Properties of Thrombin- and Elastase-Modified Human Antithrombin III†

Peter Gettins* and Brad Harten

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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ABSTRACT: Proteolytically modified forms of human antithrombin III have been prepared by reaction of native antithrombin with thrombin, human neutrophil elastase, or porcine pancreatic elastase. These forms have two chains disulfide linked and are of the same molecular weight as native antithrombin III. ^{1}H NMR spectroscopy has been used to characterize these proteins and to compare them to one another and to native antithrombin III. The three modified proteins have very similar NMR spectra and histidine residues with identical pH titration parameters, and they undergo the same spectral changes upon binding heparin. They differ from native antithrombin III in all of these respects. In addition, the proteins are much more stable than native antithrombin III. The three modified proteins behave identically as a function of temperature; at 372 K, 44 K above the unfolding temperature for native antithrombin III, the proteins are still folded and possess approximately 70 unexchanged amide protons even after several hours. The unfolding of the heparin binding domain at low concentrations of deuteriated guanidine hydrochloride seen in native thrombin III is absent in the modified forms. It is concluded that the thrombin- and elastase-modified forms of antithrombin have identical structures when allowance is made for the slightly different sites of cleavage by the two types of elastase and by thrombin. This structure is very different from that of native antithrombin III. The increase in thermal stability upon proteolysis parallels that of α_1-antitrypsin and supports the hypothesis of Carrell and Owen [Carrell, R. W., & Owen, M. C. (1985) *Nature (London) 317*, 730–732] that both proteins undergo a transition from strained to relaxed conformation upon proteolysis and that they share a common structure in the C-terminal region.

Antithrombin III (ATIII)[1] is an abundant serum glycoprotein, M_r 58 000 (Petersen et al., 1979; Chandra et al., 1983), which inhibits all the serine proteases of the intrinsic pathway of the blood coagulation cascade (Rosenberg, 1977). In particular, it is thought that its principal target is thrombin (Travis & Salvesen, 1983), with which it forms a stable, covalent 1:1 complex through formation of a carboxylic ester between Arg-393 of the inhibitor and the active-site of thrombin (Longas & Finlay, 1980; Björk et al., 1982). It is well-known that heparin can greatly accelerate the rate at which antithrombin inactivates thrombin and that it does so catalytically (Rosenberg, 1977). However, it has been found that high-affinity heparin can also promote the formation of a reactive-site-cleaved form of antithrombin that is uncomplexed to thrombin (Fish et al., 1979; Björk & Fish, 1982). By maintenance of an ionic strength of 0.01 or less, large amounts (up to 12 mol) of uncomplexed, cleaved antithrombin are produced per mole of thrombin used (Olson, 1985). Recently, it has been shown that catalytic amounts of high-affinity heparin promote the inactivation of antithrombin by human neutrophil elastase (Jordan et al., 1987). This inactivation involves cleavage of antithrombin at the Val^{389}-Ile^{390} and Ile^{390}-Ala^{391} positions (Carrell & Owen, 1985), i.e., only a few residues away from the reactive-site Arg–Ser bond.

Our interest in these proteolytically modified forms of human antithrombin III arises from the possible structural homology between antithrombin and α_1-antitrypsin and the existence of a crystal structure to 3-Å resolution of the proteolytically modified form of α_1-antitrypsin (Löbermann et al., 1984). A structural similarity between these two serine protease inhibitors was first proposed by Hunt and Dayhoff (1980) on the basis of 30% sequence homology in the region that extends from the C-terminus 340 residues back to approximately position 48 in α_1AT and position 74 in ATIII. Alignment of the sequences gives equivalent positions to the reactive-site Arg-Ser and Met-Ser sites of ATIII and α_1AT, respectively (Carrell et al., 1982).

A striking feature of the structure of protease-modified α_1AT is that Met-358 and Ser-359, which previously constituted the reactive-site bond, are separated by 67 Å and are at opposite extremes of the molecule. It has been proposed that both native α_1AT and ATIII are in a stressed conformation and that reaction with protease causes relaxation to a more ordered form (Carrell & Owen, 1985). In support of this, there is evidence to suggest much greater thermal stability for both of these inhibitors in the cleaved forms compared with the native state. A similar increase in stability upon limited proteolysis has been known for ovalbumin for many years (Linderström-Lang & Ottesen, 1947).

In this paper, we use high-resolution ^{1}H NMR spectroscopy to characterize thrombin- and elastase-modified forms of ATIII and to compare their properties with those of native ATIII. It is shown that the conformations of the three protease-modified forms, as judged by nonexchangeable amide proton and upfield-shifted methyl resonances, are nearly identical. Considering the complete ^{1}H spectra, it is seen that native and modified ATIIIs are substantially different. These differences are also manifested in significant alteration of the pK_a's and chemical shifts of some of the five histidine residues. The thermal stability of both modified proteins is dramatically

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* Address correspondence to this author.

[1] Abbreviations: ATIII, antithrombin III; α_1AT, α_1-antitrypsin; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; SDS, sodium dodecyl sulfate; Gdn-DCl, deuteriated guanidine hydrochloride; HNE, human neutrophil elastase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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increased such that unfolding does not take place below 372 K, the highest temperature employed in this study. The domain of antithrombin that is sensitive to guanidine hydrochloride at 0.6 M in the uncleaved form (Villanueva & Allen, 1983; Gettins & Wooten, 1987) no longer unfolds in the modified forms at denaturant concentrations up to 1.8 M. Although heparin still binds to modified ATIII, the ^{1}H NMR perturbations seem to be different from those of native ATIII and may reflect perturbation of contact residues rather than a more extensive change in conformation. It is concluded that the properties of proteolytically modified ATIII support the proposed structural similarity of the C-terminal portion of ATIII to that of proteolytically cleaved α_1AT.

Materials and Methods

Human antithrombin III was isolated from outdated plasma by a modification of the method of Thaler and Schmer (1975) described in detail elsewhere (Gettins, 1987). The plasma was obtained from the Vanderbilt Hospital blood bank.

Ultrapure ammonium sulfate and guanidine hydrochloride were purchased from Schwarz/Mann. Other reagents were obtained from Sigma. The protons of guanidine hydrochloride were exchanged for deuterium by two cycles of freeze-drying concentrated solutions from D_2O.

Thrombin-modified antithrombin was prepared from antithrombin by reaction with thrombin in the presence of heparin at low ionic strength (Olson, 1985). The modified inhibitor was purified by elution from a heparin–Sepharose column (10 mL) by a NaCl step gradient. Modified inhibitor was retained at 0.15 M NaCl but eluted at 0.5 M NaCl. Unmodified inhibitor and inhibitor–thrombin complex were eluted at higher NaCl concentration. The modified protein had the expected electrophoretic properties under reduced and nonreduced conditions (Olson, 1985), migrating faster and slower, respectively, than native ATIII.

Although heparin accelerates the rate of reaction between human neutrophil elastase and ATIII (Jordan et al., 1987), the reaction will proceed in its absence (Carrell & Owen, 1975). Human neutrophil elastase (Athens Research and Technology, Athens, GA) (1:800 w/w) was reacted with human ATIII for 1 h at room temperature in 10 mM HEPES–150 mM NaCl, pH 7.4. The modified ATIII was isolated from a heparin–Sepharose column by NaCl step-gradient elution, as for the thrombin-modified protein. The modified inhibitor gave a single band by gel electrophoresis with properties similar to those of thrombin-modified ATIII.

The porcine elastase-modified ATIII was prepared in the same way, except that it was found that 5-min reaction was sufficient to give the desired cleavage.

Prothrombin was isolated from outdated human plasma according to the procedure of Miletich et al. (1980). α-Thrombin was formed from prothrombin (Lanchantin et al., 1973) by reaction with venom from *Oxyuranus scutellatus* (Owen & Jackson, 1973) (Sigma). Thrombin prepared in this way was pure and free from proteolytically cleaved forms, as judged by gel electrophoresis. The enzyme was stored at −70 °C in 0.6 M NaCl–0.1 M sodium acetate, pH 7.0, at a protein concentration of 2–3 mg/mL until needed and was used immediately after thawing.

Antithrombin samples for NMR analysis were prepared by five cycles of dilution with the D_2O buffer and concentration in an Amicon ultrafiltration unit fitted with a PM30 membrane. Protein concentrations were determined spectrophotometrically using $A^{0.1\%}_{280nm} = 0.65$ (Nordenman et al., 1977) and a molecular weight of 58 000 (Petersen et al., 1979; Chandra et al., 1983) for native ATIII. Björk and Fish (1982) have shown an increase in the extinction coefficient of 7.5% for thrombin-modified bovine AIII. A similar percentage increase was assumed here, and $A^{0.1\%}_{280nm} = 0.70$ was used for proteolytically modified human ATIII. SDS–polyacrylamide gel electrophoresis was carried out in 7% slab gels according to Laemmli (1970).

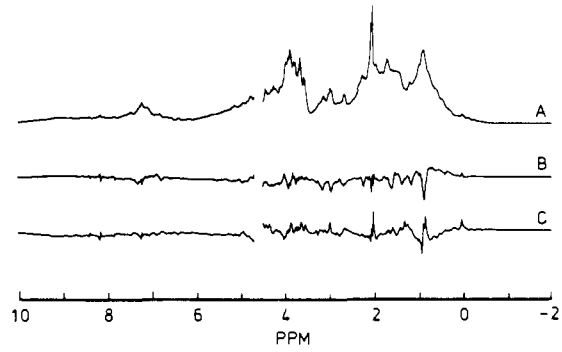

FIGURE 1: (A) 400-MHz ^{1}H NMR spectrum of thrombin-modified ATIII. (B) Difference spectrum between thrombin-modified and HNE-modified ATIII. (C) Difference spectrum between HNE-modified and porcine elastase-modified ATIII. The vertical scale of (B) and (C) is 4 times that of (A) to permit the small perturbations to be seen. The protein concentrations were 0.42, 0.67, and 0.89 mM for thrombin-, HNE-, and porcine elastase-treated ATIII, respectively. The pH was 6.19. 3000 scans were recorded at 310 K. Spectra were carefully normalized prior to computer subtraction to allow for differences in protein concentration.

^{1}H NMR spectra were recorded at 400 MHz on a Bruker AM400 narrow-bore spectrometer. A Carr–Purcell–Meiboom–Gill (CPMG) pulse train $[90°_x-(\tau-180°_y-\tau)_n]$ was used for the pH titration studies to facilitate resolution of the histidine resonances, with $\tau = 1$ ms and $n = 6$. Other spectra were recorded with a 60° pulse angle. All spectra were recorded with selective saturation of residual water proton intensity with a low-power pulse of 0.5-s duration. A sweep width of 6024 Hz and a block size of 8K points zero-filled to 16K prior to Fourier transformation were employed. pH values are reported as direct pH meter readings uncorrected for deuterium isotope effects and were measured in the NMR tube using an Ingold 3-mm diameter combination electrode and an Orion 501 pH meter. pH was adjusted by direct addition of NaOD or DCl solutions. Chemical shifts are given relative to external DSS at 0 ppm.

Histidine pK_a values were calculated by assuming simple ionization behavior and obtaining the best fit of the data to the Henderson–Hasselbalch equation. Since the complete titration range was not observed for two of the histidines, $\Delta\sigma$ (the titration range in ppm) was a variable and was chosen to give the best fit to the theoretical analysis. The values found, 0.95–1.13 ppm, are similar to those found in other proteins (Markley, 1975; Jordan et al., 1985). The error limits are ±0.04 pH unit.

Results

^{1}H Spectra of Modified and Native ATIII. The 400-MHz ^{1}H NMR spectrum of thrombin-modified ATIII is shown in Figure 1A. Beneath this are shown difference spectra between thrombin-modified and HNE-modified ATIIIs (Figure 1B) and between HNE-modified and porcine-elastase modified ATIIIs (Figure 1C). Downfield from the water resonance in Figure 1B, the principal features are negative peaks at 6.85 and 7.10 ppm of comparable intensity, estimated to be of two protons' intensity (from normalization relative to the whole aromatic region spectral envelope). A very slight pH difference of 0.02 pH unit accounts for the appearance of the histidine C(2) protons at 7.84, 8.15, and 8.40 ppm. In the aliphatic

Table I: Titration Parameters for Histidine C(2) Protons in Native and Modified ATIII at 298 K

histidine	native[a]			modified			difference[b]
	pK_a	σ^c	$\Delta\sigma^d$	pK^a	σ	$\Delta\sigma$	$\Delta\sigma$
1	6.90	8.59	1.07	6.90	8.56	1.13	−0.087
3	5.93	8.61	0.89	6.15[e]	8.65	1.03	−0.041
5	5.13	8.65	0.90	5.55	8.58	0.95	+0.028

[a] Taken from Gettins (1987). [b] Difference in chemical shift in ppm at pH 6.19 for histidine C(2) resonances. Native minus modified. [c] Chemical shift of protonated imidazole ring in ppm. [d] Titration range in ppm. [e] Resonance 3 is a composite of resonances 2, 3, and 4 and does not fit well to a single ionization. The data given are for the best fit to a single ionization and are possibly in error by as much as ±0.15 pH unit in pK_a.

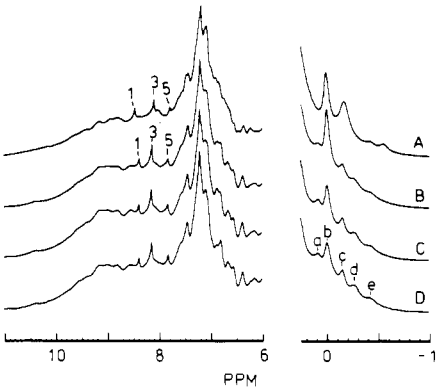

FIGURE 2: Downfield and upfield regions of the ^1H NMR spectra of the three modified ATIIIs and of native ATIII. (A) Native ATIII, 0.80 mM; (B) porcine elastase-modified ATIII, 0.89 mM; (C) HNE-modified ATIII, 0.67 mM; (D) thrombin-modified ATIII, 0.42 mM. Spectra are normalized. The histidine resonances from residues designated 1, 3, and 5 are indicated. Five upfield-shifted methyl resonances that are clearly seen at all temperatures are labeled a–e.

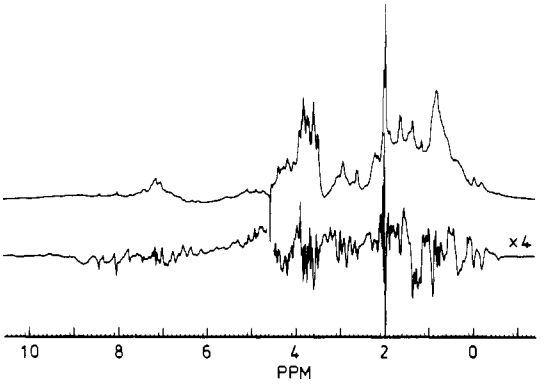

FIGURE 3: ^1H NMR spectrum of (0.58 mM) native ATIII at 303 K and pH 6.35 (top) and difference spectrum with 0.23 mM thrombin-modified ATIII (latter minus former). Spectra were normalized prior to subtraction. The vertical scale of the difference spectrum is 4 times that of the absorption spectrum.

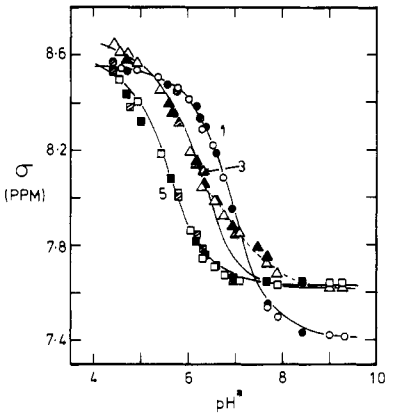

FIGURE 4: Chemical shifts of histidine C(2) protons as a function of pH*. The numbers correspond to those used in the text. Open symbols are for elastase-treated ATIII, closed symbols are for thrombin-treated ATIII, and hatched symbols are for HNE-treated ATIII. CPMG spectra were used. The temperature was 298 K.

region, there is much greater intensity, particularly between 0.8 and 1.8 ppm, the region of methyl group resonances.

If the sites of cleavage by HNE and porcine elastase were the same, their difference spectrum (Figure 1C) should be flat. This is clearly not the case. There is intensity roughly the same as in Figure 1B, though Figure 1B and Figure 1C are quite different in appearance. Thus, the two forms of elastase must cleave at different sites. Overall, the differences represent only a small change between the spectra of the three modified species, involving no more than 40 protons. In the regions of the spectrum that most clearly show the effects of structural differences (the upfield methyl region, the aromatic region—particularly upfield-shifted resonances between 6.8 and 6.2 ppm—and the amide NH region, in which only slowly exchanging protons can be seen), there is a near identity between the spectra of the three proteins (Figure 2B,C,D), suggesting great similarity of structure. In marked contrast is the spectrum of native ATIII (Figure 2A) and the difference between it and the spectrum of thrombin-modified ATIII (Figure 3). Both the absorption spectrum and the difference spectrum are on comparable vertical expansions to those of Figure 1. At least 3 times as many protons (120) differ in absorption position between these two forms than between the three protease-modified forms. In addition to the many more protein aromatic, aliphatic, and amide resonances that differ, there is in the difference spectrum evidence for a significant perturbation of the carbohydrate ring and methyl resonances. The histidine C(2) resonances, from five histidines well distributed in the primary structure, are also very different in position (see below), in contrast to their identity in the three modified ATIIIs.

Histidine Titration Behavior. Although the resonances from all five histidine C(2) protons are resolved in native ATIII (Gettins, 1987), this is not so in modified ATIII, where only three peaks are seen, with one resonance always much more intense than the other two. This is the same situation as with native ATIII complexed to heparin (Gettins, 1987) where resonances 2 and 4 overlap with resonance 3 at most pH values. The composite peak, comprising resonances 2, 3, and 4 in the native ATIII–heparin complex, seems to be equivalent to the most intense peak in protease-modified ATIII. The numbering of the three resolvable histidine resonances here will thus be 1, 3, and 5 to use the same designations as in the earlier study (Gettins, 1987), where peak 3 represents a superpositioning of peaks 2, 3, and 4.

The titration behavior of these resonances as a function of pH* is shown in Figure 4 for the thrombin- and two elastase-modified ATIIIs. The sets of curves for the three proteins are superimposable. Both resonance 1 and resonance 5 can be satisfactorily fitted to a single ionization. The composite resonance 3 does not give such a good fit between pH 6 and pH 8, perhaps reflecting differences in pK_a and $\Delta\sigma$ for the three constituent resonances. The best-fit parameters are given

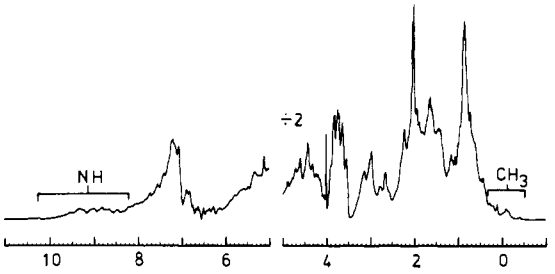

FIGURE 5: ^{1}H NMR spectrum of 0.48 mM thrombin-modified ATIII at 372 K. The aliphatic region is at half the vertical scale of the amide and aromatic regions. Regions in which amide protons and upfield-shifted methyl groups resonate are indicated by "NH" and "CH_3", respectively.

in Table I and compared with those for native ATIII. Resonance 5 has a significantly raised pK_a, resonance 3 has a smaller increase in pK_a, and histidine-1 has only a slight change in titration range when compared to native ATIII. Table I also gives the change in chemical shift of each resonance at pH 6.19 and shows that the chemical shift of resonance 1 is most strongly affected, being shifted upfield by 0.087 ppm.

Thermal Stability. Native ATIII denatures irreversibly at 328 K. This is clearly seen in the ^{1}H NMR spectrum as a collapse of the spectrum to that of a random-coil polypeptide, with loss of all amide NH resonances, upfield-shifted aromatic resonances, and upfield-shifted methyl resonances, together with a general broadening, indicating aggregation (Gettins & Wooten, 1987). Upon cooling, there is no recovery of the original folded structure (Busby et al., 1981; Gettins & Wooten, 1987). The three modified forms of ATIII behave very differently. As the temperature is raised, resonances became sharper and better resolved. Although the temperature was gradually brought to 372 K for the modified proteins, the tertiary structure remained intact, and there was no evidence for any unfolding of a major segment of the proteins. The spectrum of thrombin-modified ATIII at 372 K (Figure 5) shows many unexchanged amide proton resonances (approximately 70 ± 10 compared to about 140 protons at 303 K) as well as 11 upfield-shifted methyl peaks. The appearance of both elastase-modified ATIIIs at this temperature is comparable. Five of the upfield-shifted methyl resonances (a–e of Figure 2) can be resolved throughout the temperature range examined. The chemical shift of each of these resonances is 0.7–1.6 ppm upfield from the frequency expected for a methyl-containing amino acid in a small peptide (Bundi & Wüthrich, 1979) and depends critically on the precise relative positioning of adjacent perturbing groups, particularly aromatic side chains. The greater the upfield shift, the more sensitive will be the chemical shift to alterations in local tertiary structure. Despite this expectation, the most upfield-shifted resonance, e, is almost invariant in absorption position with temperature, indicating that its local environment is strictly unchanged. The other four resonances show a small trend toward diminution of the upfield shift perturbation (0.1 ppm), probably as a result of greater motion of aromatic side chains, giving an averaged, smaller, ring current shift. There is again no indication of unfolding in the vicinity of these groups.

Effect of Guanidine Deuteriochloride. Guanidine hydrochloride (or deuteriochloride) at a concentration of 0.6–0.8 M causes unfolding of one domain of ATIII (Villanueva & Allen, 1983; Fish et al., 1985; Gettins & Wooten, 1987). Subsequent removal of the denaturant fails to restore the original structure and results only in aggregates (Fish et al., 1985). We have previously noted the discontinuous titration behavior of histidine-5 as a function of Gdn-DCl concentration

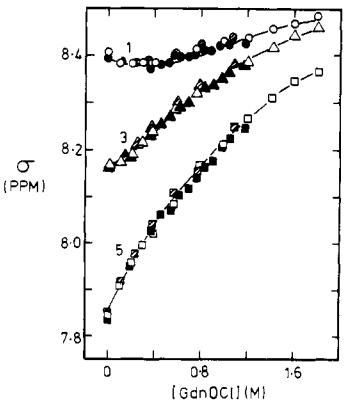

FIGURE 6: Variation in chemical shift of histidine C(2) proton resonances in porcine elastase-modified ATIII (open symbols), thrombin-modified ATIII (closed symbols), and HNE-treated ATIII (hatched symbols) as a function of added guanidine deuteriochloride. Spectra were recorded at 298 K. Initial protein concentrations were 0.30 mM for thrombin-, 0.52 mM for porcine elastase-, and 0.36 mM for HNE-modified ATIII.

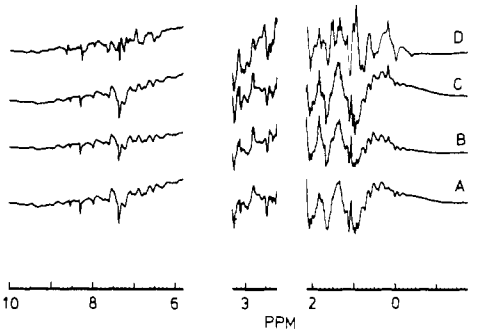

FIGURE 7: ^{1}H NMR difference spectra of ATIII species with heparin minus same sample without heparin. (A) Thrombin-modified ATIII, 0.42 mM; (B) HNE-modified ATIII, 0.67 mM; (C) porcine elastase-modified ATIII, 0.89 mM; (D) native ATIII, 0.80 mM. Spectra were recorded at 310 K and pH 6.19. 3000 scans were recorded for each spectrum. Spectra were normalized. Sufficient heparin was added to saturate the binding sites, being added at 1:1 assuming a conservative value of 1 ATIII binding site per 40 sugar residues in heparin.

as an indication of this unfolding transition (Gettins & Wooten, 1987). The resonance shows an abrupt change of +0.4 ppm[2] at the transition. The titration behavior of the histidine resonances of thrombin- and elastase-modified ATIII (Figure 6) differs from that of native ATIII. All three modified proteins behave the same, but resonance 5 now shows a smooth shift to lower field, without any evidence of an abrupt conformational change. Interestingly, the slight upfield shift of resonance 1 at low Gdn-DCl concentrations and reversal at higher concentrations, with a turning point at about 0.4 M denaturant, is similar to the behavior of native ATIII, though the turning point for the modified ATIIIs is at 0.4 M compared with 0.6 M for native ATIII. Also, resonance 3 shows a biphasic downfield shift with a change in slope at 0.3 M that was not seen with native ATIII (Gettins & Wooten, 1987). This biphasic behavior is more likely to indicate the effects of a weak binding site on ATIII for guanidine deuteriochloride (Gettins & Dwek, 1981) than an abrupt unfolding.

Heparin Binding. The difference spectra between ATIII species complexed and uncomplexed to unfractionated heparin are shown in Figure 7. The difference spectra for thrombin-modified and the two elastase-modified ATIIIs (Figure 7A–C) are almost indistinguishable, indicating a common

[2] A positive chemical shift change indicates a shift to lower field.

Table II: Perturbation of Histidine C(2) Protons in Native and Modified ATIII by Heparin Binding at pH 6.19

histidine	native	modified[a]
1	−0.024[b]	+0.037
3	+0.028	+0.022
5	+0.143	+0.125

[a] Similar values were found for all three modified ATIII species. [b] Change in chemical shift in ppm upon binding heparin. The accuracy is ±0.005 ppm (±2Hz).

binding mode. They are, however, quite dissimilar from the difference spectrum of native ATII (Figure 7D). A prominant feature of Figure 7A–C is the predominant negative intensity of the peaks, resulting from a broadening of resonances in the heparin-bound species. Such a broadening may represent a specific retardation of motion of certain, probably contact, residues. The difference spectrum with ATIII (Figure 7D) is similar to those of Figure 7A–C in intensity, though different in precise resonance chemical shifts. In the aromatic region, this involves about 20–25 protons. In the aliphatic region, however, the resonance intensity corresponds to 70–80 protons in both native and modified species, though again the resonances positions differ between native and modified forms. Since the only individual resonances that can be identified in complexed and uncomplexed ATIII species are the histidine resonances, it is instructive to compare the changes produced by heparin binding to native and to the modified ATIIIs. These values are given in Table II. Perturbations to histidine-1 are −0.024 ppm in native ATIII but +0.037 ppm in modified ATIII. There is also a major difference in perturbation of histidine-5, +0.143 ppm in native versus +0.125 ppm in modified ATIII. In contrast, histidine-3 is affected very similarly in native and modified ATIII by heparin binding.

Discussion

Similarity of Modified ATIII Species. By five criteria considered here, it has been shown that the three modified forms of ATIII generated by nicking native ATIII with thrombin, human neutrophil elastase, or porcine elastase have nearly indistinguishable properties. The high-resolution ^1H NMR spectra of the three proteins (Figure 2) show the same unexchanged amide resonances, the same upfield-shifted aromatic resonances, and the same upfield-shifted methyl resonances (of which there are at least 11). All of these types of resonance depend upon the particular tertiary structure for their resonance position and, for the amide protons, for their exchange rate with solvent. The five histidine residues give rise to three resolved C(2) proton resonances in the spectrum of each protein, with the same chemical shifts, pK_a's, and titration ranges in each (Figure 4). Heparin binding, although of greatly reduced affinity compared to native ATIII (Björk & Fish, 1982), is still in the 10^4–10^5 M^{-1} range and results in perturbation of the same, comparatively few aromatic and aliphatic resonances (Figure 7). Both proteins exhibit the same resistance to thermal denaturation and are capable of withstanding a temperature of at least 372 K without unfolding (Figure 5). The spectra of the two proteins show the same features in the three structure-dependent regions (see above) at each temperature studied. Finally, the titration behavior of the three histidine resonances as a function of guanidine deuteriochloride concentration shows the same pattern in the two modified proteins. Given the diversity of structural reporter groups examined, it is concluded that the tertiary structures are identical with the small exception of local differences caused by small differences in the site of proteolysis for the three enzymes. Since there are small differences in the spectra of HNE- and porcine elastase-treated ATIII, it must be concluded that the sites of cleavage are not identical. Given the preference for cleavage adjacent to alanine residues exhibited by porcine elastase (Nakajima et al., 1979) and the known susceptibility of the Ala^{384}–Ser^{385} bond to proteolysis by *Crotalus* venom (Virca et al., 1982), it is likely that porcine elastase cleaves between these two residues. The sites of cleavage by HNE are between residues Val^{389} and Ile^{390} and between Ile^{390} and Ala^{391} (Carrell & Owen, 1985).

Comparison of Native and Modified ATIII. By each of the criteria used to show the similarity between the three modified forms of ATIII, native ATIII is seen to differ markedly in properties from the modified inhibitors. A minimum of 120 protons differ enough in properties between thrombin-modified ATIII and native ATIII to give rise to intensity in the difference spectrum (Figure 3). Where resonances overlap severely (as here) or shift less than their line width, estimates of intensity in difference spectra give only a lower limit to numbers of protons perturbed. These differences are particularly pronounced in the aliphatic region, including strong intensity in the high-field methyl region. There are also lesser differences in the amide and upfield-shifted aromatic regions of the spectrum.

Of the three resolvable histidine C(2) proton resonances, 1 is most closely similar in pK_a in native and modified ATIII, while 5 is most different. The latter shows an increase in pK_a of 0.42 pH unit, which is, interestingly, very similar to the increase in pK_a of the same histidine side chain caused by heparin binding to native ATIII (Gettins, 1987). Resonance 1 is, however, most strongly perturbed in chemical shift (Table I).

Whereas native ATIII undergoes irreversible thermal denauration at 328 K, modified ATIII has a T_d raised by over 44 K. This is an astonishing increase in stability and perhaps the strongest argument for a major structural change between native and modified forms of ATIII. The increase in stability is also manifested by the failure of a domain, sensitive to low levels of guanidine hydrochloride in native ATIII, to unfold in modified ATIII in the concentration range studied (up to 1.8 M) (Figure 6).

The difference spectra formed upon binding heparin to the modified ATIIIs are dissimilar from that with native ATIII (Figure 7 and Table II). Of course, given the changed conformation and resulting changed chemical shift of many resonances in the two forms of the inhibitor, a given group might be perturbed in the same way by heparin binding to each form of ATIII and yet contribute intensity to the difference spectrum at different frequencies. This notwithstanding, the dissimilar heparin perturbations seen for the histidine resonances (Table II) are unlikely to be the only real differences in chemical shift alterations produced by heparin binding.

Other data that support alteration of conformation between the two forms of ATIII, at least in the heparin binding region, are the reduced affinity for heparin (Björk & Fish, 1982) and the change in environment of Trp-49 (Peterson & Blackburn, 1987) for which there is good evidence to suggest a location in the heparin binding region (Blackburn et al., 1984).

Analogies between Modified ATIII and α_1AT. Two specific results obtained here and one general conclusion argue in support of homologus tertiary structures for the C-terminal portions of ATIII and α_1AT, in their proteolytically cleaved forms. First, the enormous increase in thermal stability of ATIII upon cleavage by thrombin, HNE, and porcine elastase has now been shown to be of the same magnitude as seen previously with α_1AT reacted with papain (Carrell & Owen,

1985). Second, the only differences in the spectra of thrombin- and elastase-modified ATIII are comparatively few sharp aliphatic resonances and small, broader differences in the aromatic region. In cleaved α_1AT, scission of the reactive-center Met–Ser bond results in movement of these residues by 67 Å. The amino acids before and after these residues in the sequence are located in two different regions of β-sheet, which cannot have been true before bond scission.

Since thrombin cleaves at a homologous site in ATIII, a similar β-bonded structure for the two new ends could exist. According to the numbering system of Löbermann et al. (1984), residues Ile^{390}-Ala^{391}-Gly^{392}-Arg^{393} would lie at the end of β-strand A4 and be hydrogen bonded in antiparallel fashion to Ser^{365}-Val^{364}-Tyr^{363}-Leu^{362} at the end of strand A5. Residues Ser^{394}-Leu^{395} etc. would be located in sheet C, strand 1. For human neutrophil elastase-treated ATIII, the residues Ile^{390}-Ala^{391}-Gly^{392}-Arg^{393} would still be attached to the reactive-site serine and, since they might then protrude beyond the end of sheet C into solution, would be very mobile and give rise to much sharper resonances than in thrombin-treated ATIII. In addition, since strand A4 would be four residues shorter, the adjacent residues in strand A5 (Ser^{365}-Val^{364}-Tyr^{363}-Leu^{362}) would have different environments in thrombin-treated and human neutrophil elastase-treated ATIII. The observed sharp aliphatic resonances between 0 and 2 ppm in the difference spectrum between the two modified inhibitors (Figure 1) occur at or close to the expected chemical shifts for the residues isoleucine, alanine, and arginine (Bundi & Wüthrich, 1979).

Finally, the general conclusion of a large conformational difference between native and protease-modified ATIII is as expected if the formerly joined arginine and serine residues (in thrombin-cleaved ATIII) were to become separated by 67 Å. Proteolytic cleavage of ATIII at close but distinct sites results in ATIII species with very similar properties that differ substantially from those of native ATIII. This common transformation is as predicted on the strained (S) to relaxed (R) conversion model of Carrell and Owen (1985) in which scission of the exposed loop of peptide that encompasses the various sites of cleavage results in the same S → R transition.

Summary

We have provided strong evidence for the near identity of structure of thrombin-modified, human neutrophil elastase-modified, and porcine elastase-modified ATIII species as evidenced by similarity of NMR resonances, stability to heat and denaturant, perturbation by heparin binding, and properties of the histidine residues. In contrast, native ATIII differs markedly from the modified proteins in each of these properties, indicating a major structural difference between the two forms. The properties of modified ATIII and the major change in conformation upon cleavage with proteases are as expected if the structure of modified ATIII were homologous to that of modified α_1AT in the C-terminal region and underwent an S → R transition upon cleavage of an exposed "active center"-containing stretch of peptide.

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Registry No. ATIII, 9000-94-6; elastase, 9004-06-2; thrombin, 9002-04-4.

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