A Peptide Model for the Heparin Binding Site of Antithrombin III†

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ABSTRACT: A peptide model for the heparin binding site of antithrombin III (ATIII) was synthesized to elucidate the structural consequences of heparin binding. This peptide [ATIII(123–139)] and a sequence-permuted analogue (ATIII random) showed similar conformational behavior (as analyzed by circular dichroism spectroscopy) in aqueous and organic media. In the presence of heparin, however, the peptide ATIII(123–139) assumed a stable conformation, whereas peptide ATIII random did not. Complex formation was saturable and sensitive to salt. The ATIII(123–139)–heparin complex contained β-structure, rather than helical structure. This finding is incompatible with current models of heparin binding and suggests that heparin binding may induce nonnative structures at the binding site which could, in turn, lead to activation of ATIII. The peptide ATIII(123–139) was able to inhibit the binding of ATIII by heparin, consistent with the notion that this peptide may be a model for the heparin binding site.

Heparin is a heterogeneous, highly sulfated proteoglycan, the saccharide portion of which is widely used in the treatment of thrombosis (deProst, 1986; Linhardt & Loganathan, 1990). The anticoagulant activity of heparin results from its activation of several serine protease inhibitors (serpins) which are critical for the regulation of the blood-clotting cascade (Furie & Furie, 1988). One of these serpins, antithrombin III (ATIII),[1] undergoes a conformational change upon binding to a specific sequence within heparin (Bock, 1990). Binding results in a 1000-fold increase in the rate at which ATIII neutralizes the proteases thrombin and factor X_a (Jordan et al., 1980). The elucidation of the molecular details of this conformational change is necessary to design synthetic anticoagulants which act in a more specific manner than naturally derived heparin.

The polysaccharide chains of heparin are comprised of repeating 1,4-linked dimeric units: [α-D-uronic acid(β1→4)D-2-glucosamine]$_n$ (see Figure 1). Heterogeneity is generated after chain polymerization by partial epimerization of D-glucuronic acid to L-iduronic acid and incomplete *N*- and *O*-sulfation (Kusche et al., 1988). Studies of the heparin sequence requirements for the activation of ATIII revealed that a pentasaccharide sequence which occurs infrequently in heparin is sufficient to activate ATIII (Atha et al., 1984; Uhlrich et al., 1986). A bioactive pentasaccharide with this sequence has been prepared by chemical synthesis (Choay, 1983), and its conformation has been studied by ^{1}H NMR (Torri et al., 1985). The results of these and other studies suggest that sulfation at the 3-position of glucosamine and the occurrence of a flexible L-iduronic acid residue are crucial for the anticoagulant activity (Atha et al., 1987; Ferro et al., 1990; Lindahl et al., 1980).

ATIII is a glycoprotein of 432 amino acids which inhibits the plasma proteases thrombin and factor X_a via formation of a slowly reversible complex (Longstaff & Gaffney, 1991). The structure of ATIII has not been determined. Since heparin is polysulfated, the heparin binding site of ATIII is assumed to involve a region on the surface of the protein having a high density of basic residues. Three regions in the ATIII sequence have been proposed to be associated with heparin binding. However, the available data do not differentiate between regions which interact directly with heparin and those that are indirectly affected by heparin binding. One such region includes amino acids 105–150. Four lysine residues in this region, K107, K114, K125, and K136, are protected from chemical modification by heparin binding (Chang, 1989; Liu & Chang, 1987; Peterson et al., 1987). Arginine labeling experiments show that Arg 129 and Arg 145 may be involved in binding as well (Sun & Chang, 1990). Proteolytic fragments of ATIII containing this region of the sequence (114–156) have been shown to have a high affinity for heparin (Smith & Knauer, 1987). In addition, polyclonal antibodies raised against a synthetic peptide based on the sequence 124–145 have been show to block heparin binding and to partially activate ATIII for thrombin inhibition (Smith et al., 1990). Reduction of the Cys 128–Cys 8 disulfide bond prevents activation of ATIII (Sun & Chang, 1989). Finally, heparin binds more strongly to an ATIII variant lacking *N*-glycosylation at Asn 135 than to the native glycoprotein (Brennan et al., 1987). The convergence of the above evidence led to the focus of the studies reported herein on the ATIII-(123–139) sequence. The ATIII sequence around Arg 47 may comprise a second heparin binding region, as it also has a high concentration of positively charged residues. Naturally occuring ATIII variants having mutations at R47 (R47H, R47C) and P41 (P41L) have been isolated from patients with clotting disorders, and heparin binding has been shown to be affected (Borg et al., 1987; Chang & Tran, 1986; Duchange et al., 1987; Korde et al., 1984). A third region on the ATIII sequence comprising residues 281–292 has also been cited as a possible heparin binding region (Villanueva, 1984).

The interaction between heparin and ATIII has been studied by a variety of spectroscopic methods. These studies suggest that heparin binding induces and/or stabilizes structure in ATIII (Gettins, 1987; Gettins & Choay, 1989; Olson & Shore, 1981; Villanueva & Allen, 1983). However, these methods

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[1] Abbreviations: ATIII, antithrombin III; NMR, nuclear magnetic resonance spectrsocopy; CD, circular dichroism spectroscopy; FTIR, Fourier transform infrared spectroscopy; FAB, fast atom bombardment; FMOC, 9-fluorenylmethoxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DMF, dimethylformamide; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; RPHPLC, reverse-phase high-performance liquid chromatography.

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are not capable of elucidating the molecular structure of the complex. In order to model the heparin binding site on ATIII, several groups have used the crystal structure of the proteolytically cleaved α_1-antiproteinase serpin molecule (Grootenhuis & van Boeckel, 1991; Huber & Carrell, 1989; Loebermann et al., 1984). A disadvantage of this approach is that it ignores the possibility that the surfaces of proteins can be flexible and that structures which do not exist in the crystalline protein may be induced in the protein–ligand complex. Furthermore, it has been demonstrated that the affinity of cleaved ATIII for heparin is reduced over 500-fold relative to native ATIII (Bjork & Fish, 1982). We report herein an alternative approach which is based on the idea that peptides can be used to model conformationally mobile regions on the protein surface. We have synthesized two peptides derived from the sequence 123–139 of human ATIII. The conformational behavior of these peptides in the presence and absence of heparin and other polysulfated polysaccharides provides clues regarding those conformations which can be induced by heparin binding and the features of the peptide, as well as the polysaccharide, which contribute to the induction and stabilization of structure. The properties of the two synthetic peptides in a variety of aqueous and organic media were explored by circular dichroism spectroscopy (CD). Mixtures of each of the peptides with heparin and other sulfated polysaccharides were also analyzed by CD. Fluorescence spectroscopy was used to monitor the competition for heparin binding between each peptide and the protein ATIII. Existing models for the binding event are inconsistent with our findings.

Materials and Methods

Materials. FMOC amino acids were purchased from Fisher Biotech. Heparin (porcine intestinal mucosal sodium salt), chondroitin 6-sulfate (shark cartilidge sodium salt), and dextran sulfate were purchased from Sigma Chemical Co. BOP was purchased for Richelieu Biotechnologies. Human antithrombin III was a generous gift of Dr. R. D. Rosenberg.

Peptide Synthesis and Purification. The peptides were synthesized manually in a stepwise fashion on Du Pont Rapid Amide resin using an FMOC amino-terminal protection scheme (Fields & Noble, 1990). Amino acid side chains were protected as Ser(*O*-*tert*-butyl), Tyr(*O*-*tert*-butyl), Cys(S-S-*tert*-butyl), Lys(Boc), and Arg(Mtr). The syntheses were performed according to the Du Pont RaMPs Multiple Peptide Synthesis Systems instruction manual with the exception that the coupling was achieved in 1–2 h with 3 equiv of BOP and 5.3 equiv of DIEA for each 2.5 equiv of incoming amino acid in DMF. Couplings were monitored using the Kaiser test (Kaiser et al., 1970). After coupling the final residue, the amino terminus was deprotected and acetylated with 10 equiv of acetic anhydride and 5 equiv of DIEA in methylene chloride for 30 min. The resin-bound peptide was dried in vacuo. Commercial grade HPLC solvents were used. DIEA and piperidine were freshly distilled from ninhydrin at reduced pressure.

The peptide was cleaved from the resin and deprotected by stirring in a solution of 90% TFA, 5% 1,2-ethanedithiol, 4% H_2O, and 1% thioanisole for 24–36 h at room temperature. This procedure leaves the cysteine residue protected. The resin was filtered through a scintered glass funnel and washed with minimal TFA. The crude peptide was precipitated from the filtrate, washed 3× with cold diethyl ether, dissolved in distilled H_2O, and used directly for RPHPLC purification.

The crude peptide mixtures and the pure peptides were analyzed on a Waters DeltaPak C4-300-Å reverse-phase analytical column (3.9 × 30 cm) run at 2 mL/min. The eluent was monitored at 225 nm. Gradient or isocratic conditions were used. Solvents used for both analytical and preparative conditions were (H_2O + 0.1% TFA) and (CH_3CN + 5% TFE + 0.1% TFA). HPLC grade solvents were filtered and degassed before use. Peptides were purified using a shallow gradient on a Waters Deltapak C4-300Å semipreparative column (19 × 30 cm) run at 15 mL/min.

Characterization of Peptides. Quantitative amino acid analysis was performed on the resin-bound peptides, the crude cleavage products, and the pure peptides. Peptide samples (either free or resin-bound) were hydrolyzed with 6 N HCl + 0.1% phenol at 110 °C for 24 h. Norleucine or valine was used as an internal standard. The hydrozylates were labeled with phenylisothiocyanate and analyzed on a Waters Picotag amino acid analysis system.[2,3] The purity of each peptide was determined to be ≥95% by analytical RPHPLC under isocratic conditions. For each peptide, the correct molecular ion was observed, either by fast atom bombardment mass spectrometry performed by Dr. I. Papayannopoulos of the Massachusetts Institute of Technology Mass Spectrometry Facility (NIH Grant No. RR00317) or plasma desorption mass spectrometry performed by Mr. Martin Lacey of the Procter and Gamble Co.[2,3] No other parent ions were detected, verifying the purity of the samples. ^{1}H NMR spectra of the peptides in D_2O were obtained on the Varian XL-300 MHz spectrometer at the Chemistry Department Mass Spectrometry Laboratory, MIT.[2,3] These spectra were consistent with the proposed structure.

N-Deacetylation of Heparin. Crude porcine heparin was *N*-deacetylated with hydrazine and 1% hydrazine sulfate for 12 h at 100 °C and subsequently treated with HIO_3 to hydrolyze any hydrazides that may have formed (Shaklee & Conrad, 1984). The reaction product was purified on Bio-Gel P-2. Deacetylation was complete as judged by ^{1}H NMR analysis.

Circular Dichroism Spectroscopy. CD spectra were obtained on an AVIV 60DS spectropolarimeter in the laboratory of Dr. Robert Sauer, Department of Biology, MIT. Strain-free quartz cells of 0.1-cm path length were purchased from Hellma. Samples were prepared by diluting aliquots of peptide or polysaccharide stock solutions into degassed solvents. Concentrations of peptide stock solutions were determined by tyrosine absorbance (ϵ_{276nm} = 1470) (Edelhoch, 1967). Concentrations of polysaccharide solutions were determined by the carbazole test for uronic acid (Bitter & Muir, 1962) or by weight, in the case of the dextran sulfate solutions. Samples were scanned four times from 250 to 195 nm in 0.5-nm steps with 1.0-s averaging. Individual wavelengths were recorded for 90 s in 1-s intervals, and the signal was averaged over this

[2] ATIII(123–139). Amino acid analysis: N, 2.1 (2 expected); S, 1.8 (2); R, 1.9 (2); A, 2.0 (2); Y, 0.9 (1); L, 1.9 (2); F 1.0 (1); K 3.1 (4). FABMS: 2157.1 $(M + H)^+$; MW = 2155.2. ^{1}H NMR: (D_2O, 300 MHz) δ 7.2 (m, 5 H, Phe), 6.9 (d, 2 H, Tyr 2,6H), 6.7 (d, 2 H, Tyr 3,5H), 4.3 (m), 4.1 (m), 3.8 (m), 3.1–2.5 (m), 1.8 (s, 3 H, *N*-acetyl), 1.7–1.1 (m), 1.1 [s, 9 H, Cys(S-S-*tert*-butyl)], 0.7 (m, 12 H, 2 Leu δ-CH_3). HPLC: [78% H_2O/22% (95% CH_3CN + 5% TFE)/0.1% TFA], 2 mL/min, Waters C4–300-Å 3.9 cm × 30 cm column, elution volume = 24 mL.

[3] ATIII random. Amino acid analysis: N, 2.0 (2 expected); S, 1.8 (2); R, 2.0 (2); A, 2.0 (2) Y, 0.9 (1); L, 1.9 (2); F, 0.9 (1); K, 2.9 (4). FABMS: 2157.1 $(M + H)^+$; MW = 2155.2. ^{1}H NMR: (D_2O, 300 MHz) δ 7.2 (m, 5 H, Phe), 6.9 (d, 2 H, Tyr 2,6H), 6.7 (d, 2 H, Tyr 3,5H), 4.3 (m), 4.1 (m), 3.8 (m), 3.1–2.5 (m), 1.8 (s, 3 H, *N*-acetyl), 1.7–1.1 (m), 1.1 [s, 9H, Cys(S-S-*tert*-butyl)], 0.7 (m, 12 H, 2 Leu δ-CH_3). HPLC: [79% H_2O/21% (95% CH_3CN + 5% TFE)/0.1% TFA], 2 mL/min, Waters C4–300-Å 3.9 cm × 30 cm column, elution volume = 10.4 mL.

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ATIII: ...EKTSDQIHFFFAKLNCRLYRKANKSSKLVSANRLFGD...

ATIII(123-139): CH_3CONH-FAKLNCRLYRKANKSSK-$CONH_2$

ATIII Random: CH_3CONH-FKAKNCRLYRAKSSNLK-$CONH_2$

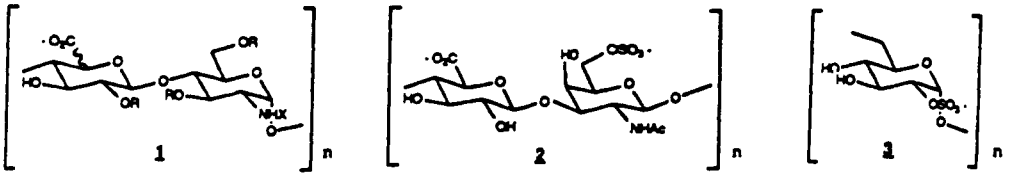

FIGURE 1: (Above) Partial sequence of human antithrombin III and the sequences of the synthetic peptides ATIII(123–139) and ATIII random. In the native protein, ATIII, Cys 128 is disulfide bonded to Cys 8. The cysteine residues of each peptide are protected as the S–S-*tert*-butyl derivative. (Below) Structures of heparin (1, R = SO_3^-, H; X = SO_3^-, H, Ac), chondroitin 6-sulfate (2), and dextran sulfate (3).

time. Solvent backgrounds were subtracted from all data. All spectra were obtained at 25 °C and have been generated in triplicate. Analysis of the CD data was performed using PROSEC (Chang et al., 1978).[4]

Fourier Transform Infrared Spectroscopy. Solution FTIR spectra were obtained in 99.9% D_2O in 0.15-mm path length CaF_2 cells on a Mattson Cygnus 100 FTIR spectrophotometer at the Spectroscopy Laboratory of the Department of Chemistry at MIT. Spectra were corrected for solvent background.

Fluorescence Spectroscopy. Fluorescence spectroscopy was performed on a Perkin-Elmer LS 50 in the laboratory of Dr. Paul Schimmel, Department of Biology, MIT. All samples were excited at 300 nm with a 2.5-nm bandwidth. Emission spectra were scanned 4× from 310 to 410 nm at a 5.0-nm bandwidth. Readings were obtained at 340 nm and integrated over 20 s. All samples were corrected for any solvent contributions.

Results

Design and Preparation of Peptides ATIII(123–139) and ATIII Random. Two synthetic peptides were studied. The peptide ATIII(123–139) represents the native sequence 123–139 of human antithrombin III (see Figure 1). It has been proposed that this region of the protein is helical when bound to heparin because all of the positively charged amino acid side chains would be found on the same face of the helix, thereby providing a linear array of cations with which the heparin could interact (Cardin & Weintraub, 1989; Villanueva, 1984). In order to test this proposal, a second peptide, designated ATIII random, was synthesized. This peptide has the same amino acid composition as ATIII(123–139), but the sequence was changed such that the basic residues would be evenly distributed around a helical cylinder. Both peptides were synthesized with blocked termini (N-acetyl and C-terminal amide) in order to avoid ionic interactions that would not exist in the native protein. The cysteine residues were left protected as the *tert*-butyl disulfide derivatives as this is representative of the native protein in which Cys 128 is disulfide-bonded to Cys 8 (Sun & Chang, 1989).

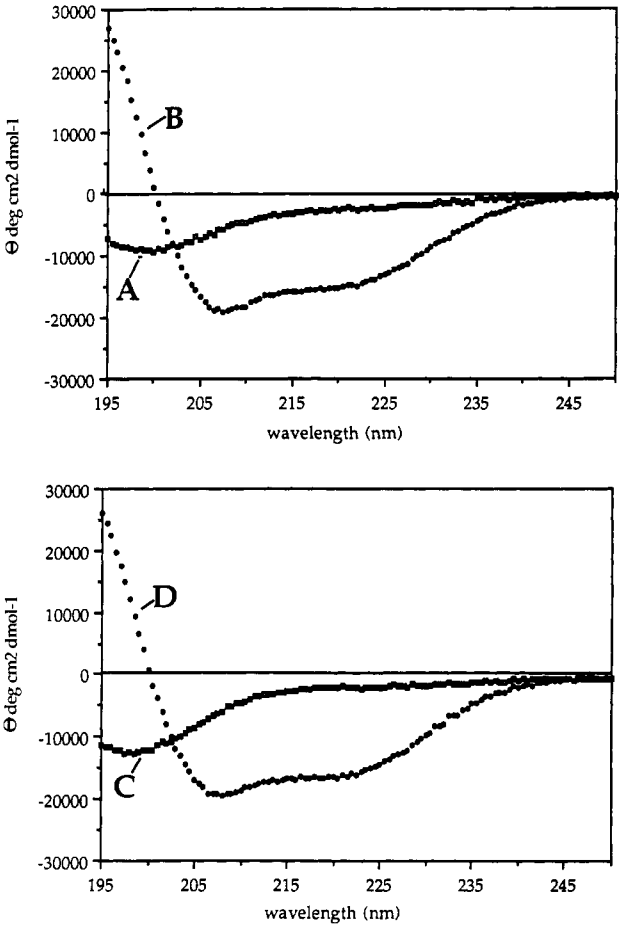

FIGURE 2: CD spectra of the peptides. (A) 50 μM ATIII(123–139) in 10 mM sodium phosphate and 50 mM KF, pH 7. (B) 50 mM ATIII(123–139) in 89% (v/v) TFE. (C) 50 μM ATIII random in 10 mM sodium phosphate and 50 mM KF, pH 7. (D) 50 μM ATIII random in 89% (v/v) TFE.

Peptides ATIII(123–139) and ATIII Random Exist as a Mixture of Conformers in Aqueous Media but Take On a Helical Conformation in Nonpolar Media. The CD spectrum of each peptide in aqueous buffer had a minimum signal at 200 nm (see Figure 2), while the FTIR spectrum showed an amide I absorption band centered around 1650 cm^{-1}. These spectra are characteristic of peptides existing as a random ensemble of conformations and indicate that neither peptide has a preferred secondary structure in pH 7 phosphate buffer. The peptides showed no concentration-dependent behavior in the range tested (20–200 μM). In order to probe the effect

[4] A curve-fitting program was applied to the CD data shown in Figure 5. The program PROSEC (Chang et al., 1978) ran through 15 cycles to optimize the data fit. The CD spectrum for the ATIII(123–139)–heparin complex was found to be the sum of 8% α-helix, 80% β-sheet, 9% β-turn, and 3% random structure. The ATIII(123–139)–chondroitin 6-sulfate complex was found to contain 23% α-helix, 35% β-sheet, and 42% β-turn, and the ATIII(123–139)–dextran sulfate complex was found to contain 6% α-helix, 51% β-sheet, 19% β-turn, and 24% random structure.

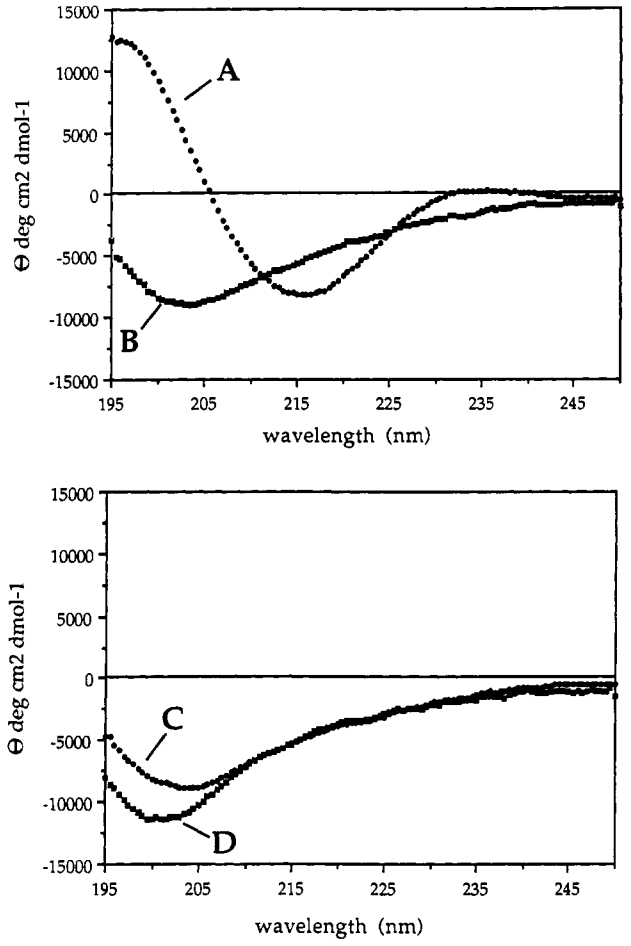

FIGURE 3: CD spectra of the peptides and heparin. All samples were in 10 mM sodium phosphate and 50 mM KF, pH 7. (A) 50 μM ATIII(123–139) and 2.0 mM heparin. (B) The sum of the spectrum of 50 μM ATIII(123–139) and the spectrum of 2.0 mM heparin. (C) 50 μM ATIII random and 2.0 mM heparin. (D) The sum of the spectrum of 50 μM ATIII random and the spectrum of 2.0 mM heparin.

of nonspecific charge neutralization on the conformation of the peptides, CD spectra of 50 μM peptide samples were obtained in 1 M solutions of NaCl, sodium sulfate, and lithium perchlorate, as well as in 0.1 M NaOH (pH 13). The CD spectra under these conditions showed no significant change in conformation. Addition of trifluoroethanol to aqueous solutions of both peptides gave rise to signals indicative of α-helical structure (minima at 208 and 222 nm, see Figure 2). At 90% (v/v) TFE, ATIII(123–139) was 46% helical and ATIII random was 48% helical (Greenfield & Fasman, 1969; Morrisett et al., 1973). A 10% solution of sodium dodecyl sulfate in 50 mM phosphate buffer (pH 7) was also capable of inducing helical structure in both peptides.

Peptide ATIII(123–139) Forms a Structured Complex with Heparin, Whereas Peptide ATIII Random Does Not. In order to determine the effect of heparin upon the conformation of the peptides, CD spectra were obtained of samples containing 50 μM peptide and 2 mM polysaccharide in phosphate buffer. These spectra were compared to the sum of the spectra of the peptide and that of the polysaccharide (Figure 3).[5] The CD

[5] CD spectra of heparin, chondroitin 6-sulfate, and dextran sulfate were measured from 250 to 195 nm. Both heparin and chondroitin 6-sulfate had negative signals at 210 nm. The mean residue ellipticity at 210 nm was −1250 and −4500 for heparin and chondroitin 6-sulfate, respectively. Dextran sulfate has no CD signal in the region between 250 and 195 nm. The CD spectra of heparin and chondroitin 6-sulfate were insensitive to ionic strength.

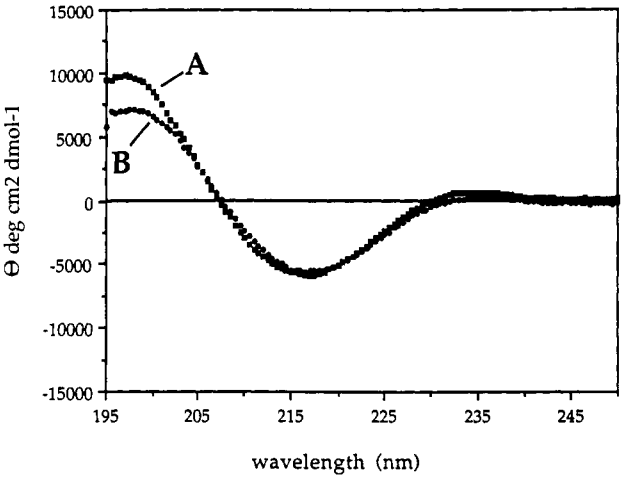

FIGURE 4: CD spectra of the peptide complexes with heparin and *N*-deacetylated heparin. Each sample contained 50 μM ATIII-(123–139) and 2 mM of either heparin or *N*-deacetylated heparin in 10 mM sodium phosphate and 50 mM KF, pH 7. (A) Heparin. (B) *N*-Deacetylated heparin. The CD contributions of each polysaccharide have been subtracted out of the spectra shown.

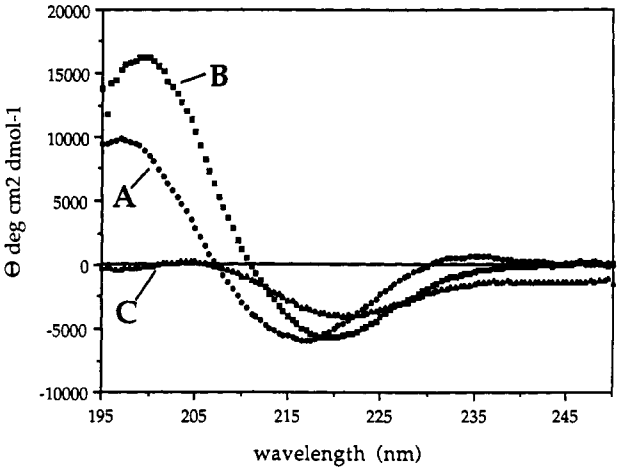

FIGURE 5: CD spectra of 50 μM ATIII(123–139) and 2.0 mM polysaccharide in 10 mM sodium phosphate and 50 mM KF, pH 7. The CD contributions of each polysaccharide have been subtracted out of the spectra shown. (A) Heparin. (B) Chondroitin 6-sulfate. (C) Dextran sulfate.

spectrum of the mixture of ATIII(123–139) and heparin had a distinctive signal indicative of stable complex with defined secondary structure, while the spectrum of the mixture of ATIII random and heparin resembled the summed spectra of the components (see Figure 3). In order to address the possibility that the induced signal observed for the ATIII(123–139)–heparin complex was derived from the heparin rather than the peptide backbone, *N*-deacetylated heparin was prepared (Shaklee & Conrad, 1984). *N*-deacetylated heparin has a lower mean residue ellipticity (−350 deg cm^2 dmol^{-1} vs −1250 deg cm^2 dmol^{-1}) at 210 nm than heparin. The complex of ATIII(123–139) and deacetylated heparin afforded a very similar, but not identical, CD spectrum to the peptide–heparin complex (after subtraction of the appropriate polysaccharide CD spectrum, see Figure 4). A difference in the CD spectral behavior between ATIII(123–139) and ATIII random was also observed with chondroitin 6-sulfate and dextran sulfate. These CD experiments indicate that the complexes of ATIII(123–139) and each of these polysaccharides had distinctive secondary structures while ATIII random showed little or no structural change in the presence of any of the polysaccharides tested. The CD spectra of the ATIII(123–139)–heparin and

ATIII(123–139)–chondroitin 6-sulfate complexes (corrected for their respective polysaccharide contributions) are similar in shape but not identical (Figure 5).[4] The spectra vary in the relative intensities of their signals and in the position of the single minimum. The spectrum of the ATIII(123–139)–dextran sulfate complex is very different in that there is no positive signal in the region around 200 nm. None of the complexes seem to contain primarily α-helical structure.[4]

Solutions of 50 μM ATIII(123–139) and 1 mM heparin were prepared in buffers with NaCl concentrations varying from 50 mM to 1 M. The CD signal of each sample at 217 nm was used to determine the percentage of the complex at a given salt concentration. A full CD scan (250–200 nm) was also obtained for each sample, to ensure that a one-step dissociation was being followed. The magnitude of the negative ellipticity (217 nm) decreased with increasing salt concentration, indicating dissociation of the complex. The heparin–ATIII(123–139) complex was 50% dissociated at 0.6 M NaCl. The chondroitin 6-sulfate–ATIII(123–139) and dextran sulfate–ATIII(123–139) complexes, monitored at 222 nm, were 50% dissociated at 0.2 M NaCl and 0.6 M NaCl, respectively.

Complex Formation Is Saturable. In order to determine the amount of crude heparin needed to induce structure in a given amount of peptide, solutions were prepared containing 50 μM ATIII(123–139), and the effect of increasing polysaccharide concentration over a range of 100 μM to 4 mM (based on monosaccharide) was monitored by following the large positive CD signal at 200 nM (similar trends were observed by following the smaller negative signal at 217 nm). In the case of heparin, the signal is maximized at 1000 μM, corresponding to an optimal ratio of one ATIII(123–139) molecule for every 20 saccharide residues of heparin. Saturation was also observed using chondroitin 6-sulfate (1000 μM) and dextran sulfate (400 μM), yielding ATIII(123–139) to saccharide ratios of 1 to 20 and 1 to 8, respectively.

Peptide ATIII(123–139) Inhibits the Heparin–ATIII Interaction. The binding of the active heparin sequence to ATIII results in a fluorescence enhancement in ATIII due to a conformational change which effects one or more of the four Trp residues in ATIII (Olson & Shore, 1981). The inhibition of this effect was used as a measure of the relative affinity of peptides ATIII(123–139) and ATIII random for the active sequences in heparin. Control experiments showed that the peptides had no effect on the fluorescence emission spectrum of ATIII or heparin. A solution containing 3.4 μM human ATIII and 200 μM heparin was prepared in 50 mM sodium phosphate, 50 mM NaCl (pH 7). The Trp residues were excited at 300 nm, and the fluorescence emission spectrum of the sample was measured from 310 to 410 nm. Emission spectra were collected before and after the addition of heparin. The emission intensity at 340 nm of the ATIII alone was designated as 100% free, while the emission intensity of ATIII + heparin was designated as 100% bound. Small aliquots of peptide were titrated into this sample over the concentration range from 3 to 35 μM peptide, and the emission intensity was measured at 340 nm. Addition of >35 μM peptide caused precipitate to form in the sample cell. These results demonstrate that the peptide ATIII(123–139) was more effective than ATIII random in blocking the binding of ATIII to heparin. The ATIII–heparin complex was 46 ± 2% dissociated by 26 μM ATIII(123–139) (peptide to ATIII ratio = 7.4); whereas, at 26 μM ATIII random, 8 ± 2% dissociation was observed.[6]

Discussion

The interaction of heparin and antithrombin III may be representative of many proteoglycan–protein interactions which regulate protein function in vivo (Ruoslahti, 1989). Binding of a rare oligosaccharide sequence within heparin induces an undefined conformational change in ATIII which increases the rate of formation of the inhibitory complex 1000-fold (Jordan et al., 1980). In order to design anticoagulants which are specific for ATIII and lack the side effects of heterogeneous heparin, one must elucidate the bioactive conformation of both heparin and its binding site on ATIII. The most direct approach to this problem is to solve the crystal structure of the ATIII–heparin complex. However, this complex has not been crystallized, and neither the structure of ATIII nor that of any of the active serpin homologues has been solved, possibly due to the inherent strain in these molecules (Bock, 1990; Carrell & Owen, 1985). Theoretical models of the heparin binding site on ATIII have been derived from examination of the crystal structure of the proteolyzed form of the homologous serpin, α_1-antiproteinase inhibitor (Huber & Carrell, 1989; Loebermann et al., 1984). It is important to note that the structure of heparin and ATIII in the complex may not resemble the favored conformation of the isolated components in solution or the crystalline state. This point has been demonstrated in many cases involving enzyme–inhibitor complexes (Alber et al., 1981), protein–DNA complexes (Wolberger et al., 1988), and peptide–antibody complexes (Stanfield et al., 1990). Therefore, approaches based on the structures of ATIII and heparin in isolation may impose artificial limitations on the flexibility of these molecules; that is, the model is less flexible than the real system (Grootenhuis & van Boeckel, 1991; Huber & Carrell, 1989). The approach described herein suffers from the opposite problem; that is, the model is more flexible than the real system.

The region of ATIII encompassing residues 123–139 has been implicated in heparin binding by several experimental approaches. The basic residues in this sequence are distributed such that, in a helical conformation, the positively charged side chains would be segregated along one surface. Such a helix could be stabilized by charge neutralization that would accompany binding of the anionic ligand heparin. This model for heparin binding was first suggested with regard to the 278–293 sequence of ATIII (Villanueva, 1984) and later as a general heparin-binding motif (Cardin & Weintraub, 1989). The peptide ATIII random was designed as a negative control to test this idea. The helical binding site model is compatible with the analogy to the cleaved α_1-antiproteinase structure in which a portion of the sequence which is homologous to the ATIII(123–139) sequence comprises the C-terminal portion of helix D (Huber & Carrell, 1989). CD studies of polylysine have shown that nonspecific charge neutralization such as increased pH will induce α-helical structure in these homopolymers (Townend et al., 1966). Other studies have shown that heparin and chondroitin sulfate can also induce α-helical structure in polylysine (Blackwell et al., 1977; Gelman et al., 1972). The two peptides discussed herein were expected to exhibit similar conformational behavior under conditions of nonspecific charge neutralization (high pH, salt) but behave differently in the presence of heparin, assuming that specific interactions are important.

[6] At 16 μM peptide, 24 ± 2% dissociation of the ATIII–heparin complex was observed with ATIII(123–139), while no dissociation was observed with ATIII random. At 36 μM peptide, 77 ± 1% and 27 ± 2% dissociation were observed with ATIII(123–139) and ATIII random, respectively.

Peptides ATIII(123–139) and ATIII random were both found to exist as a mixture of conformers in aqueous solution, and neither peptide was affected by charge-neutralizing aqueous media such as high salt or high pH, which demonstrates that nonspecific charge neutralization is not sufficient to induce structure. Both peptides assumed helical structure in nonpolar (trifluoroethanol, see Figure 2) and amphiphilic media (aqueous sodium dodecyl sulfate). In the presence of heparin, peptide ATIII(123–139) assumed a distinctive structure as detected by CD, whereas the conformational state of peptide ATIII random was apparently unchanged (see Figure 3). The difference in behavior of the two peptides suggests that a geometrically specific heparin–peptide interaction is involved in complexation. Unlike the previous studies with polylysine, the CD spectrum of the ATIII(123–139)–heparin complex showed none of the characteristics that are typical of α-helices but rather resembled the spectra of peptides in the β-sheet or β-turn conformation (Figures 3, 4, and 5) (Woody, 1985).[4] Stoichiometric studies of formation of the ATIII(123–139)–heparin complex showed that complex formation was saturable at ratios of ca. 1 peptide to 20 monosaccharide units. Given that the peptide binds less tightly to heparin than the protein does, the sequence within heparin which is responsible for induction of structure within peptide ATIII(123–139) is apparently more common than the sequence required to activate ATIII (Linhardt et al., 1989). This result indicates that the sequence requirements for peptide complexation are different or less stringent than those for ATIII complexation by heparin. The peptide–heparin complex was sensitive to salt (0.6 M NaCl was required to observe 50% dissociation), suggesting that ionic interactions are responsible for binding.

In order to further probe the polysaccharide sequence requirements for structure induction in the model peptide, the effects of two other polysulfated polysaccharides which have relatively low anticoagulant activity were tested. Like heparin, both chondroitin 6-sulfate and dextran sulfate were able to induce structure in the peptide ATIII(123–139) but not in the peptide ATIII random. However, the structures of these ATIII(123–139)–polysaccharide complexes were distinct from each other and from the structure of the ATIII(123–139)–heparin complex (Figure 5).[4] The chondroitin 6-sulfate complex was much more sensitive to salt than the heparin complex, consistent with the lower charge density of the chondroitin 6-sulfate polymer. The complexation of dextran sulfate and peptide ATIII(123–139) was saturated at a higher peptide to monosaccharide ratio (1:8 vs 1:20), suggesting that fewer saccharide units are required for complexation in this case or that a rare sequence is involved in the complexation of heparin and/or chondroitin 6-sulfate.

The peptide ATIII(123–139) was able to compete with the protein ATIII for binding to the active heparin sequence at a molar ratio of 7.4 peptide to 1 ATIII. The peptide ATIII random also inhibited the ATIII–heparin interaction, albeit at a higher peptide to protein ratio (possibly due to nonspecific binding). This result provides further evidence that the peptide ATIII(123–139) is a legitimate model for a portion of the heparin binding site on ATIII. Because of the interference of nonspecific binding modes and the heterogeneity of binding sites on heparin, the affinity of each peptide for heparin could not be measured.

Two general models of the conformational change induced by heparin binding can be considered. The first model supposes that a particular conformation of a specific sequence in heparin recognizes preexisting stable structures on the ATIII surface (Huber & Carrell, 1989; Lucas et al., 1990). Binding to heparin changes the relative orientation of the surface structures, forcing a conformational change in the protein. This model justifies the interest in the crystal structure of the proteolyzed ATIII homologue, α_1-antiproteinase. However, analogies between native ATIII and the cleaved form of that molecule must be made with caution. We believe that secondary structural elements on the surface of some proteins are flexible and may explore many conformations at equilibrium (Stanfield et al., 1990; Stein et al., 1990). This assumption leads to a second general model in which a particular conformation of a specific sequence in heparin recognizes and stabilizes a nonnative conformation at one or more sites on the ATIII surface. This conformational change could be communicated to the rest of the protein via tertiary interactions or via a disulfide bond. The approach discussed in this paper, that is, a dissection of the ATIII molecule in order to isolate the surface conformation(s) involved in binding, is ideally suited for the elucidation of binding events as presented in the second model. However, it is important to note that the two models are not mutually exclusive and that the actual event may have characteristics of both.

A peptide derived from the ATIII sequence 123–139 is capable of forming an α-helical structure under certain conditions; however, the preferred structure of this peptide in the presence of heparin resembles a β-sheet rather than an α-helix.[4] This finding emphasizes the advantage of the peptide modeling approach and calls into question previous structural models for heparin binding (Cardin & Weintraub, 1989; Grootenhuis & van Boeckel, 1991). The interaction between heparin and the ATIII(123–139) peptide is ionic in nature and is dependent on the peptide sequence. Polysaccharides which have low anticoagulant activity are capable of inducing different structure in the peptide ATIII(123–139).[4] These results suggest that the interaction modeled by the ATIII(123–139)–heparin complex represents a necessary, but not sufficient, condition for the activation of ATIII by heparin. The actual binding site on the ATIII surface may involve additional residues from distal portions of the protein sequence. This possibility is being investigated using the peptide model approach. Studies are in progress to identify the detailed molecular structure of the ATIII(123–139)–heparin complex.

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