Antithrombin III and Its Interaction with Heparin. Comparison of the Human, Bovine, and Porcine Proteins by ^{1}H NMR Spectroscopy†

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ABSTRACT: ^{1}H NMR has been used to characterize and compare the structures of antithrombin III from human, bovine, and porcine plasma as well as to investigate the interactions of each of these proteins with heparin fragments of defined length. The amino acid compositions of the three proteins are very similar, which is reflected in the gross features of their ^{1}H NMR spectra. In addition, aromatic and methyl proton resonances in upfield-shifted positions appear to be common to all three proteins and suggest similar tertiary structures. Human antithrombin III has five histidine residues, bovine has six, and porcine has five. The C(2) proton from each of these residues gives a narrow resonance and titrates with pH; the pK_a's are in the range 5.15–7.25. It is concluded that all histidines in each protein are surface residues with considerable independent mobility. The carbohydrate chains in each protein also give sharp resonances consistent with a surface location and motional flexibility. The ^{1}H spectra are sensitive to heparin binding. Although heparin resonances obscure protein resonances in the region 3.2–6.0 ppm, difference spectra between antithrombin III with and without heparin show clear perturbation of a small number of aromatic and aliphatic protein protons. These resonances include those of histidine C(2) and C(4) protons, of 10–20 other aromatic protons, of a methyl group, and also of protons with chemical shifts similar to those of lysine and/or arginine side chains. For human antithrombin III, it was shown that heparin fragments 8, 10, and 16 sugar residues in length result in almost identical perturbations to the protein. In contrast, tetrasaccharide results in fewer perturbations. Significantly, intact high molecular weight heparin causes the same spectral perturbations as the 16-residue fragment. These data are discussed in terms of requirements for heparin binding.

In recent years, much effort has been directed at understanding the nature of the interaction between antithrombin III and heparin. This is because of the role that antithrombin III plays in inhibiting blood coagulation and the clinically important enhancement of its rate of reaction with coagulation proteases that is effected by heparin.

Human plasma antithrombin III is a glycoprotein of molecular weight 58 000, consisting of a single polypeptide chain of 432 residues (Petersen et al., 1979; Chandra et al., 1983) and four asparagine-linked carbohydrate chains (Petersen et al., 1979). The carbohydrates are similar to the complex-type structures found in proteins such as C1q, transferrin, and plasminogen (Mizuochi et al., 1980) and constitute approximately 15% of the total molecular weight. The protein was first isolated from human plasma in pure form by Rosenberg and Damus (1973), who showed that it forms a 1:1 complex with thrombin via an interaction between an arginine residue of the inhibitor and the active-site serine of the enzyme. In addition to inactivating thrombin, antithrombin III can also inhibit factors IXa, Xa, and XIa (Yin et al., 1971; Damus et al., 1973; Kurachi et al., 1976a), plasmin (Highsmith & Rosenberg, 1976), and trypsin and chymotrypsin (Aubry & Bieth, 1972). However, it has been suggested, from consideration of relative rates of inactivation of proteases by the various plasma protease inhibitors, that the only physiologically important target of antithrombin III is thrombin (Travis & Salvesen, 1983).

The enhancement of the rate of inactivation of thrombin by antithrombin III is a catalytic rather than a stoichiometric process (Björk & Nordenman, 1976). Two principal mechanisms of action have been proposed for this heparin enhancement. The first involves binding of heparin to antithrombin III, resulting in a conformational change of the latter and a greater reactivity toward thrombin. There are kinetic (Olson et al., 1981; Fletcher & Nelsestuen, 1982), spectroscopic (Olson & Shore, 1981; Stone et al., 1982), and chemical modification studies (Rosenberg & Damus, 1973; Björk & Nordling, 1979; Villanueva et al., 1980; Blackburn et al., 1981) that support this model. Other kinetic data, however, favor a second mechanism in which the rate enhancement results from binding of both thrombin and antithrombin to the same heparin molecule. This has been termed the template model by Griffith (1982). It has been pointed out, however, by Olson et al. (1981) that the affinity of antithrombin III for heparin depends markedly on the conformation of the former. Thus, the first model can readily accommodate the catalytic activation of heparin by a conformationally modulated change in the affinity of the heparin/antithrombin III interaction in the binary and ternary complexes. Such catalytic activation is not so readily explained by the second mechanism.

This paper concentrates on the interaction between antithrombin III and heparin. High-resolution ^{1}H NMR has been used both to characterize certain aspects of the antithrombin molecule and also to probe the nature of heparin-induced perturbations in the protein.

Experimental Procedures

Materials. Antithrombin III (ATIII)[1] was isolated from plasma by a modification of the method of Thaler and Schmer

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[1] Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill; ATIII, antithrombin III; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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(1975). The precipitation step with poly(ethylene glycol) (PEG) was omitted as it was found by ^{1}H NMR that at least one molecule of high molecular weight PEG remains per molecule of antithrombin III, even after ammonium sulfate precipitation and extensive dialysis of the isolated protein. Instead, fractions were collected during the final elution of antithrombin from the heparin–Sepharose affinity column, and only those that contained significant amounts of antithrombin were pooled. It was consistently found that antithrombin III eluted immediately after the main peak of protein. By discarding this main peak, many of the contaminants were removed. A two-stage ammonium sulfate fractionation, at 50% and 95%, then yielded antithrombin III pure by SDS–polyacrylamide gel electrophoresis. Human ATIII was isolated from outdated plasma obtained from the Vanderbilt Hospital blood bank. Bovine ATIII and porcine ATIII were isolated from freshly collected citrated blood obtained from a local slaughterhouse.

Porcine intestinal heparin was purchased from Sigma. Lower molecular weight fragments were generated by reaction with heparinase, which has specificity for the glycosidic linkage between the glucosamine and α-L-idopyranosyluronic acid 2-sulfate (Silva & Dietrich, 1975). This cleavage leaves the chemical structure of the glucosamine reducing end unaltered (Linker & Hovingh, 1979) but introduces a $\Delta^{4,5}$ site of unsaturation in the terminal uronic acid residue (Linker & Hovingh, 1972a,b). Since only very small amounts of heparinase were available (purchased from Miles Biochemicals), the degradation was performed in a dialysis bag with a nominal molecular weight cutoff of 3500, and the dialysate was collected. The reaction was allowed to proceed at room temperature at pH 7.0 for 4 days, after which the reaction had nearly stopped, as monitored by the change in absorbance at 235 nm (Linker & Hovingh, 1972a). The dialysate was freeze-dried and redissolved in 5 cm^3 of 0.5 M ammonium bicarbonate. All the heparin fragments dissolved, but much of the sodium acetate buffer was left behind. This solution was applied to a 120 cm × 2.5 cm Bio-Rad P6 superfine column equilibrated in 0.5 M ammonium bicarbonate. Fractions were monitored at 235 nm. The last, and largest, peak was identified as disaccharide fragments from its ^{1}H (Linker & Hovingh, 1984) and ^{13}C NMR spectra. Earlier peaks differ in size by pairs of residues as a result of the specificity of the heparinase cleavage. Oligosaccharides up to 22 sugars long were separated in this way, though there was incomplete resolution from adjacent peaks for the longest sugars.

Ultrapure ammonium sulfate was purchased from Schwarz/Mann. Other reagents were obtained from Sigma.

Methods. Antithrombin samples for NMR analysis were prepared by dissolution of the freeze-dried protein in D_2O. The protein had been freeze-dried at 10 mg/mL in 30 mM NaCl/4 mM phosphate buffer such that the NMR samples, at approximately 50 mg/mL, were in 150 mM NaCl/20 mM phosphate. It has been found previously (Thaler & Schmer, 1975) and confirmed for these samples that freeze-drying from buffer solutions does not lead to loss of inhibitory activity. Protein concentrations were determined spectrophotometrically by using $A^{0.1\%}_{280nm}$ = 0.65 (Nordenstrom et al., 1977) and a molecular weight of 58 000 (Petersen et al., 1979; Chandra et al., 1983) for human antithrombin III and 0.60 and 56 600, respectively, for bovine antithrombin III (Kurachi et al., 1976b). The same values as for bovine antithrombin III were used for the porcine protein. Amino acid compositions were determined on an Applied Biosystems 120A PTH analyzer.

Table I: Amino Acid Compositions of Human, Bovine, and Porcine Antithrombin III

amino acid	human[a]	bovine		porcine
aspartic acid	46	45.5[b]	42.6[c]	41.5
glutamic acid	51	43.9	53.0	52.5
serine	32	33.9	30.5	29.2
glycine	18	18.1	16.9	22.0
histidine	5	6.4	6.2	5.3
arginine	22	23.2	22.8	24.0
threonine	23	28.1	25.7	24.2
alanine	31	29.0	27.9	31.1
proline	21	18.2	19.8	21.9
tyrosine	10	9.6	10.0	10.9
valine	28	29.2	30.7	31.6
methionine	12	8.9	10.6	9.8
isoleucine	22	24.5	24.4	24.2
phenylalanine	26	34.0	27.1	24.2
tryptophan	4	5.8	nd[d]	nd
lysine	35	32.0	30.4	31.1
cysteine	6	6.3	nd	nd
leucine	40	42.4	44.0	41.4

[a] From amino acid sequence (Petersen et al., 1979). [b] From Kurachi et al. (1976b). [c] This paper. [d] nd, not determined.

Protein samples were hydrolyzed in 6 M HCl at 110 °C for 24 h. SDS–polyacrylamide gel electrophoresis was carried out in 10% slab gels according to Laemmli (1970).

^{1}H NMR spectra were recorded at 400 MHz on a Bruker AM 400 narrow-bore spectrometer equipped with a 5-mm ^{1}H probe. Residual water proton intensity was diminished by presaturation of this resonance. Carr–Purcell–Meiboom–Gill spectra (Meiboom & Gill, 1958), using a $90°_x-(\tau-180°_y-\tau)n$ pulse train, were recorded with a τ value of 1 ms and n = 6. The probe temperature was maintained at 298 ± 1 K. A sweep width of 6000 Hz and a data block size of 8K points zero-filled to 16K points prior to Fourier transformation were employed. pH* values are reported as pH meter readings uncorrected for deuterium isotope effects and were measured in the NMR tube using an Ingold 3-mm diameter combination electrode. The pH* was adjusted by direct additions of 0.1 or 0.5 M solutions of NaOD or DCl. 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 most of the histidines examined, $\Delta\sigma$, the titration range in ppm, was a variable and was chosen to give the best fit to the theoretical analysis. The values determined, 0.78–1.11 ppm, with many around 0.90 ppm, are similar to those found in other proteins (Markley, 1975; Jordan et al., 1985). The error limits are ±0.07 pH unit.

Results

Amino Acid Composition of Porcine ATIII. Although the complete amino acid sequence of human ATIII is now known (Petersen et al., 1979) and the amino acid composition of bovine ATIII has been reported previously (Kurachi et al., 1976b), this is the first report of a characterization of porcine ATIII. The amino acid compositions of this and also bovine ATIII prepared here are given in Table I together with the composition of human ATIII and the earlier reported bovine ATIII for comparison. The bovine and porcine data given here are based on similar chain lengths to human ATIII as judged from the similar electrophoretic mobility in SDS–PAGE gels (Figure 1).

The two sets of data for bovine ATIII are in good agreement except for glutamic acid (nine residues different) and phenylalanine (seven residues). In each case, the new data are

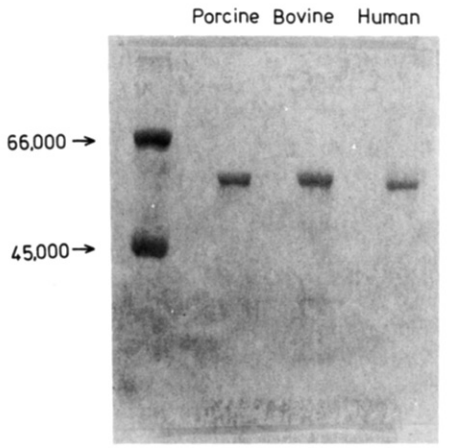

FIGURE 1: SDS–10% polyacrylamide gel of porcine, bovine, and human antithrombin III. The left-hand land has ovalbumin and bovine serum albumin as molecular weight standards.

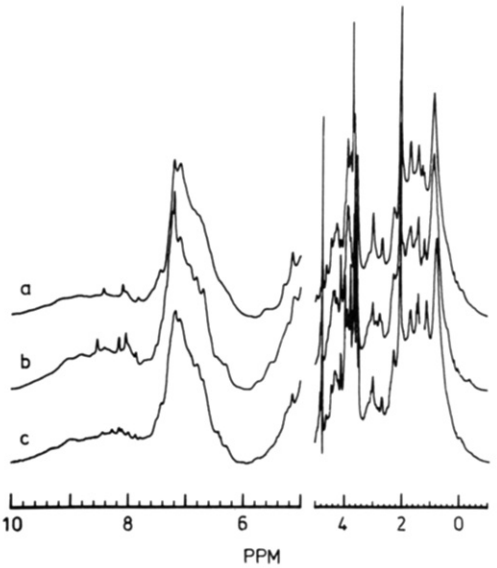

FIGURE 2: 400-MHz ^{1}H NMR spectra of human, bovine, and porcine antithrombin III. (a) Human ATIII, 0.93 mM, pH 6.31, 4000 scans; (b) bovine ATIII, 0.95 mM, pH 6.10, 4000 scans; (c) porcine ATIII, 0.61 mM, pH 6.29, 4000 scans. All spectra were recorded at 298 K with presaturation of the solvent water resonance. Note the different horizontal scales for the aromatic and aliphatic regions. The vertical scale for the aromatic region is 4 times that of the aliphatic region.

closer to the composition in human ATIII, and since most of the other amino acids differ from the human composition by no more than two or three residues, it is likely that the present data are closer to the true composition. Using this composition for bovine ATIII, it can be seen that all three proteins are very similar in composition. Indeed, Kurachi et al. (1976b) determined the amino-terminal sequences, up to residue 17, for human, bovine, and equine ATIIIs and found them to be identical in 15 of the positions, including an amino-terminal histidine.

^{1}H NMR Spectra of Antithrombins. Figure 2 shows the ^{1}H NMR spectra of the three antithrombins. The spectra are not greatly different from one another. This similarity is especially noticeable in the aromatic region between 6 and 8 ppm. The chemical shifts of aromatic amino acid ring protons in small peptides are in the range 6.8–7.6 ppm (Bundi & Wüthrich, 1979). The occurrence of significant resonance intensity between 6.2 and 6.8 ppm for these three antithrombins reflects perturbations of the normal chemical shifts of ring protons arising from secondary interactions within the folded protein. Similarly, upfield-shifted methyl resonances

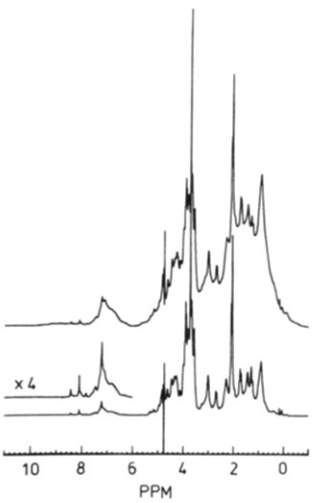

FIGURE 3: Carr–Purcell–Meiboom–Gill spectrum of human ATIII (bottom) and normal ^{1}H spectrum of the same sample (top). Details of the CPMG pulse sequence are given under Experimental Procedures.

at −0.1 and 0.05 ppm occur in all three spectra. Such perturbations of the normal chemical shift in globular proteins arise from local electric and magnetic field effects. The most studied, and perhaps the most important in instances where there are no paramagnetic centers present, are ring current shifts from the aromatic amino acids. The effect that such residues have on a nearby nucleus is very sensitive to geometry. Both upfield and downfield shifts can result and may be of magnitude 1 ppm or more (Johnson & Bovey, 1958; Perkins & Dwek, 1980). In addition, carbonyl groups and charged amino acids may have a significant effect. In the region between 3.5 and 4.5 ppm, there is a collection of resonances with line widths that seem to be narrower than the majority of other resonances in these spectra. These sharp resonances arise from the carbohydrate chains attached to the protein (Mizuochi et al., 1980). The sharp resonance at 2.0 ppm also arises from the carbohydrate, being the CH_3 resonances from the *N*-acetylglucosamine and *N*-acetylneuraminic acid residues (Vliegenthart et al., 1983).

Use of a CPMG pulse sequence to observe the protons of ATIII emphasizes those resonances with longer T_2 values and thus narrower resonances (Campbell et al., 1975). CPMG spectra gave better signal to noise ratios than use of convolution difference spectra. Figure 3 shows a CPMG spectrum of human ATIII together with a normal single-pulse spectrum of the same sample. Although the carbohydrate CH and CH_2 protons constitute only 9.1% of the total aliphatic protons in the protein, their resonances between 3.5 and 4.5 ppm dominate the CPMG spectrum because of the greater mobility and therefore longer T_2 values of these protons. Similarly, the sugar acetyl resonance at 2.0 ppm is enhanced relative to the protein methyl resonances. In the aromatic region, there is a great reduction in resonance intensity of less mobile residues. This loss of intensity from the broader resonances, however, reveals a number of sharp resonances between 8 and 8.5 ppm and also around 7.4 ppm. From the titration behavior as a function of pH, these resonances were attributed to the C(2) and C(4) ring protons, respectively, of histidine residues.

pH Titration of Histidine Residues. By use of CPMG spectra, it was possible to follow the pH titration behavior of C(2) histidine resonances in human, bovine, and porcine antithrombins. In human ATIII, there are five histidine residues (Chandra et al., 1983). Although three of these histidines have very similar titration behavior and do not give well-separated resonances, all five histidines give observable resonances and

Table II: Titration Parameters for Histidine C(2) Protons in Human, Bovine, and Porcine Antithrombins at 298 K

	human			human + heparin[a]			bovine			porcine		
histidine	pK_a[b]	σ[c]	$\Delta\sigma$[d]	pK_a[b]	σ[c]	$\Delta\sigma$[d]	pK_a[b]	σ[c]	$\Delta\sigma$[d]	pK_a[b]	σ[c]	$\Delta\sigma$[d]
1	6.90	8.59	1.07	6.90	8.63	1.07	7.25	8.70	1.03	6.90	8.62	1.08
2	6.00	8.61	0.87				7.15	8.64	1.11	6.40	8.62	0.78
3	5.93	8.61	0.89	6.00	8.61	0.90	6.10	8.67	0.89	6.50	8.57	0.88
4	5.75	8.62	0.90				5.80	8.74	0.98	5.90	8.71	0.90
5	5.13	8.65	0.90	5.45	8.65	0.90	5.60	8.66	0.90	5.45	8.70	0.90
6							5.35	8.63	0.90			

[a] Human ATIII with 1 equiv of bound heparin hexadecasaccharide. [b] The error is ±0.07 for all except histidine-3 of the human ATIII sample with bound heparin, for which it is ±0.15. [c] Chemical shift of protonated imidazole ring in ppm. [d] Titration range in ppm.

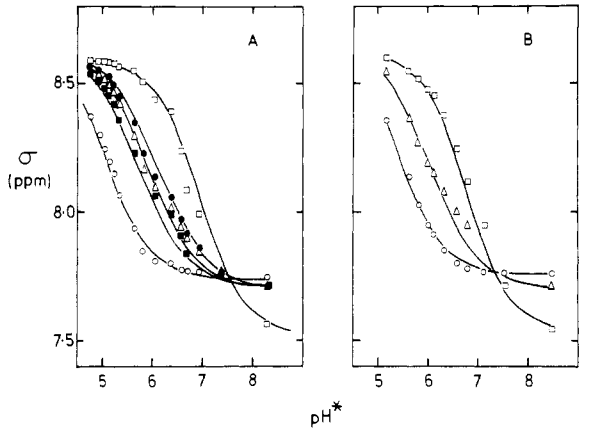

FIGURE 4: pH vs. chemical shift behavior of histidine C(2) protons in human antithrombin III with and without bound heparin. Chemical shifts were obtained from CPMG spectra. The curves shown are theoretical fits to the Henderson–Hasselbach equation with parameters given in Table II. (A) Human ATIII, 1.06 mM: (□) histidine-1; (●) histidine-2; (△) histidine-3; (■) histidine-4; (○) histidine-5. (B) The same sample as for (A) but after addition of 1 equiv of heparin hexadecasaccharide. The same symbols are used as in (A), but only data for histidines-1, -3, and -5 are shown. Spectra were recorded at 298 K.

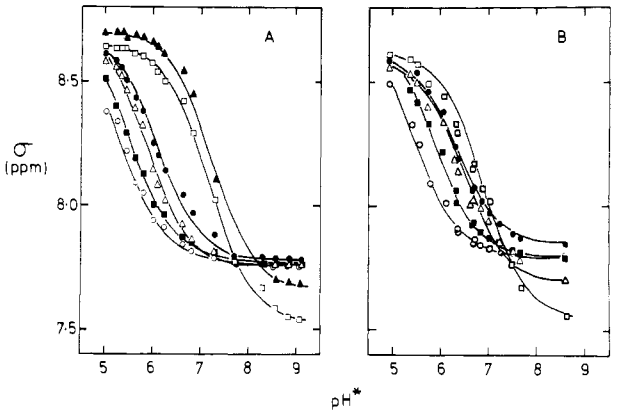

FIGURE 5: Histidine C(2) proton chemical shifts as a function of pH for bovine and porcine antithrombin III. (A) Bovine ATIII, 0.95 mM; (▲) histidine-1; (□) histidine-2; (●) histidine-3; (△) histidine-4; (■) histidine-5; (○) histidine-6. (B) Porcine ATIII, 0.71 mM; (□) histidine-1; (●) histidine-2; (△) histidine-3; (■) histidine-4; (○) histidine-5. Spectra were recorded at 298 K.

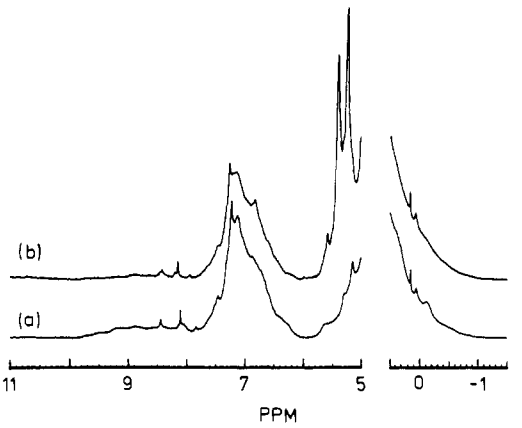

FIGURE 6: Effect of heparin hexadecasaccharide on the ^1H NMR spectrum of human ATIII. (a) 0.93 mM human ATIII, pH 6.31, 4000 scans; (b) sample (a) after addition of 1 equiv of heparin hexadecasaccharide. Only the aromatic region and the upfield methyl regions are shown. The two prominent resonances in (b) at 5.5 and 5.3 ppm arise from heparin anomeric protons from the glucosamine and L-iduronate residues, respectively.

titrate with pH in the pH range from 5 to 9. The titration behavior is shown graphically in Figure 4A, and the pK_a's and chemical shift extrema are summarized in Table II. Histidine in simple peptides such as glycylhistidylglycine has a pK_a of 6.38 and a titration range of 0.98 ppm, from 7.64 to 8.62 ppm (Tanokura et al., 1976). One of the histidines in human ATIII (peak 1) has a pK_a and titration range much greater than this, and two have pK_a's much less than this (number 4 with a pK_a of 5.75 and number 5 with a pK_a of 5.13).

Bovine ATIII has six titratable C(2) histidine protons and gives somewhat better resolved spectra over much of the titration range than does human ATIII. The chemical shifts are plotted in Figure 5A, and the pK_a's and titration ranges are given in Table II.

Porcine ATIII gives the best-resolved histidine C(2) resonances, with five resonances visible. The data are given in Figure 5B and Table II. Although the range of pK_a's and chemical shifts is similar for all three proteins, no histidine seems to be exactly equivalent in its NMR properties for the three systems.

Binding of Heparin Fragments to Human ATIII. Addition of 1 equiv of heparin, 16 sugars in length, to human ATIII results in perturbation of a number of the protein's aromatic and aliphatic proton resonances (Figure 6 and Figure 7b). The area represented by the difference spectrum is only a small fraction of the total resonance intensity and might therefore be strongly affected by any slight misalignment or incorrect choice of scaling factor for the initial and final spectra used in generating the difference. However, the difference spectrum was exactly reproducible in both aromatic *and* aliphatic regions, using human ATIII from both the same and different preparations. Therefore, the effects on both regions of the spectrum may be considered as genuine perturbations.

Resonances from the added heparin occur between 3.5 and 6 ppm and obscure any perturbation to antithrombin resonances in this region of the difference spectrum, particularly to the carbohydrate moiety. The sharp resonances in the difference spectrum at 8.45, 8.15, and 7.87 ppm arise from histidine C(2) protons. Although addition of the heparin fragment to ATIII resulted in a fall in the sample pH by 0.07 unit, the pH was brought back to within 0.01 pH unit of the starting value with NaOD. The histidine resonance pertur-

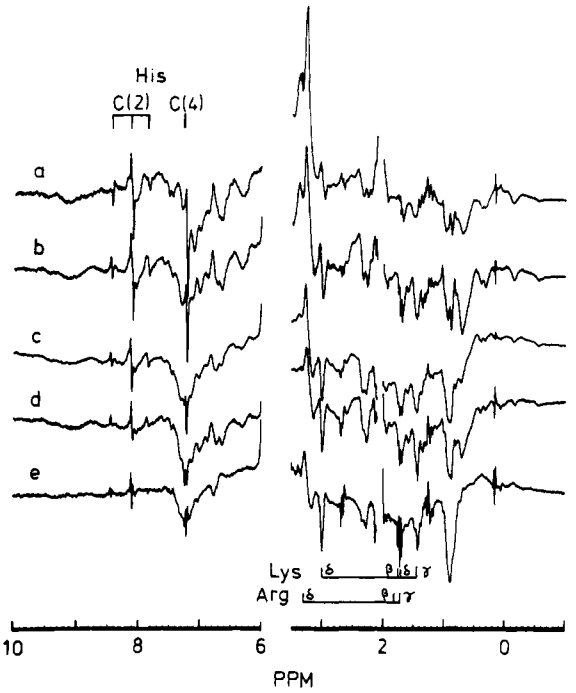

FIGURE 7: ^{1}H NMR difference spectra obtained after binding heparin and heparin fragments to human ATIII at pH 6.3. (a) Difference spectrum with 1:1 unfractionated heparin. The amount of heparin added was based on an assumed average mass of 25 000 daltons; (b) 1:1 hexadecasaccharide; (c) 1:1 decasaccharide; (d) 1:1 octasaccharide; (e) 1:1 tetrasaccharide. For b–e, the amount of heparin added was based on the extinction coefficient at 235 nm of the α,β-unsaturated linkage, which occurs in each saccharide at the nonreducing end. The vertical scale in the region 6–10 ppm is twice that of the region −1–3.5 ppm. The large resonances at 3.3 and 2.1 ppm (the latter is not plotted) in (a) arise from heparin, which is present in higher w/v amounts than for the spectra b–e. The region from 3.5 to 6 ppm is not shown since it is dominated by the heparin resonances, making it impossible to isolate the perturbations to antithrombin III resonances. The expected positions of lysine and arginine side-chain resonances are indicated. The histidine C(2) protons are also identified.

bations thus do not represent a pH effect but rather a perturbation in the pK_a and/or chemical shift range. The specific changes in chemical shift upon adding heparin at this pH are from 8.436 to 8.449 ppm for histidine-1, from 8.142 to 8.186 ppm for histidine-2, from 8.109 to 8.151 ppm for histidine-3, from 8.066 to 8.111 ppm for histidine-4, and from 7.844 to 7.910 ppm for histidine-5. To determine the perturbation in titration parameters for each histidine, a pH titration was performed. The chemical shifts are given in Figure 4B, and the parameters are summarized in Table II. Only the C(2) proton resonances from histidines-1 and -5 are clearly resolvable over most of the titration range. The resonances from histidines-2, -3, and -4 overlap more than in the absence of heparin. For this reason, only data for peak 3 from this group are given, and there is greater uncertainty in its chemical shift than in those from histidines-1 and -5. Histidine-1 is only slightly affected by heparin binding; its pK_a and titration range are the same, but the chemical shift of the protonated species is shifted 0.04 ppm downfield. In contrast, the pK_a of histidine-5 is increased by 0.32 pH unit, but with no change in the titration range or chemical shift of the protonated species. Histidines-2, -3, and -4 are all affected slightly, probably with a small increase in pK_a, though it was not possible to determine this accurately. The peaks in the difference spectrum at 7.25 and 7.32 ppm arise from the C(4) protons of the histidines.

Other peaks between 7.6 and 6.8 ppm fall in the region of resonances from aromatic amino acid ring protons in small peptides, whereas peaks from 6.8 to 6.0 ppm are either from

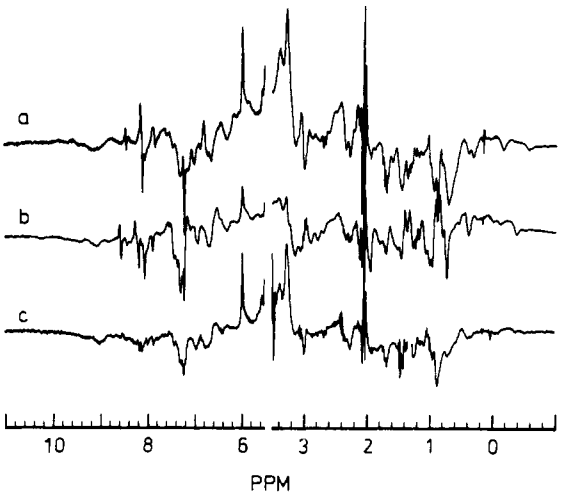

FIGURE 8: ^{1}H difference spectra between antithrombin III with and without bound hexadecasaccharide. (a) Human ATIII, pH 6.31, 0.93 mM; (b) bovine ATIII, 0.95 mM, pH 6.10; (c) porcine ATIII, 0.16 mM, pH 6.29. Note the different horizontal scales for the aromatic and aliphatic regions. The vertical scale of the aromatic region is 2 times that of the aliphatic region.

upfield-shifted aromatic protons or from downfield-shifted α-CH protons. The intensities of these resonances are such that they probably correspond to one or two protons each, though there is the problem in difference spectra of resonances moving less than their line width and consequently giving very small intensity in the difference spectrum. Excluding histidine protons, the number of aromatic protons in the difference spectrum may be estimated to be 10–20, or 5–10% of the total number of aromatic protons.

Much of the intensity in the aliphatic region of the difference spectrum upfield from 3.2 ppm seems to be negative and may well result from resonance broadening in addition to shifting upon heparin binding. Because so many types of resonance occur in this region of the spectrum, identifying the nature of the perturbed resonances is at best speculative. However, resonances at 0.9 ppm and also to higher field are almost certainly from methyl groups. Also, it is interesting that peaks at 3.0, 1.7, and 1.45 ppm are at the positions expected for lysine methylene groups, since there is chemical modification evidence implicating lysine residues in antithrombin/heparin interactions (Rosenberg & Damus, 1973). Approximately 35–40 aliphatic protons are perturbed by heparin binding. A difference spectrum between antithrombin with and without bound intact heparin is shown in Figure 7a. This is almost identical with the hexadecasaccharide difference spectrum.

Difference spectra were also obtained between human antithrombin III and decasaccharide, octasaccharide, and tetrasaccharide heparin fragments. The aromatic and aliphatic regions are shown in Figure 7, together with the hexadecasaccharide difference spectrum for comparison. The aromatic region differences are nearly identical for the 16, 10, and 8 sugars but differ substantially in magnitude of perturbation and chemical shift of resonances for the tetrasaccharide. The aliphatic difference spectra are also very similar for the 16, 10, and 8 sugars. The major difference is the variation in relative intensity of the methyl peaks at 0.90 and 0.75 ppm. The tetrasaccharide again has fewer resonances perturbed, though the large methyl resonance at 0.9 ppm found with the 8 and 10 sugars is present at full intensity.

Binding of Hexadecasaccharide to Bovine and Porcine Antithrombins. Figure 8 shows the difference spectra obtained between hexadecasaccharide heparin fragments and human, bovine, and porcine antithrombins. Approximately the same

fraction of resonances is affected in the aliphatic region of each spectrum. Also all six of the histidine C(2) protons in bovine and all five in porcine are affected by heparin binding; this is more clearly seen in the original spectra, since some of the changes involve substantial resonance broadening. The changes in chemical shift are all downfield, as was seen with human ATIII, and vary in magnitude from 0.03 to 0.08 ppm in bovine ATIII and from 0.01 to 0.03 ppm in porcine ATIII. However, the positions of perturbed resonances in the aromatic region are quite different for all three, and the number of aromatic protons perturbed in porcine ATIII is less than that for bovine or human ATIII. In the aliphatic region, the features corresponding to the chemical shifts of lysine methylene protons are common to all three spectra. The appearance of the methyl region shows significant differences. The porcine ATIII difference spectrum in this region is quite similar to that of the human with 10 and 8 sugars. The bovine difference spectrum, in contrast, has positive intensity at 0.85 ppm, and two new negative peaks are on either side at 0.75 and 0.95 ppm.

Discussion

Comparison of the Three Antithrombins. The amino acid analyses demonstrate great similarity in composition between all three antithrombins studied. For most of the different amino acids, there is less than 10% variation in the number of residues in each protein. This results in very similar proportions of charged, hydrophobic, and hydrophilic residues for each of the antithrombins. Also, the absolute size of each protein is quite similar. It has been reported that the amino-terminal 17 residues of bovine and human ATIII are nearly identical in sequence (Kurachi et al., 1976b). The almost identical amino acid compositions for the three antithrombins studied suggest that the sequence homology extends much further and may result in very similar tertiary structures. Such a similarity is also supported by the NMR data presented here. There are regions of the proton NMR spectra of these three proteins that have resonances not coinciding with those of amino acids in small peptides and therefore reflect perturbations of the normal resonance frequencies resulting from through-space interactions with other parts of the protein. These perturbations arise principally from proximity to aromatic residues, i.e., ring current shifts, or to other groups with anisotropic magnetic susceptibilities. The sensitivity of the perturbation to geometry means that similar structures for different proteins should result in similar NMR spectra. However, the reverse conclusion need not hold, since a given chemical shift perturbation may arise in more than one way as the sum of many separate contributions. In the three antithrombins studied here, the upfield-shifted aromatic protons in the region 6.8–6.0 ppm are similar in number and chemical shift. Also, there are two upfield-shifted methyl resonances at similar, though not identical, chemical shifts in each protein. As discussed below, some of the histidine residues appear to be homologous in the three proteins. This comparison of NMR data does not, per se, prove similar tertiary structures but, when considered with the very similar amino acid compositions and the limited available sequence data, does support it.

The size of each of these proteins is such that protons from residues having no motion independent of the whole protein should have NMR line widths of the order of 20–40 Hz. This broadness seems to hold for most of the protons, but with a few exceptions. The carbohydrate chains in all three proteins show much narrower resonances, consistent with a surface location and greater freedom of motion. Also, all the histidine residues (five each in human and porcine and six in bovine) give C(2) proton resonances with line widths of 6–12 Hz. This narrowness and their titration with pH indicate that all are surface residues accessible to solvent and free to rotate about C_α–C_β and C_β–C_γ bonds.

Though there is no exact equivalence of histidines between the three proteins, there are a number of similarities. Thus, according to the numbering in Table II, histidine-1 in human ATIII has a near counterpart, in titration range and chemical shift and pK_a, in histidine-1 of porcine ATIII and in histidine-2 of bovine ATIII. Similarly, histidines-5, -5, and -6 of human, porcine, and bovine ATIII, respectively, are similar in all three parameters. Also, histidine-3 in human and bovine and histidine-4 in porcine are quite similar. The remaining histidines, while not having an obvious counterpart in each protein, do not differ greatly from one antithrombin to another.

In the absence of a structure for human ATIII, any suggested assignment of histidine resonances to particular residues must be considered highly speculative. Histidines occur at positions 1, 65, 120, 319, and 369 in the sequence of human ATIII (Petersen et al., 1979; Chandra et al., 1983). Villanueva (1984) has predicted the secondary structural elements for human ATIII using the Chou and Fasman procedure (Fasman et al., 1976). This structure has the amino-terminal histidine in an unordered region, histidine-120 in a region of β structure, and the remaining three residues in α-helical regions, with His-65 and His-369 capable of forming intrahelical salt bridges with aspartate residues. Since His-1 is adjacent to the positively charged amino terminus, assignment of resonance 5, with the low pK_a of 5.15, to this residue is reasonable. Kurachi et al. (1976b) found an amino-terminal histidine in human, bovine, and equine ATIII. If this finding extends to porcine, there should be a histidine with similar properties in human, porcine, and bovine ATIII, which there is. A likely candidate for resonance 1, pK_a 6.90 in human ATIII, is either His-65 or His-369, since these are predicted by Villanueva (1984) to be in positions to form salt bridges and thereby have elevated pK_a's. However, only one such high pK_a residue is seen so that it is unlikely that both of these histidines form the intrahelical salt bridges proposed by Villanueva (1984). Something that may help in these assignments and subsequently aid in investigation of the structure and mechanism of action of ATIII is the suggestion of a two-domain structure in the protein (Villanueva & Allen, 1983a,b). On the basis of the disulfide linkage distribution, it might be expected that histidines-1, -65, and -120 would be in one domain and His-319 and -369 in the second domain. Since these domains appear to unfold at quite different and discrete levels of guanidinium chloride (Villanueva & Allen, 1983a), it may be possible to use this denaturant to discriminate between the ^{1}H resonances of one domain vs. the other. These experiments are in progress.

Effect of Heparin Chain Length on Perturbation of Human ATIII. The effects on the ^{1}H NMR spectrum of human ATIII of binding heparin fragments 4, 8, 10, and 16 sugars long have been shown above in Figures 6–8. It is clear that fragments 8 sugars long or greater result in almost identical changes. The major difference seems to be in the intensity of perturbed methyl resonances at 0.9 ppm. This finding is in accord with earlier studies that showed similar antithrombin CD difference spectra with 8-, 10-, 12-, and 14-residue-long heparin fragments (Stone et al., 1982), as well as with other binding studies showing that the smallest heparin fragment with high affinity for ATIII is an octasaccharide (Thunberg et al., 1982). However, these and other authors (Riesenfeld et al., 1981; Choay et al., 1980; Ooster et al., 1981; Atha et al., 1984) suggest that the actual minimum length interacting oligo-

saccharide is four or five residues in length and that heparin octasaccharide fragments are the smallest heparinase degradation products to contain the specific interaction sequence. Indeed, Choay and his colleagues have synthesized the putative pentasaccharide binding sequence (Sinaÿ et al., 1984) and shown that it binds tightly to antithrombin III. It would be most revealing to compare the ^{1}H NMR difference spectra obtained here with 8-, 10-, and 16-residue oligosaccharides binding to ATIII with one obtained for this pentasaccharide binding to ATIII.

It has been claimed by Stone and co-workers (Stone et al., 1982) that intact heparin with molecular weights of 6500 (22 sugars) or larger has interactions with antithrombin in addition to those that are present with the smaller chain length fragments. This claim is based on differences between CD difference spectra, which show altered intensities in the difference spectrum maxima and minima, though with similar peak positions. The present NMR data show not only that heparin fragments 8–16 residues in length perturb the same ^{1}H resonances to the same extent but also that hexadecasaccharide and intact heparin result in similar difference spectra. It is possible that there are additional interactions between intact heparin and ATIII that cause little or no perturbation of the ^{1}H NMR spectrum and thus go undetected here. However, a more resonable conclusion is that intact heparin binds ATIII in the same way as an octasaccharide fragment.

From the NMR difference spectra, it is possible to be more descriptive of the nature of the perturbations resulting from heparin binding than it is with CD or fluorescence changes. One obvious effect of binding is the downfield shift of all five histidine C(2) protons in human ATIII at constant pH. The changes are small, but reproducible, and correspond to perturbation of the pK_a's by +0.07 to +0.32 pH unit. These changes are perhaps too small to result from direct interactions between any of these histidine residues and negatively charged sulfate or carboxyl groups on the heparin fragment. For example, the changes in pK_a for the active-site histidines of ribonuclease upon binding mononucleotides, with interaction between histidine and phosphate, are in the range 1.2–1.8 pH (Meadows et al., 1969). However, addition of the tetrasaccharide does not cause any alteration in chemical shift of these histidines, only a broadening, so that the observed changes with larger fragment are likely the result of specific heparin/antithrombin interactions.

Another obvious feature of the difference spectra is the perturbation of 10–20 non-histidine aromatic protons. Earlier chemical modification studies have implicated tryptophan-49 in the heparin binding site (Björk & Nordling, 1977; Blackburn & Sibley, 1980; Villanueva et al., 1980; Blackburn et al., 1981, 1984). Also, fluorescence, CD, and solvent perturbation spectra have suggested alteration of the environment of one or more tryptophan residues and two tyrosine residues upon heparin binding (Villanueva & Danishefsky, 1977; Nordenman & Björk, 1978; Olson & Shore, 1981). The present NMR data are consistent with the magnitude of these perturbations and, therefore, are almost certainly monitoring the same changes.

In the aliphatic region of the difference spectra, there are resonances corresponding in position to the β-, γ-, δ-, and ϵ-methylene protons of lysine side chains and/or of arginine β-, γ-, and δ-methylenes. It has been mentioned above that lysine side chains have been implicated in heparin binding, from chemical modification studies. However, a mutant ATIII, having a single Arg → Cys substitution at position 47, has been reported that possesses normal thrombin reactivity but lacks heparin cofactor activity (Koide et al., 1984). These authors suggest that the heparin binding site lies in this region, noting that tryptophan-49 is only two residues removed and also that there is a lysine at position 53. Also, a significant degree of sequence homology has been noted between residues 24–79 of ATIII and residues 1–64 of histidine-rich glycoprotein, a protein with high affinity for heparin (Koide et al., 1986). In contrast, Villanueva (1984) favors a heparin binding site involving residues 279–292. This region is predicted to be α-helical and has three lysine residues that might be positioned to interact with the sulfate groups of a heparin octasaccharide. The NMR results presented here suggest interaction with one or more lysine and possibly arginine side chains but do not at present permit discrimination between these two sites of binding.

Comparison of the Heparin Binding Sites of Human, Bovine, and Porcine Antithrombins. There are two main conclusions from a comparison of the ^{1}H difference spectra obtained from binding heparin hexadecasaccharide to human, bovine, and porcine antithrombins. First, similar numbers of protons seem to be perturbed in each case, with the exception of fewer aromatic protons for porcine ATIII. The spectra include resonances at the chemical shifts of lysine side chains and involve perturbation of some or all of the histidine residues. The common features argue for a similar general mechanism of interaction in each case, which is not unexpected. However, the second conclusion is that the difference spectra are far from being identical. The methyl resonance(s) around 0.9 ppm in the bovine difference spectrum is (are) quite different from the same region of the porcine or human spectrum. Also, in the aromatic region, the chemical shifts of perturbed resonances are not the same for each, though this may simply be due to slightly different chemical shifts for homologous residues in the unliganded proteins resulting from small local variations in sequence. Without at least primary sequence data on all three antithrombins, it is impossible to interpret these similarities and differences any further at this time, though the NMR data have demonstrated the capability of observing perturbations of individual proton resonances in these proteins.

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