Distortion of the Active Site of Chymotrypsin Complexed with a Serpin[†]

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ABSTRACT: There is no complete understanding of how serine protease inhibitors of the serpin family inhibit their target enzymes. Structural and biochemical studies have suggested that serpins utilize a mechanism that is distinct from the standard mechanism of inhibition proposed for most small protein protease inhibitors. Proton nuclear magnetic resonance spectroscopy was used in the present study to demonstrate a fundamental difference in the atomic environment of the catalytic triad of enzyme in complex with serpins when compared to uncomplexed enzyme and enzyme in complex with standard mechanism inhibitors. This work demonstrates that the active site of chymotrypsin is distorted when complexed to a serpin and makes tenable a mechanism of inhibition in which the serpin induces a conformational change in the enzyme that dramatically reduces or completely abrogates the catalytic activity of the protease.

Serpins comprise a large family of regulatory proteins that include a subfamily of inhibitors of serine proteases from which the family derives its name. The mechanism of inhibition of a serine protease by a serpin is controversial. Many properties of the interaction between serpins and serine proteases are not easily interpreted by the standard mechanism model (Laskowski & Kato, 1980). The standard model was developed from studies on the interaction of proteases with relatively small proteins of the Kazal and Kunitz inhibitor families. This model is based on the strong complementary binding of both the intact and hydrolyzed inhibitors to the active site of the target protease. The key features of this model are that the structure of the reactive loop of the inhibitor is held in a β-strand conformation that is complementary to the enzyme binding cleft and that this conformation is not significantly altered by proteolysis of the active site, P1−P1′ residues. Strong intramolecular associations between the reactive loop and body of the inhibitor constrain the structure of the loop to the β-strand or canonical conformation (Bode & Huber, 1992).

In marked contrast to the reactive loop conformation of standard mechanism inhibitors, X-ray crystallographic methods have demonstrated that the conformation of the reactive loop of uncomplexed and uncleaved (native) serpins is a distorted helix held well away from the body of the serpin by two extended strands with no stabilizing intramolecular contacts (Wei et al., 1994; Song et al., 1995). Therefore, a considerable alteration in the structure of the reactive loop of a native serpin is required for it to adopt a canonical conformation (Wei et al., 1994). Furthermore, unlike standard mechanism inhibitors, serpins are irreversibly inactivated when the reactive loop is cleaved. This is a consequence of a large conformational change and an increase in conformational stability (Carrell & Owen, 1985). The reactive loop residues P14−P1 insert into the body of the protein, converting a five-stranded A-β-sheet into a six-stranded sheet as well as separating the P1 and P1′ residues by approximately 70 Å (Löbermann et al., 1984). Certain biochemical characteristics of serpin−protease interactions are also difficult to reconcile with the standard mechanism model. Some reactions between serpins and their target proteases produce a serpin with a cleaved reactive loop in a rapid hydrolysis reaction that competes with the inhibition reaction (Fish et al., 1979; Schechter et al., 1989; Rubin et al., 1990). This behavior produces an apparent stoichiometry of inhibition for the reaction that is greater than 1, and suggests that stable complex formation is dependent on a rapid isomerization of an intermediate complex form. Finally, serpin−protease complexes break down slowly producing active enzyme and cleaved-inactive serpin (Oda et al., 1977; Padrines et al., 1989; Cooperman et al., 1993) indicating that serpin inhibition is a kinetic rather than a thermodynamic phenomenon.

Information on the structure of serpin−protease complexes is limited. In contrast to the many solved structures of standard mechanism inhibitor−protease complexes, no serpin−protease complex has been crystallized. SDS−polyacrylamide gels of serpin−protease complexes indicate that

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[1] Abbreviations: SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; rACT, recombinant human α-1-antichymotrypsin; BPTI, bovine pancreatic trypsin inhibitor; SBTI, Kunitz-type soybean trypsin inhibitor; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; Chtr, α-chymotrypsin.

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the protease is covalently attached to the inhibitor, implying that complexes may be trapped in the acyl intermediate that is associated with substrate hydrolysis (Travis & Salvessen, 1983). In this study we investigate the structure of serpin−protease complexes using proton nuclear magnetic resonance (NMR) to assess the environment of the His57-Nδ1 proton. This proton participates in a hydrogen bond between Asp 102 and His57 of the catalytic triad of serine proteinases and is characterized by a unique low-field resonance signal (Robillard & Schulman, 1972; Bachovchin, 1985). We find that in a serpin−protease complex this bond is distorted, whereas in complexes with two different standard mechanism inhibitors this bond remains essentially unchanged. This finding further emphasizes the difference between serpins and standard mechanism inhibitors at the level of complex structure, and suggests that the alignment of amino acid residues responsible for protease catalysis is altered in serpin−protease complexes.

EXPERIMENTAL PROCEDURES

Proton NMR spectra were recorded using a Bruker AMX 500MHz spectrometer equipped with an Oxford magnet. Water signal suppression was obtained using a "jump−return" pulse sequence (Plateau & Guéron, 1982). Spectra are reported with chemical shift in ppm from 3-(trimethylsilyl)propionate (d_6). Data analysis was performed on a Silicon Graphics Iris running the Felix 2.30 software package. FIDs were subjected to a convolution based solvent reduction algorithm to suppress water signal and exponential multiplication with 10 Hz line broadening prior to Fourier transformation.

Sample solutions were prepared in NMR buffer: 100 mM Tris-HCl/10−20 mM sodium phosphate (pH as per experiment), 150 mM NaCl, 8%−10% D_2O. The samples were maintained at 298 °K. The pH of each sample was checked before and after spectra were obtained. Concentrations of *N*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated α-chymotrypsin (Worthington) and the recombinant serpin rACT-P3P3′ (gift from the Lexin Pharmaceutical Corporation) were determined as described previously (Rubin et al., 1994). rACT-P3P3′ is a variant of recombinant human α-1-antichymotrypsin (rACT) that forms especially stable complexes with α-chymotrypsin, i.e., more stable than wild type ACT (Rubin et al., 1994). rACT-P3P3′−α-chymotrypsin complexes were formed at room temperature, pH 7.0. An initial inhibitor to enzyme ratio of 1.2 was used to prevent cleavage of the complex by free enzyme (Cooperman et al., 1993). The same complex solution was used to obtain spectra at pH 7.0, 9.0, and 10.1. SDS−PAGE analysis was performed on solutions containing complex and demonstrated that less than 10% of the total rACT−P3P3′ was in the free-cleaved form.

rACT-P3P3′ cleaved in the reactive loop was prepared by incubating 800 μM serpin with 30 μM trypsin in pH 7.2 buffer at 23 °C. The reaction was followed by assaying aliquots of the reaction mixture for residual α-chymotrypsin inhibitory activity. The reaction was stopped when approximately 75% complete by the addition of 100 μM (final) TLCK.

Complexes of bovine pancreatic trypsin inhibitor (BPTI) and α-chymotrypsin were prepared by the addition of 4.5 mg of BPTI (Sigma) to 1.1 mL of 640 μM α-chymotrypsin in standard NMR buffer. α-Chymotrypsin was approximately 97% inhibited. The complex solution was diluted 1:1 with NMR buffer before the spectrum demonstrated in Figure 3 was obtained.

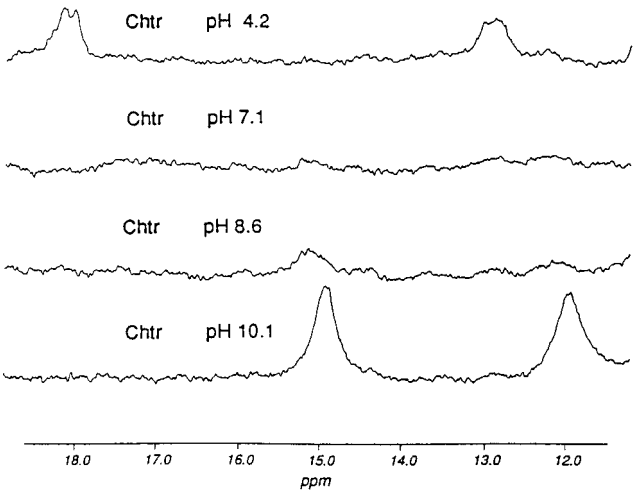


FIGURE 1: 500 MHz ^{1}H-NMR spectra of bovine α-chymotrypsin-A (Chtr) at pH 4.2−10.1. Spectra from top to bottom: 1 mM Chtr, pH 4.2, 1k scans; 1 mM Chtr, pH 7.1, 1k scans; 1 mM Chtr, pH 8.6, 1k scans; 1 mM Chtr, pH 10.1, 1k scans. The pH of each sample was checked before and after spectra were obtained.

Bovine trypsin (Sigma) stock concentrations were determined by active site titration with 4-nitrophenyl-4-guanidinobenzoate hydrochloride (Chase & Shaw, 1980). Kunitz-type soybean trypsin inhibitor (SBTI) was purchased from Sigma. The concentration of SBTI stock solutions was determined by titration of trypsin. The SBTI−α-chymotrypsin ternary complex was formed at an initial inhibitor to enzyme stoichiometry of <0.5 in order to ensure that the ternary complex (E2:I) predominated (Bösterling & Quast, 1981). The mixed α-chymotrypsin−SBTI−trypsin (E1E2:I) ternary complex was formed by the addition of an equimolar amount of α-chymotrypsin to the SBTI−trypsin complex.

RESULTS

The proton NMR properties of the His57-Nδ1 proton of α-chymotrypsin are demonstrated in Figure 1. Uncomplexed α-chymotrypsin shows a titratable peak with a chemical shift of 14.9 ppm at pH > 10 and 18.0 ppm at pH 3.6−4.5 (4 °C and 25 °C). The difference in the chemical shift of the His57-Nδ1 proton at high and low pH reflects the different environments generated by the imidazole form of His57, where Nϵ2 is unprotonated, and the imidazolium form, where Nϵ2 is protonated (Robillard & Schulman, 1972; Bachovchin, 1985). Proton NMR spectra of α-chymotrypsin, δ-chymotrypsin, trypsin, the zymogens chymotrypsinogen and trypsinogen, and the bacterial serine proteases subtilisin and α-lytic protease all demonstrate the low-field resonance signal in the range 13−18 ppm (Robillard & Schulman, 1974a; Bachovchin, 1986; Liang & Abeles, 1987). Extreme linewidth broadening obscures the low-field peak around pH 7.0 in Figure 1 (second spectrum from the top) and is believed to be a result of intermediate exchange conditions at His57-Nϵ2 (Bachovchin, 1986; Liang & Abeles, 1987).

The effect of serpin−chymotrypsin complex formation on the environment of the hydrogen bond is demonstrated in Figure 2 (second spectrum from the top, second from the

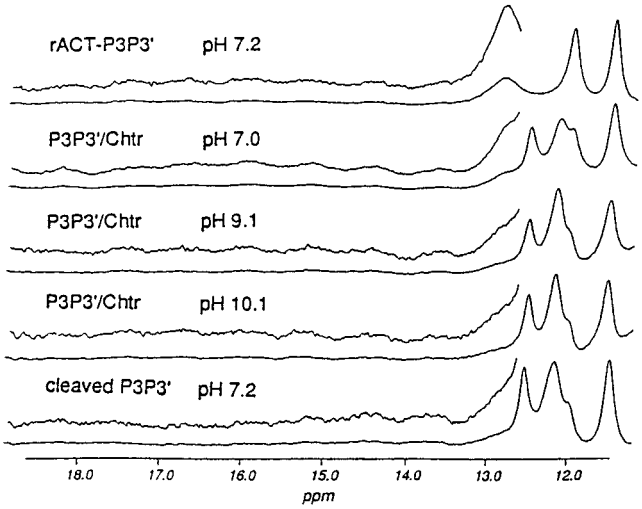


FIGURE 2: 500 MHz ^{1}H-NMR spectra of native rACT-P3P3′, rACT-P3P3′−Chtr complex, and cleaved rACT-P3P3′. Spectra from top to bottom: 1 mM rACT-P3P3′, pH 7.2, 1k scans; 0.4 mM rACT-P3P3′−Chtr complex, pH 7.0, 10k scans; 0.4 mM rACT-P3P3′−Chtr complex, pH 9.1, 2k scans; 0.4 mM rACT-P3P3′−Chtr complex, pH 10.1, 2k scans; 0.7 mM cleaved rACT-P3P3′, pH 7.2, 1k scans.

bottom). In α-chymotrypsin complexed with rACT-P3P3′ (pH 7.0−10.1) the characteristic low-field resonance of the His57-Nδ1 proton is absent. The rACT-P3P3′−α-chymotrypsin complex was studied over a pH range from 7 to 10 to exclude line-width broadening as an explanation for the loss of the low-field signal, similar to that seen with α-chymotrypsin at pH 7. The spectra of the complex did show two new peaks at 12.1 and 12.4 ppm that were not found in either the free enzyme or the uncleaved inhibitor (Figure 2, top). These peaks are not derived from the His57-Nδ1 proton. They are found in the spectrum of rACT-P3P3′ cleaved by trypsin (Figure 2, bottom) indicating that these peaks arise from serpin protons. The observation of the 12.1 and 12.4 ppm peaks in the rACT-P3P3′−α-chymotrypsin complex essentially excludes slow tumbling of the 68 kDa complex as a cause for the apparent loss of the low-field resonance signal of the His57-Nδ1 proton. The absence of the low-field peak in the serpin−protease complex represents either a disruption/distortion of the His57-Asp102 hydrogen bond or increased solvent accessibility and rapid exchange of the His57-Nδ1 proton with the solvent. In either case the active site must be altered.

In contrast to the serpin−enzyme spectra, NMR spectra (Figure 3, top) obtained of α-chymotrypsin complexed with the standard mechanism inhibitor BPTI (7 kDa) retains a low-field peak at 14.9 ppm. The peak did not change upon titration between pH 7.6 and 9.1. Robillard and Schulman (1974b) reported similar NMR findings for the BPTI−δ-chymotrypsin complex for the pH range 4.1−9.4. Not only is the peak present in the BPTI−α-chymotrypsin complex, additional experiments demonstrated decreased exchange of the His57-Nδ1 proton with solvent, this would be expected if access of water to the active site of chymotrypsin is limited in the BPTI−α-chymotrypsin complex. Presaturation experiments demonstrated that the His57-Nδ1 proton of the BPTI−α-chymotrypsin complex exchanged with solvent on a time scale >1 s while spectra of free α-chymotrypsin (pH 8.7−10.7) collected utilizing the "Watergate" solvent suppression method (Piotto et al., 1992) demonstrated exchange

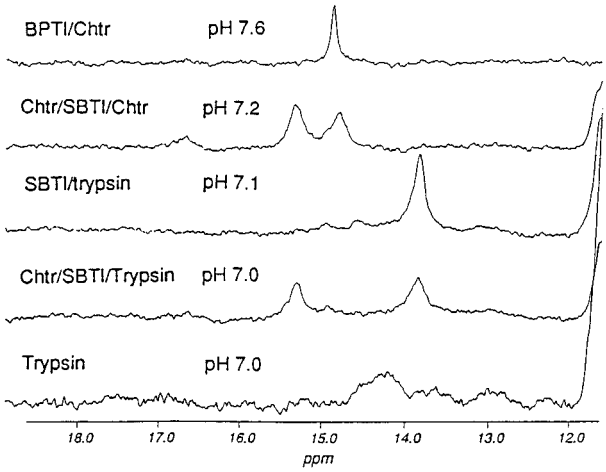


FIGURE 3: 500 MHz ^{1}H-NMR spectra of trypsin and standard mechanism inhibitor−protease complexes. Spectra from top to bottom: 0.3 mM Chtr−BPTI complex, pH 7.6, 1k scans; 0.26 mM Chtr−SBTI−Chtr complex (E_2:I), pH 7.1, 1k scans; 0.5 mM SBTI−trypsin complex (E:I), pH 7.1, 1k scans; 0.3 mM Chtr−SBTI−trypsin ternary complex (E1E2:I), pH 7.0, 1k scans; 0.8 mM trypsin, pH 7.0, 4k scans.

of the His57-Nδ1 proton on a time scale of <10 ms (data not shown).

A second standard mechanism inhibitor, SBTI (22 kDa) was studied in complexes with α-chymotrypsin and trypsin in order to further investigate the state of the low-field resonance signal in standard mechanism inhibitor−protease complex. Arg63 is the P1-residue of the reactive loop of SBTI. This site complexes with α-chymotrypsin and trypsin (DeVonis-Bildlingmeyer et al., 1972). α-Chymotrypsin can also form complexes with SBTI through a secondary, less well characterized, site in which Met84 may be the P1 residue (Bösterling & Quast, 1981). The presence of the two chymotrypsin sites allows for the formation of chymotrypsin−SBTI−chymotrypsin and mixed chymotrypsin−SBTI−trypsin ternary complexes (Bösterling & Quast, 1981). SBTI−trypsin complex (Figure 3, third from the top) demonstrated the presence of the low-field signal of the His57-Nδ1 proton at 13.9 ppm. The ternary complexes chymotrypsin−SBTI−chymotrypsin and chymotrypsin−SBTI−trypsin (Figure 3, second from the top and forth from the top) both showed two peaks, indicating that the low-field signal is retained in interactions at the secondary site. The results of these studies with SBTI−protease complexes demonstrate that the retention of the low-field signal is a common property of complexes formed by the interaction of standard mechanism inhibitors and serine proteases.

The ternary complexes between SBTI and the proteinases have a molecular weight of approximately 70 kDa, the approximate mass of an rACT-P3P3′−chymotrypsin complex. The demonstration of the low-field signal in the ternary complexes supports the conclusion that loss of the signal from the complex is not a result of slow tumbling of the large serpin−protease complex.

DISCUSSION

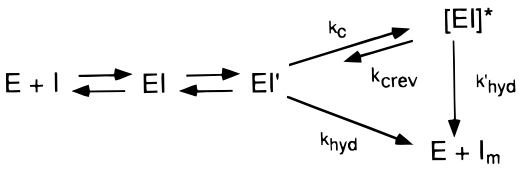
Proton NMR spectroscopic analysis of serpin−protease and standard mechanism inhibitor−protease complexes has demonstrated that the characteristic low-field reaonance signal of the His57-Nδ1 proton of free serine proteases is present in spectra of standard mechanism inhibitor−protease

complexes. Persistence of the low-field signal is consistent with the presence of a structurally intact catalytic triad and a standard mechanism model in which protease inhibition is a function of lock and key type interaction (Laskowski & Kato, 1980). X-ray crystal structures of BPTI−trypsin and SBTI−trypsin complexes demonstrate that the standard mechanism inhibitors do not appear to significantly alter their conformation or the conformation of the bound enzymes (Read & James, 1986).

We show in the present study that the low-field signal of His57-Nδ1 is absent from the spectra of serpin−protease complexes. Absence of the low-field resonance signal has been reported for some cases of proteases modified at Ser195 by small molecule inhibitors (Robillard & Schulman, 1974a; Bachovchin, 1986). The X-ray crystal structure of one such modified protease, tosyl-α-chymotrypsin, demonstrated that formation of the sulfonyl ester bond to Ser195-Oγ resulted in disruption of the Ser195-Oγ/His57-Nϵ2 hydrogen bond and a 0.3 Å rotation of His57 toward the solvent. Otherwise, minimal differences were observed between native and modified enzyme (Henderson et al., 1971). The serpin−protease complex also appears to be a covalent complex with the P1 residue of the serpin bound to Ser195 in a tetrahedral intermediate (Matheson et al., 1991) or an acyl enzyme form (Lawrence et al., 1995; Wilczynska et al., 1995). Thus, the absence of the low-field signal might be a consequence of the modification of Ser195. Alternatively, loss of the low-field signal may be a result of conformational changes in the enzyme induced by interaction with the serpin. Structural changes in the complexed enzyme have been suggested by fluorescence spectroscopy, structural stability, and proteolytic susceptibility analyses (Hervé & Ghélis, 1991; Kaslik et al., 1995). Alterations in the conformation of the enzyme may be driven by structural rearrangements in the serpin associated with insertion of a subset of the reactive loop P14−P2 residues into the A-β-sheet (Wei et al., 1994; Lawrence et al., 1995; Kaslik et al., 1995; Olson et al., 1995). Recent evidence demonstrates that P1−P1′ bond cleavage is necessary for reactive loop insertion and formation of the serpin−protease complex (Olson et al., 1995; Lawrence et al., 1995). The observation of resonance signals at 12.1 and 12.4 ppm that were present in the spectra of the rACT-P3P3′−chymotrypsin complex and trypsin-cleaved rACT-P3P3′ but not intact serpin (Figure 2) supports other evidence that complexed and cleaved serpins undergo similar conformational changes (Mast et al., 1991; Björk et al., 1993; Lawrence et al., 1995; Shore et al., 1995; Olson et al., 1995; Debrock & Declerk, 1995).

The absence of the low-field peak in the serpin−protease complex suggests a mechanism of inhibition. As noted above, the low-field signal is present in virtually every serine protease examined. Craik and co-workers (1987) found that the catalytic activity of a trypsin Asp102→Asn variant was reduced 10 000-fold compared to wild type trypsin. Similar results were reported for an Asp32→Ala variant of subtilisin by Carter and Wells (1988). It has been suggested that the His57-Asp102 hydrogen bond may enhance catalytic activity through the formation of a strong or low-barrier hydrogen bond that facilitates formation of the tetrahedral intermediates of substrate hydrolysis (Frey et al., 1994). Therefore, distortion of the His57-Asp102 hydrogen bond of chymotrypsin complexed to rACT-P3P3′, as suggested by the absence of the low-field proton signal, may indicate that the catalytic activity of chymotrypsin in complex is dramatically reduced or completely abrogated. A kinetic scheme (Scheme 1) for the serpin mechanism proposed by a number of investigators is shown (Patston et al., 1991; Björk et al., 1992; Cooperman et al., 1993; Hopkins & Stone, 1995; Olson et al., 1995).

Scheme 1



The stable inhibitory complex is represented by [EI]*. The kinetic scheme reflects the observation that some serpin−protease interactions clearly demonstrate a rapid substrate-like hydrolysis reaction, k_{hyd}, competing with [EI]* formation. k_c is the conformational change in the complex that prevents rapid substate like hydrolysis. The kinetic scheme proposes two pathways k_{crev} and k'_{hyd} by which [EI]* breaks down slowly to produce active enzyme and cleaved inhibitor, I_m. The present study suggests a molecular mechanism for these two potential pathways. k_{crev} represents a reversal of the conformational change that altered the catalytic activity of the protease. In this case the rate of complex breakdown would reflect the ratio of [EI]*/EI′, k_c/k_{crev}. The second or k'_{hyd} pathway may represent the slow turnover of serpin by a catalytically challenged protease; i.e., the rate of deacylation is dramatically decreased by the altered His57-Asp102 hydrogen bond.

In summary, the data presented here demonstrate that the structure of the active site in serpin−protease complexes is different from either uncomplexed enzyme or enzymes complexed to standard mechanism inhibitors. The distortion of the His57-Asp102 hydrogen bond in a serpin−protease complex may result in a catalytically challenged protease and explain the formation of covalent complexes that break down on time scales of hours or days.

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