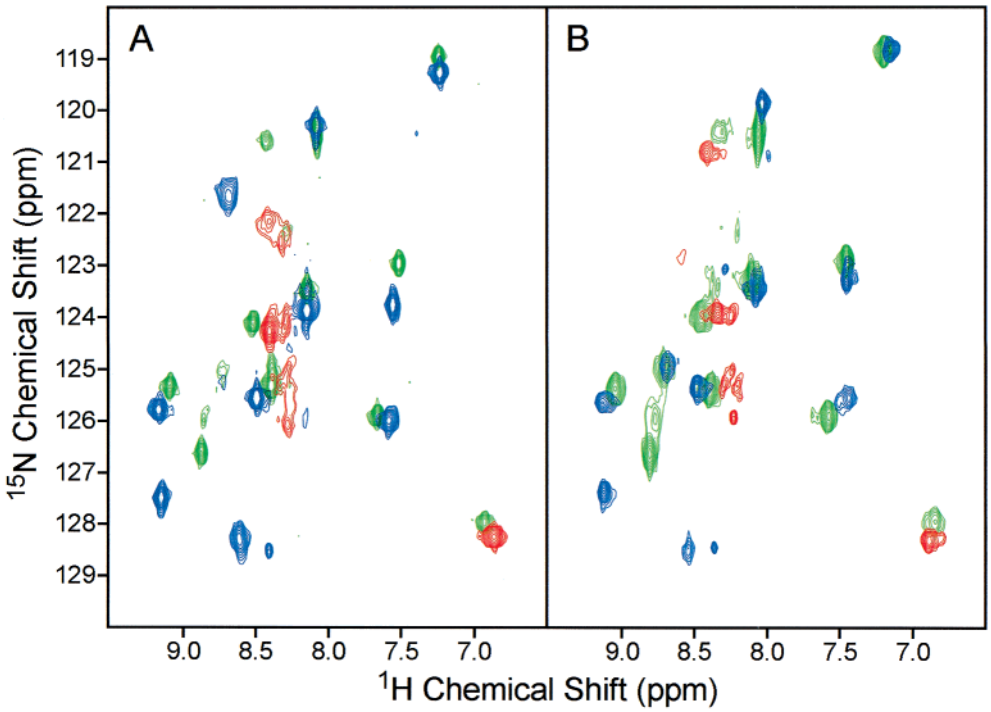

FIGURE 5: [^{1}H-^{15}N]-HSQC spectra of ^{15}N-alanine-labeled bovine β-trypsins overlaid by spectra of their zymogen forms. Panel A, S195A trypsin and trypsinogen; panel B, S195A/D189S trypsin and trypsinogen. The same color key is used for both panels. The zymogen spectrum is shown in green and the β-trypsin spectrum in blue and red. The 10 resonances shown in blue are ones whose chemical shifts are insensitive to noncovalent complex formation, whereas those in red are from the remaining 5 alanines, which can show large perturbations in chemical shift and, as a result of exchange, in line width to the point of invisibility. This same blue/red color scheme for β-trypsin is also used in Figures 6 and 7.

which the active site serine has been converted chemically to dehydroalanine by β-elimination of a phenylmethylsulfonyl fluoride adduct (*22*). X-ray structures are available for complexes of anhydrotrypsin with BPTI (*23*) (1TPA) and of rat S195A trypsin with pancreatic trypsin inhibitor (3TGJ). It is also known that anhydrotrypsin binds tightly ($K_d \sim 5$ nM) and noncovalently to α_1PI Pittsburgh (*17*).

The HSQC spectrum of ^{15}N-alanine-labeled S195A trypsin with BPTI gave 14 well-resolved resonances, including a trace of resonance 5, with 10 resonances in positions identical to ones in the spectrum of uncomplexed S195A trypsin (Figure 6B). The remaining resonances showed quite large changes in position, and disappearance for one of them. The complex between S195A/D189S trypsin and BPTI behaved analogously, though here no resonances were broadened beyond detection and resonance 5 was very clearly defined. Thus, the spectrum of the S195A/D189S−BPTI complex shows all 15 resonances, as well-defined, homogeneous peaks (Figure 6F), with the same 10 resonances (shown in blue) unperturbed by complex formation and the remaining 5 perturbed by significant amounts, depending on what the correspondence is between the red resonances in Figures 6E and F.

Complexes of S195A trypsin and S195A/D189S trypsin with the larger inhibitor SBTI resulted in similar types of spectral changes to complexes with BPTI. Thus, for S195A trypsin, the complex spectrum (Figure 6C) is very similar to that in Figure 6B, with the blue resonances being

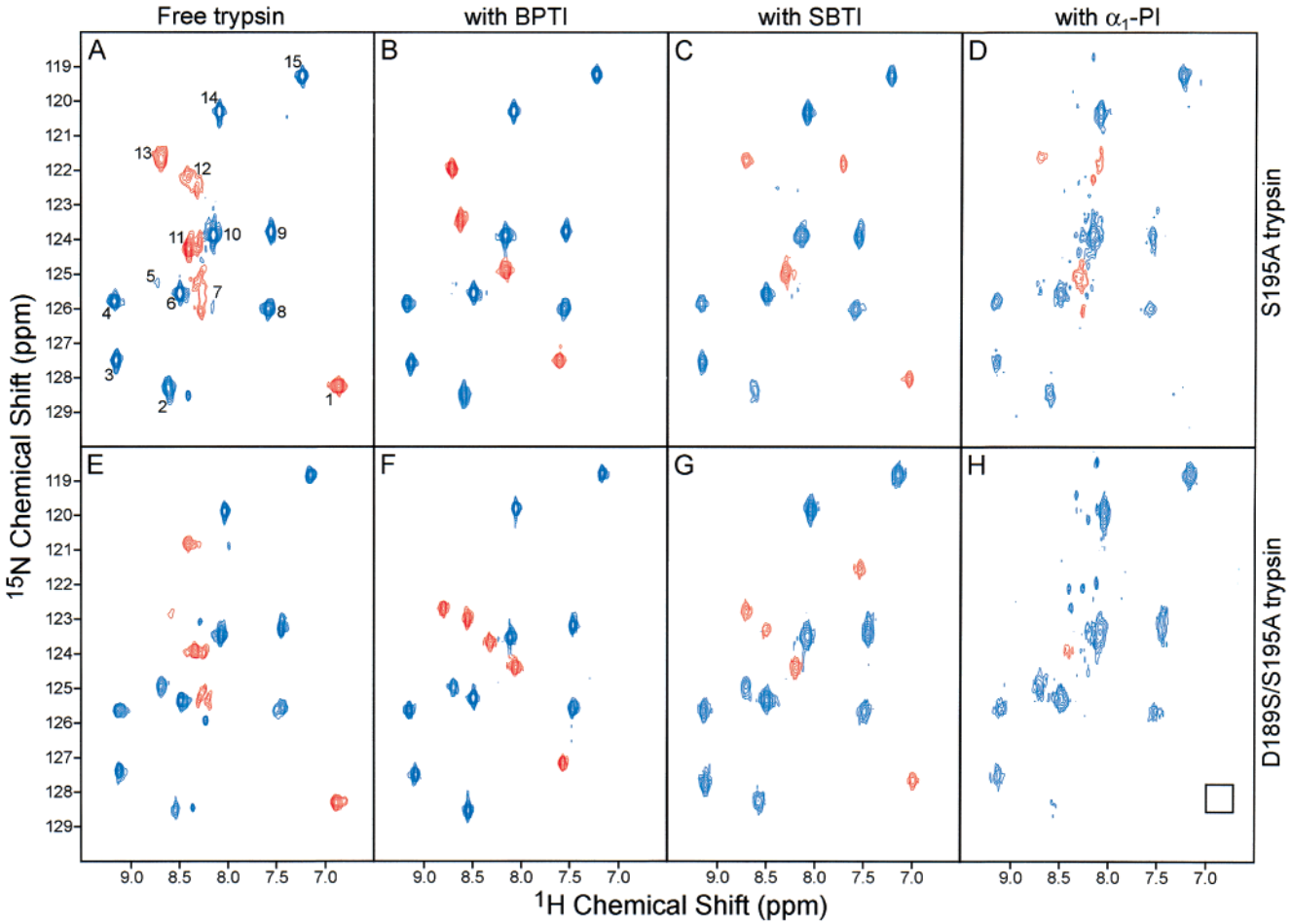

FIGURE 6: Effect of formation of noncovalent complexes on the [^{1}H-^{15}N]-HSQC spectra of ^{15}N-alanine-labeled bovine β-trypsins. Top panels, S195A trypsin; bottom panels, S195A/D189S trypsin. Left to right: trypsin alone, molecular mass 24 kDa (panels A and E); trypsin−BPTI, molecular mass 31 kDa (panels B and F); trypsin−SBTI, molecular mass 44 kDa (panels C and G); trypsin−α_1PI Pittsburgh, molecular mass 69 kDa (panels D and H). Coloring of resonances is as in Figure 5. Note the invariance in positions of the blue resonances within a horizontal series of spectra, but the large variation in position and intensity for the red resonances. The box in panel H identifies the location of the resonance from A56, which is visible only at a lower contour level.

unperturbed and the red resonances again being at different positions compared with uncomplexed trypsin. Two of the peaks marked as red, however, were at quite different positions in spectra of the two complexes. The same pattern was seen for S195A/D189S trypsin, with the same 10 blue resonances being unaffected by complex formation and the remaining 5 being at different positions, both compared to uncomplexed trypsin and compared to the complex with BPTI. For both trypsins, this thus shows that those peaks that are sensitive to complex formation are affected differently by different inhibitors.

The spectra obtained for the two noncovalent complexes of the much larger inhibitor α_1PI Pittsburgh with S195A trypsin or S195A/D189S trypsin were of slightly inferior quality, but still well enough defined to permit useful comparison with spectra of the other complexes and with spectra of uncomplexed trypsin. For each complex with α_1-PI, the same pattern of unperturbed and perturbed resonances was seen as for the equivalent complexes with BPTI and SBTI. Thus, for S195A in complex with α_1PI, the same nine well-defined and one faint unperturbed (blue) resonances were present, while three of the five complex-sensitive (red) resonances were clearly visible at modestly different chemical shifts from each of the other two complexes (Figure 6D). The complex of α_1PI Pittsburgh with S195A/D189S had all 10 unperturbed (blue) resonances present, while only 2 of the complex-sensitive resonances were discernible (Figure 6H) (1 is not seen at the contour level used in the figure and is boxed).

Selected Alanine Assignments by Site-Directed Mutagenesis. To better define the origins of the complex-sensitive (red) and complex-insensitive (blue) resonances in the above spectra, we prepared a set of three variants for each of the wild-type and D189S trypsins to permit assignment of the alanines in and near the active site, at positions 55, 56, and 195. For each trypsin, these variants were S195G, A55G, and A56G. HSQC spectra were recorded for each protein alone and in complex with BPTI to permit assignments in both uncomplexed and complexed states. The resulting spectra are shown for the D189S set in Figure 7, and all of the assignments for both D189S and wild-type trypsin are included in Table 1.

Interpretation of the spectra was not as straightforward as hoped, since the mutations themselves had some secondary effects on other of the red alanine resonances. In particular, change of A55 to glycine appeared to have a major effect on the resonances of A56 and A195. However, since for both uncomplexed and BPTI-complexed trypsin none of the mutations led to either loss or perturbation of any of the 10 blue resonances, we can be sure that the 5 red, complex-

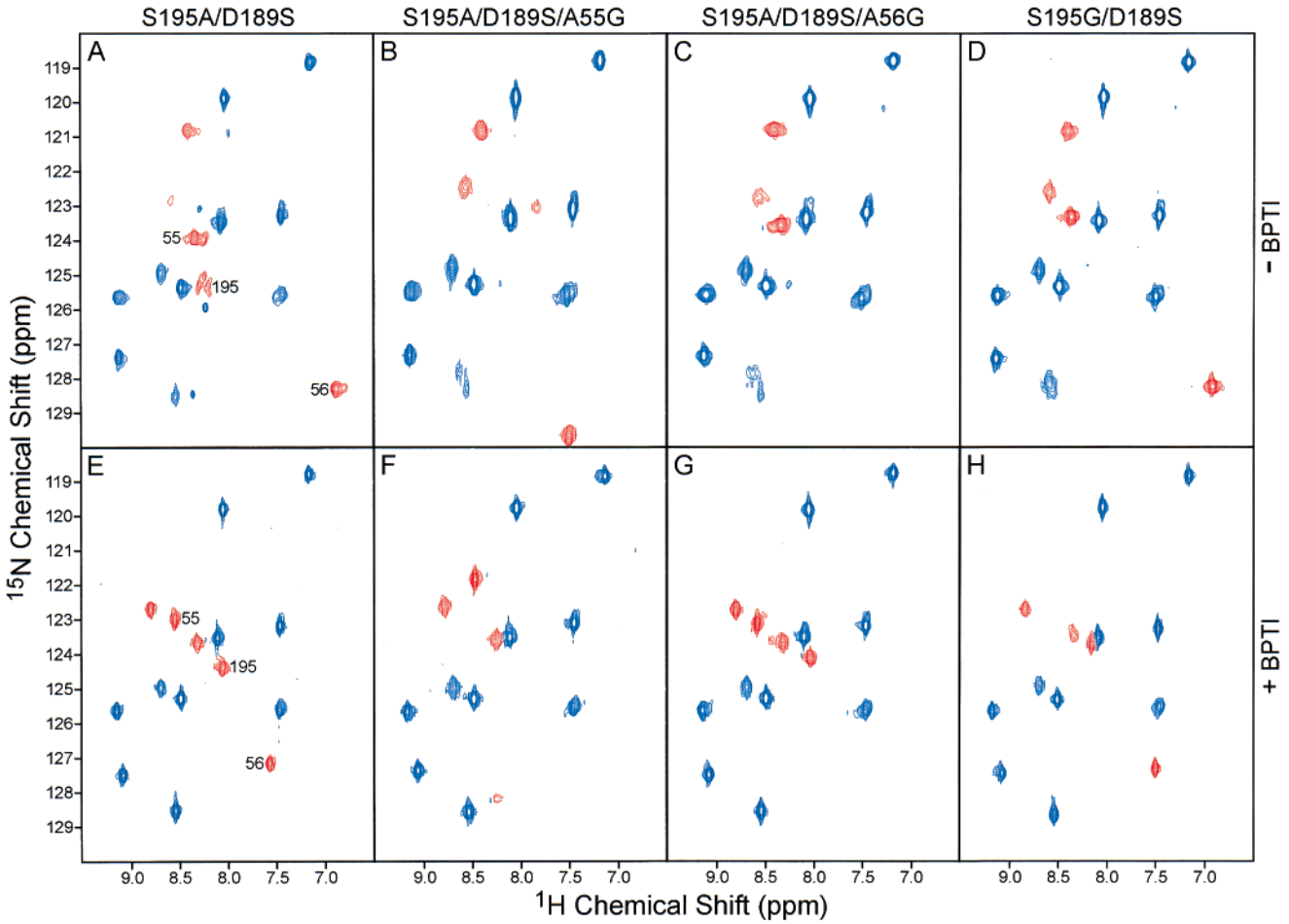

FIGURE 7: [^{1}H-^{15}N]-HSQC spectra of ^{15}N-alanine-labeled bovine D189S-containing β-trypsins used for assignment of A55, A56, and A195. Top panels, trypsin alone; bottom panels, noncovalent complexes with BPTI. From left to right: S195A/D189S trypsin (panels A and E); S195A/D189S/A55G trypsin (panels B and F); S195A/D189S/A56G trypsin (panels C and G); S195G/D189S trypsin (panels D and H).

Table 1: Assignments for Alanines in S195A and S195A/D189S Trypsins and Their Complexes with BPTI

	^{15}N chemical shift (ppm)			^{1}H chemical shift (ppm)		
species	A55	A56	A195	A55	A56	A195
S195A	124.3	128.3	125.6	8.41	6.86	8.27
+BPTI	123.5	127.6	125.0	8.62	7.61	8.16
S195A/D189S	124.0	128.4	125.3	8.34	6.90	8.25
+BPTI	123.1	127.2	124.5	8.55	7.57	8.05

sensitive resonances include the active site 195 and the nearby alanines 55 and 56. Inspection of the structure of trypsin (Figure 4) shows that there are only three other alanines that are possible candidates for local perturbation either by the mutations introduced or else by formation of complex with proteinase inhibitors. These are alanines 160, 183, and 221, which are 8.4, 9.3, and 4.7 Å, respectively, from the Cα of residue 189, which lies at the bottom of the S1 specificity pocket and which forms a salt bridge with the P1 lysine or arginine side chain in wild-type trypsin−inhibitor complexes. In the uncomplexed state, it is clear from comparison of the S195A and A56G spectra of D189S trypsin that A56 is at 128.4/6.9 ppm. Similarly, it is clear from comparison of the S195A and S195G spectra that A195 is at 125.3/8.3 ppm (Figure 7), though with possibly compound appearance of the resonance. The most perturbing mutation was the A55→G replacement, which appeared to shift both the A195 and A56 resonances markedly. The A55G spectra, for both uncomplexed (Figure 7B) and BPTI-complexed trypsin (Figure 7F), are therefore most perturbed relative those of the S195A forms (Figures 7A and D).

DISCUSSION

We have described the first successful large-scale expression and refolding of bovine trypsinogen and the extension of this to the incorporation of specifically ^{15}N-labeled alanine. This has allowed us to collect high-quality, well-resolved [^{1}H-^{15}N]-HSQC NMR spectra of both the trypsinogen and trypsin forms of the protein and hence to monitor the structural changes that occur both upon zymogen activation and upon noncovalent complex formation with three protein proteinase inhibitors of different sizes and mechanistic classes. The sensitivity of our 2D NMR approach is well demonstrated by the perturbation of most of the resonances of both S195A trypsinogen and S195A/D189S trypsinogen upon activation to trypsin. X-ray crystallography shows that the overall folds of trypsinogen and trypsin are the same, but that activation results in structural changes in the N-terminal activation domain and consequent ordering of the 186−194 loop, thereby giving access to the substrate binding cleft and fully forming the oxyanion hole needed for stabilization of the tetrahedral intermediate of the proteolysis reaction (*24*). These relatively modest structural changes are nevertheless sufficient to perturb most of the alanine resonances in the HSQC spectrum, although some of the perturbations are very small.

Spectra of complexes of S195A trypsin with BPTI and SBTI confirmed that, with the exception of active site residues 195, 55, and 56, and 2 other alanines probably close to the bottom of the S1 specificity pocket, there are no structural perturbations within the remaining body of the proteinase, by virtue of the chemical shift invariance of resonances from the remaining 10 alanines, which are well distributed throughout the protein. These results provide a validation of the linkage between X-ray and NMR results that then permits interpretation in structural terms of the NMR spectra obtained with the noncovalent α_1PI−trypsin complex, for which no X-ray structures are available. We can thus be confident that the invariance of the same 10 resonances in the complexes of either S195A trypsin or S195A/D189S trypsin with α_1PI Pittsburgh requires that the global structure of the trypsin must be the same in these complexes as in the complexes with BPTI and SBTI. Taken together with our previous NMR study of the same noncovalent α_1PI Pittsburgh−trypsin complex examined by ^{15}N-alanine label in the serpin, which showed no structural change in the *serpin* (*8*), the present results show that formation of the noncovalent serpin−proteinase complex must involve the same type of docking, without structural change in either proteinase or inhibitor as occurs for more traditional inhibitors of the Kunitz, Kazal, and Bowman−Birk families (*25*). Such a conclusion is mechanistically important for two reasons. First, the structure is likely to serve as a good model for the initial noncovalent Michaelis-like complex that serpins form with target proteinases en route to the covalent kinetically trapped complex. Second, there is an intriguing report of a serpin apparently acting as an inhibitor of the single-chain zymogen form of urokinase (scuPA) by a reversible noncovalent mechanism (*26*), which might resemble the type of complex examined here.

Effect of the D189S Mutation. Besides providing information on the nature of the noncovalent serpin−trypsin complex, the present studies have also provided insight into the effects of the D189S mutation on the trypsin structure. An earlier structure of the complex of the D189S variant of *rat anionic* trypsin with BPTI had shown that the trypsin moiety was essentially identical to wild-type trypsin (*27*). In contrast, a more recent structure of uncomplexed D189S rat anionic trypsin has shown much larger structural differences from wild-type trypsin (*19*), which would require significant structural changes, particularly in the activation domain, upon complex formation with BPTI. Our NMR results, on bovine cationic trypsin, support the structural differences in uncomplexed D189S trypsin versus wild-type trypsin, but do not support the proposal that there is a major structural change upon complex formation with BPTI. Instead, the differences in the body of the trypsin that are already present in uncomplexed bovine cationic D189S trypsin are retained in complexes with BPTI, SBTI, and α_1-PI. It seems possible that the X-ray structure of the D189S−BPTI complex was misleading with regard to the normal structure of the complex. In that study, the authors had "rescued" the activity of the D189S variant against arginine-containing substrates by addition of 3 M acetate. This was very effective at restoring catalytic activity. The X-ray structure of the complex was from crystals grown in high acetate with the result that acetate was found at the bottom of the S1 pocket, effectively replacing the carboxyl of the normal aspartate. It therefore seems possible that the acetate not only provided a carboxyl to salt-bridge to the P1 side chain, but also restored the conformation of the rest of the trypsin to that of wild-type trypsin.

Conformational Heterogeneity in Trypsin. It was surprising, given the well-defined resonances that were always present for some amides and sometimes present for other amides, that there was clear evidence of different conformational states shown by heterogeneity or broadening of other resonances. Thus, one can contrast the spectra of S195A/D189S trypsin in complex with either BPTI or SBTI, each of which has 15 well-shaped resonances, with spectra of the equivalent complexes with S195A trypsin, which are missing 2 of the resonances that are known to be present in the spectrum of the uncomplexed S195A trypsin. Likewise, the spectrum of uncomplexed S195A trypsin shows splitting of the resonances from A195, A55, and the unassigned, but complex-sensitive, resonance labeled 12 in Figure 6A. Even for the set of 10 invariant resonances, there is some evidence for conformational interchange. Thus, the resonance marked 5 in Figure 6A has very low intensity, but appears to correspond to an equivalent full intensity resonance in the spectrum of the S195A/D189S variant (Figure 6D). There may thus be some plasticity in conformation, particularly affecting the active site and its immediate vicinity, that can result in adoption of different conformations, even in complexes with tight-binding inhibitors such as BPTI or SBTI.

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