^{1}H NMR Spectroscopic Studies on the Interactions between Human Plasma Antithrombin III and Defined Low Molecular Weight Heparin Fragments†

Angela Horne and Peter Gettins*

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

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ABSTRACT: The effects of length and composition upon the antithrombin-binding properties of heparin have been investigated for two series of structurally related heparin oligosaccharides. Each series consists of a tetrasaccharide, hexasaccharide, and octasaccharide heparin fragment composed of alternating hexuronic acid (either iduronate 2-sulfate or glucuronate) and glucosamine 6,*N*-disulfate residues. These two series represent dominant structural motifs in intact heparin and differ from each other by the presence of a glucuronic acid in one series in place of an iduronate 2-sulfate residue penultimate to the reducing end of the fragment. Perturbations to the ^{1}H resonances in the NMR spectrum of antithrombin upon binding of the two series of heparin fragments are compared to those generated by intact heparin binding, as well as to the effects of binding of a synthetic high-affinity pentasaccharide. All of the heparin fragments examined appear to bind to antithrombin at the same site. Three of the heparin fragments (hexasaccharide-2, octasaccharide-2, and octasaccharide-1) produce almost identical perturbations in the antithrombin ^{1}H NMR spectrum compared to binding of intact heparin, including perturbations of resonances from tryptophan 49. This indicates that neither the glucuronic acid nor the trisulfated glucosamine residue (structural elements known to be part of the high-affinity heparin motif) are necessary for the majority of the conformational changes induced upon heparin fragment binding to antithrombin. However, the low anticoagulant activity of these fragments indicates that the changes in protein conformation upon fragment binding, as manifested by these ^{1}H resonance perturbations, are not sufficient for catalytic activation of the inhibitor. Since there are few differences between the difference spectra generated by active heparin (either pentasaccharide or the intact species) as compared to the inactive heparin fragments, further limited conformational changes that arise from the presence of elements of the high-affinity motif that are absent in the heparin fragments considered here must be necessary for inhibitor activation by heparin.

The plasma glycoprotein antithrombin is the key regulatory element in the blood coagulation cascade, acting as an inhibitor of thrombin and factor Xa, as well as of other serine proteinase factors involved in the formation of a blood clot [for a review, see Pratt and Church (1991)]. The rate of inactivation of thrombin is relatively slow, having a second-order rate constant of 7×10^3–1.1×10^4 M^{-1} s^{-1} (Björk & Danielsson, 1986). However, the presence of catalytic amounts of the sulfated glycosaminoglycan heparin can increase the rate of inhibition 1000-fold, to give a bimolecular rate constant of $(1.5–4) \times 10^7$ M^{-1} s^{-1} (Jordan et al., 1979; Griffith, 1982). Specific sequences in the heparin polysaccharide are important for antithrombin binding and activation. Fractionation by affinity chromatography demonstrated that approximately one-third of the heterogeneous heparin polymer contains anticoagulant activity (Lam et al., 1976). Based on the identification of the essential residues necessary for antithrombin activation, Choay et al. (1983) synthesized a pentasaccharide with high affinity for antithrombin. Significantly, this fragment was able to enhance the inhibition of factor Xa but was ineffective against thrombin; longer heparin fragments (18 residues and greater) containing the high-affinity sequence were required for enhanced inhibition of thrombin (Lane et al., 1984; Olson & Björk, 1991; Olson et al., 1991). A trisulfated glucosamine residue as well as an unsulfated glucuronic acid (two structural features resulting from infrequent events during heparin biosynthesis) are necessary for catalytic activity of the heparin fragments (Walenga et al., 1988). More recent studies examined variations on the original high-affinity pentasaccharide motif, indicating that the spacial arrangement of the sulfate groups and the positions of the "flexible" iduronate and "rigid" glucuronate residues within the fragment play an important role in positioning the pentasaccharide within the primary heparin-binding site of antithrombin (Kusche et al., 1991; Petitou et al., 1991; van Boeckel et al., 1991).

While previous NMR studies have examined the interactions between antithrombin and heterogeneous pools of tetrasaccharides, octasaccharides, and longer fragments (Gettins, 1987), no systematic comparison has been made of the effects that defined differences in heparin length and composition have upon perturbations in antithrombin structure. In this paper, perturbations to the ^{1}H resonances in the NMR spectrum of antithrombin upon binding of two series of structurally related heparin fragments are compared to those generated by intact heparin binding, as well as to data in the literature on the effects of binding of a synthetic high-affinity pentasaccharide (Gettins & Choay, 1989; Crews et al., 1992). The two series of heparin fragments examined (Figure 1) are composed of alternating sulfated uronate and disulfated glucosamine residues. The series 1 heparin fragments differ from their series 2 counterparts by the substitution of an unsulfated glucuronic acid for the iduronate 2-sulfate residue penultimate to the

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* Address correspondence to this author at the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146.

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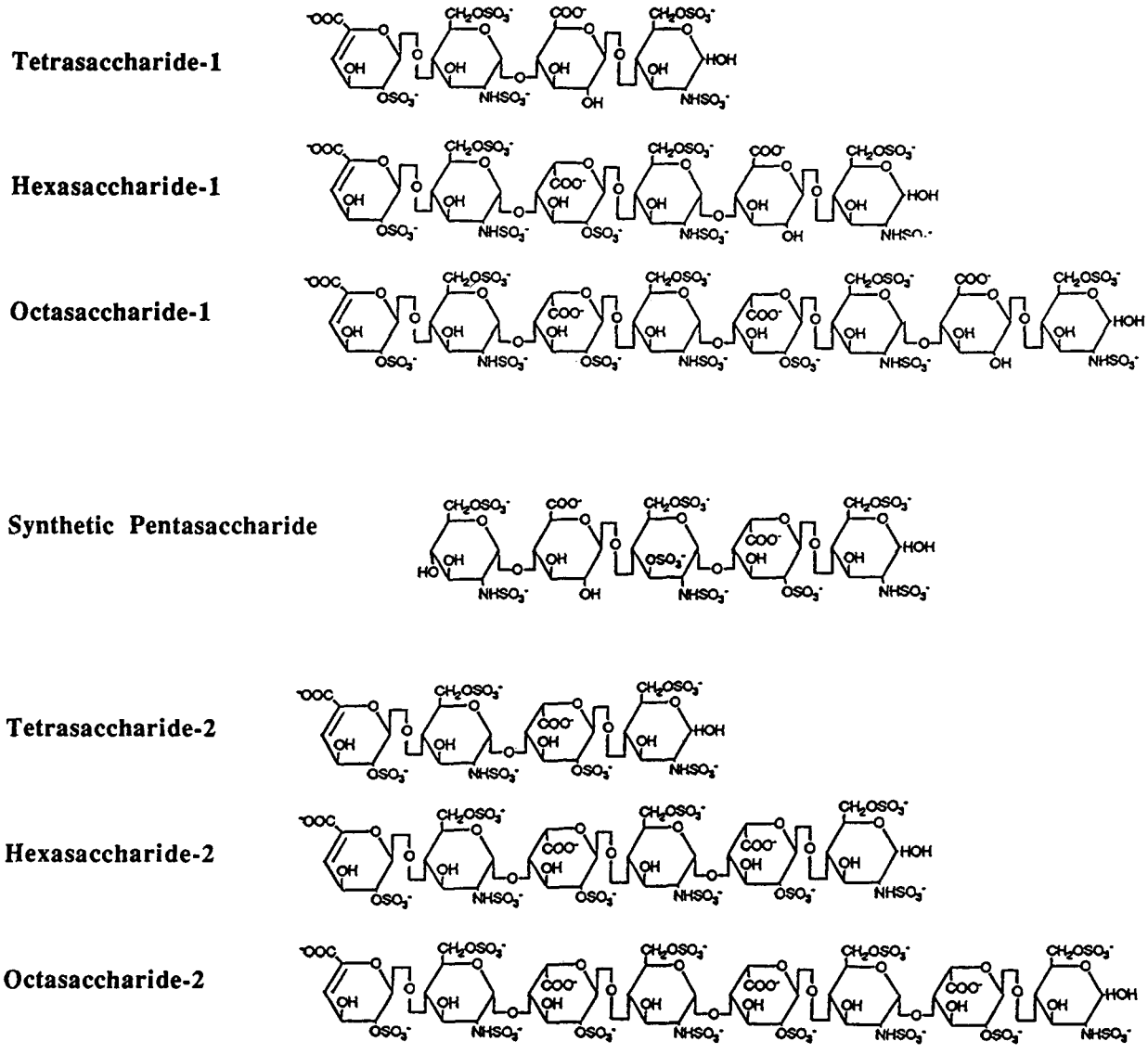

FIGURE 1: Structures of the six oligosaccharides used in the NMR binding studies, grouped according to series, and the structure of the high-affinity heparin pentasaccharide (Choay et al., 1983) for comparison.

reducing end of the oligosaccharide. These fragments represent common sequences in heparin.

Experimental Procedures

Materials. Heparinase-cleaved heparin fragments were isolated and characterized as described (Horne & Gettins, 1991). In brief, porcine intestinal mucosal heparin was cleaved with heparinase (EC 4.2.2.7, from *Flavobacterium*), separated by size using P6 gel exclusion chromatography, and further fractionated by charge by high-pressure liquid chromatography using a strong anion exchange column (Whatman Partisil SAX). For a given fragment size pool, species eluted from the HPLC column according to their total negative charge. Fragments containing *N*-acetyl groups therefore eluted earlier than the highly sulfated species examined in this study. The purity of all species obtained in this way was greater than 90% (Horne & Gettins, 1992). Since the all-iduronate and glucuronate fragments of a given size eluted as adjacent peaks, the majority of the small amount of impurity in any sample is from the other member of the pair of fragments. It is therefore not possible that sufficient contaminant containing the high-affinity 3-*O*-sulfate residue was present in any of the fragments to give misleading titration results, even at the highest molar excesses (6-fold) used. The structures of the purified heparin fragments (Figure 1) were determined using two-dimensional ^{1}H NMR spectroscopy (Horne & Gettins, 1992). Outdated human plasma used in preparation of antithrombin was obtained from the Vanderbilt Hospital blood bank. Ultrapure ammonium sulfate was obtained from ICN; all other reagents were from Sigma. Heparin-Sepharose was prepared as described (Cuatrecasas, 1970; Höök et al., 1976).

Preparation of Antithrombin. Human antithrombin was prepared by affinity chromatography of plasma over a heparin-Sepharose matrix. Plasma (1.5 L) was centrifuged to remove red blood cells and then treated with dextran sulfate (0.1% v/v) and calcium chloride (0.05 M) as described by McKay (1981). After being stirred for 1 h at room temperature, the plasma was centrifuged and the supernatant loaded onto a 500-mL column of heparin-Sepharose equilibratd in 0.05 M Tris-HCl, 0.01 M sodium citrate, and 0.15 M sodium chloride, pH 7.4. Following a wash with the above buffer containing 0.6 M NaCl, the antithrombin was eluted with a salt gradient from 0.6 to 1.5 M NaCl. The antithrombin fractions were pooled and concentrated in an Amicon ultrafiltration cell using a PM-30 filter to a final protein concentration of 10 mg/mL. Protein concentrations for human antithrombin were determined spectrophotometrically, using $A_{280}^{1\%} = 6.5$ and a molecular mass of 58 000 daltons (Norden-

man et al., 1977). Impurities were removed with a 50% ammonium sulfate precipitation; the antithrombin was precipitated by increasing the ammonium sulfate concentration to 95%. Typical yields from 1.5 L of plasma were between 80 and 100 mg of antithrombin. Purity was judged to be greater than 95% from Coomassie-stained 10% SDS–polyacrylamide gels (Laemmli, 1971). The purified antithrombin was rechromatographed over heparin-Sepharose immediately prior to use, to ensure a fully active sample.

NMR Spectroscopy Studies. NMR experiments were performed on a 400-MHz Bruker narrow bore spectrometer using a high-sensitivity 5-mm ^{1}H probe. The antithrombin was exchanged into D_2O containing 20 mM sodium phosphate and 0.15 M NaCl, at pH 7.0, by several cycles of dilution and concentration using a 10-mL Amicon ultrafiltration cell. The protein concentration was adjusted to either 0.2 or 0.5 mM antithrombin for each 400-μL sample. Stock solutions of heparin (also at pH 7.0) were prepared at a concentration such that one molar equivalent of heparin could be added to the NMR sample in a volume of 1–8 μL. Heparin concentrations were determined spectrophotometrically at 232 nm, using the absorption of the α,β-unsaturated linkage at the nonreducing end of the fragments introduced during heparinase cleavage [ϵ_{232} = 5200 M^{-1} cm^{-1} (Linker & Hovingh, 1972)]. Two thousand scans at a temperature of 310 K were collected for each experiment, using a block size of 8K and a sweep width of 6024 Hz centered on the water resonance. The data were zero-filled to 16K points and a line broadening of 2 Hz was applied prior to Fourier transformation. A low-power selective presaturation pulse was used in some cases to remove resonance intensity from residual water protons. Chemical shifts are given relative to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate at 0 ppm.

Determination of Dissociation Constants. The dissociation constant (K_d) was determined for each of the heparin fragments from the NMR titration data, using the best fit to a single binding site. For each heparin fragment, aliquots were added to the antithrombin sample and NMR spectra recorded. A minimum of eight concentrations of heparin was used for each titration. For the weakly binding fragments, all antithrombin resonances were in fast exchange, so that the percentage saturation of the protein by heparin was determined from the fractional chemical shift perturbation of the resonance. To improve the accuracy, as many resolved antithrombin resonances as possible were used in the analysis. For the higher affinity fragments some antithrombin resonances were in slow exchange. The percent saturation of antithrombin with heparin was then determined from the percentage change in height of protein resonances upon heparin binding. The titrations were performed at protein concentrations (2×10^{-4} M) on the same order of magnitude as the dissociation constant for low-affinity heparin.

Antithrombin-Mediated Anti-Factor Xa Activity Assays. Bovine factor Xa was generously provided by Dr. Paul Bock, Vanderbilt University. The effects of the heparin fragments upon the second-order rate constant (k_{bi}) for the reaction of human antithrombin with bovine factor Xa were determined by monitoring the residual level of enzyme activity as a function of length of incubation with antithrombin (Craig et al., 1989). All of the reactants were prepared in 20 mM sodium phosphate, pH 7.4, containing 0.25 M sodium chloride and 0.1 mM EDTA. One thousand molar excess of the heparin fragments was preincubated with antithrombin at 298 K in plastic poly(ethylene glycol)-coated tubes; this results in 40–60% saturation of the antithrombin molecule, depending on the K_d of the heparin fragment bound. The reaction was started with the addition of factor Xa, giving final concentrations of 4×10^{-8} M factor Xa 4×10^{-7} M antithrombin, and 4×10^{-4} M heparin fragments. At various time intervals, 20 μL of the reaction mixture was quenched by dilution into 980 μL of 300 μM S2222 containing 50 μg/mL Polybrene, and the initial velocities of substrate hydrolysis were monitored at 405 nm. Pseudo-first-order rate constants were calculated from the slopes of the initial velocities of substrate hydrolysis. These values were then used to determine the second-order rate constants (k_{bi}) as a function of antithrombin concentration (Craig et al., 1989).

Table I: Physical Properties of the Heparin Fragments[a]

heparin species	dissociation constant (M)	second-order rate constant (M^{-1} s^{-1})	fold increase in antithrombin activity
intact heparin[b]	1.0×10^{-7}	4×10^6	1300
pentasaccharide[c]	1.4×10^{-7}	na	na
octasaccharide-2	$(2.3 \pm 0.6) \times 10^{-4}$	5.4×10^3	1.2
octasaccharide-1	$(1.5 \pm 1.0) \times 10^{-4}$	2.1×10^4	4.6
hexasaccharide-2	$(6.7 \pm 1.8) \times 10^{-4}$	5.0×10^3	1.1
hexasaccharide-1	$(1.6 \pm 0.6) \times 10^{-4}$	5.4×10^3	1.2
tetrasaccharide-2	$(1.1 \pm 0.3) \times 10^{-3}$	nd[d]	nd
tetrasaccharide-1	$(1.8 \pm 0.4) \times 10^{-3}$	nd	nd

[a] K_d values were determined at 310 K from NMR titration data, and k_{bi} values for inhibition of bovine factor Xa were determined at 298 K. [b] The dissociation constant and second-order rate constant for interaction with intact heparin are from Jordan et al. (1979) and Jordan et al. (1980), respectively. [c] The dissociation constant for interaction between antithrombin and the synthetic pentasaccharide is from Choay et al. (1983). The second-order rate constant for the pentasaccharide-catalyzed inhibition of factor Xa was not available (na); however, this fragment possesses 4000 anti-factor Xa units/mg as determined by amidolytic assay in Choay et al. (1983). [d] nd, not determined.

Results and Discussion

Structures and Characteristics of the Heparin Fragments. The structures of the six heparin oligosaccharides examined in this study are given in Figure 1. The series 2 heparin fragments are composed of alternating iduronate 2-sulfate and glucosamine 6,*N*-disulfate residues. The series 1 oligosaccharides, which elute from the anion exchange column at a lower salt concentration, differ from the series 2 fragments in the substitution of an unsulfated glucuronic acid for the iduronate 2-sulfate at the reducing end of the oligosaccharide. Enzymatic cleavage of heparin with the enzyme heparinase generates a double bond in the iduronate residue at the nonreducing end of the fragment. Also shown in Figure 1 is the structure of the high-affinity heparin pentasaccharide as synthesized by Choay et al. (1983). The dissociation constants (K_d), determined from the NMR data (see Experimental Procedures) for each of the fragments examined, range from 1.5×10^{-4} M for the octasaccharide-1 fragment to 1.8×10^{-3} M for the series 1 tetrasaccharide (Table I). This binding is much weaker than what is seen for high-affinity heparin (1×10^{-7} M) or the synthetic pentasaccharide (1×10^{-8} M) but is within an order of magnitude of that for low-affinity heparin (1×10^{-4} M). Activity assays were performed as described by Craig et al. (1989). The weaker binding of these fragments (as compared to high-affinity heparin) makes assessment of their antithrombotic activity difficult, due to the need for greater than stoichiometric amounts of the heparin fragment to ensure partial saturation of the antithrombin molecule at concentrations appropriate for the enzyme assays. The reaction of factor Xa with antithrombin in the absence of added heparin gave a k_{bi} value of $(2.6–4.5) \times 10^3$ M^{-1} s^{-1}, which is slightly higher than the published rate of 2.1×10^3 M^{-1} s^{-1}

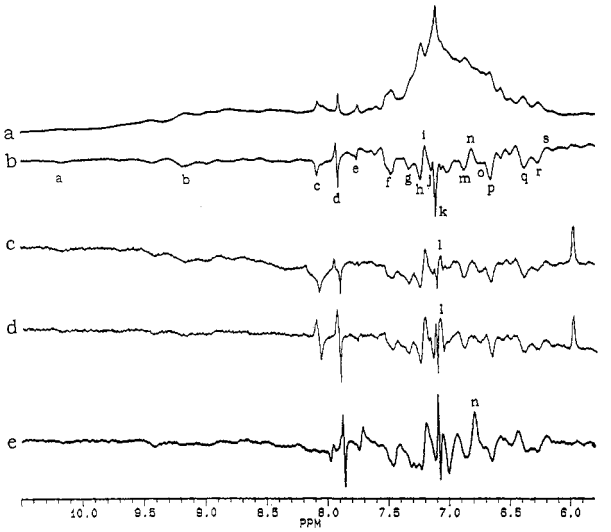

FIGURE 2: Downfield region of the 400-MHz ^{1}H spectrum of antithrombin, showing perturbations upon binding of active heparin species and the two octasaccharides: a, ^{1}H spectrum of antithrombin between 5.8 and 10.5 ppm; b, difference spectrum upon saturation with intact heparin; c, difference spectrum upon saturation with octasaccharide-1; d, difference spectrum upon saturation with octasaccharide-2; e, 500–MHz difference spectrum upon binding pentasaccharide [reproduced with permission from Crews et al. (1992)].

(Craig et al., 1989). Incubation mixtures containing a 1000-fold molar excess of heparin fragment to antithrombin, resulting in between 40–60% saturation of the antithrombin molecule (as calculated from the K_d), were assayed for enhancement of the factor Xa inhibition rate. The two octasaccharide fragments were able to enhance antithrombin activity against factor Xa by 1.2- and 4.6-fold, respectively (Table I). Enhancement of antithrombin activity decreases for each structural series with decreasing size; hexasaccharide-2 enhances antithrombin activity 1.1-fold, while hexasaccharide-1 has a 2.5-fold effect. The activities of the two tetrasaccharides could not be distinguished from that for antithrombin alone. The low activity of these heparin fragments supports previous findings by Petitou and co-workers (Petitou et al., 1988; Walenga et al., 1988) indicating that the 3-*O*-sulfate moiety of the heparin polysaccharide is necessary for high antithrombotic activity.

Binding of Intact Heparin. Intact porcine intestine mucosal heparin was titrated into a 0.2 mM human antithrombin sample at pH 7.0, and perturbations in the ^{1}H NMR spectrum were determined. Saturation was reached between 6.8 and 9.0 mg of heparin per micromole of antithrombin, representing an average of 23–30 residues of heparin per antithrombin molecule. The downfield region of the ^{1}H NMR spectrum for human antithrombin is shown in Figure 2a, and the difference spectrum containing perturbations in the antithrombin spectrum upon intact heparin binding is shown in Figure 2b. Peaks with positive intensity arise from proton resonances perturbed in the heparin-bound antithrombin spectrum; peaks with negative intensity are from the corresponding resonances in the unbound state.

The resonances between 8.2 and 10.2 ppm in the downfield region of the antithrombin spectrum (Figure 2a) arise from nonexchanged hydrogen-bonded amide protons that are involved in stable secondary structure. Perturbation of these resonances indicates changes in protein conformation, ranging from minor changes in proton environment to disruption of hydrogen bonds upon heparin binding. A minimum of 8–10 amide protons are affected by intact heparin. Antithrombin contains five histidine residues, at positions 1, 65, 120, 319, and 369 (Bock et al., 1982). These residues are located at the N-terminus, in helix A, in helix D, in strand 2C and in strand 5A, respectively, in the structure of cleaved antithrombin (Mourey et al., 1990). Assuming that the structure of native antithrombin does not differ drastically from that described for the reactive center-cleaved molecule, except for insertion of the reactive center loop into β-sheet A, histidines 1, 65, and 120 may be in or near to the proposed primary heparin binding site (Mourey et al., 1990). Resonances c, d, and e (Figure 2) represent 1, 3, and 1 histidine C(2) protons, respectively. The most upfield of these resonances (peak e) has tentatively been assigned to the N-terminal histidine in human antithrombin on the basis of its low pK_a (Gettins & Wooten, 1987). The majority of the resonances in the downfield region of the antithrombin spectrum (between 6.0 and 8.0 ppm in Figure 2a) arise from overlapping proton signals from 4 tryptophan, 10 tyrosine, and 26 phenylalanine residues as well as the five histidine C(4) protons. Recent work from this lab has made possible the assignment, by type of residue, of some of the aromatic peaks in the ^{1}H spectrum. Studies using a recombinant antithrombin in which tryptophan 49 has been changed to a lysine residue (Crews et al., 1992) indicate that peak f is in part from tryptophan 49, a residue shown by chemical modification studies to be in the heparin-binding site of antithrombin (Blackburn et al., 1984). Likewise, ^{1}H NMR studies using antithrombin produced by cells grown on ring perdeuterated tyrosine and phenylalanine[1] indicate that peak q is also in part from a tryptophan proton (though not from tryptophan 49) and peak r is from a non-tryptophan aromatic proton (i.e., it arises from either tyrosine or phenylalanine).

Table II: Changes in ^{1}H Chemical Shifts for Histidine C(2) Resonances at 310 K and pH 7.0[a,b]

heparin species	peak c	peak d	peak e
intact heparin	+0.111	+0.017	−0.010
octasaccharide-2	+0.037	+0.026	−0.010
hexasaccharide-2	+0.011	+0.011	+0.012
tetrasaccharide-2	+0.022	+0.018	+0.012
octasaccharide-1	+0.175	+0.060	+0.006
hexasaccharide-1	nc[c]	+0.013	+0.006
tetrasaccharide-1	nc	+0.010	nc

[a] The estimated error in the resonance assignments is ±0.002 ppm. Downfield shifts are designated by (+), upfield shifts by (−). [b] Chemical shifts given are for the antithrombin molecule upon saturation; for the tetrasaccharide fragments, these values were extrapolated to 100% saturation of the antithrombin molecule. [c] nc, no change in resonance position upon binding of the heparin fragment.

The perturbations seen in the downfield region of the antithrombin spectrum (at pH 7.0) are similar to those previously published for human antithrombin at pH 6.3 (Gettins & Wooten, 1987) and at pH 6.5 (Gettins & Choay, 1989). The major differences noted are in perturbations of the histidine C(2) and C(4) resonances, which might be expected given their sensitivity to pH. In the present study, histidine peak c moves downfield 0.111 ppm, peak d titrates downfield 0.017 ppm, and peak e (tentatively assigned to the N-terminus) titrates 0.010 ppm upfield (Table II). This differs from the results seen by Gettins and Choay (1989), in which peak c shifts upfield by 0.017 ppm, while peaks d and e move 0.042 and 0.091 ppm downfield. These studies demonstrate that all five histidines C(2) resonances are affected when intact heparin interacts with antithrombin. Similar changes are seen when comparing perturbations in the nonexchanged amide protons (peaks a and b) as well as for several resonances in the

[1] Brenda Crews, Gerd Zettlmeissl, Jean Choay, and Peter Gettins (manuscript in preparation).

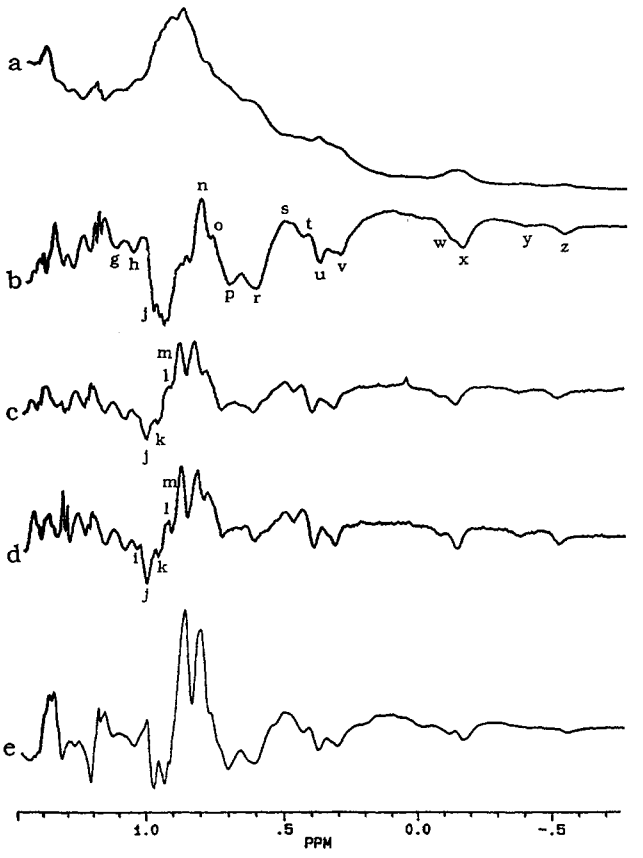

FIGURE 3: Upfield region of the 400-MHz ^{1}H spectrum of antithrombin, showing perturbations upon binding of active heparin species and the two octasaccharides: a, ^{1}H spectrum of antithrombin between −0.75 and 1.5 ppm; b, difference spectrum upon saturation with intact heparin; c, difference spectrum upon saturation with octasaccharide-1; d, difference spectrum upon saturation with octasaccharide-2; e, 500-MHz difference spectrum upon binding pentasaccharide [reproduced with permission from Crews et al. (1992)].

overlapping region of the aromatic protons (peaks f through r). The resonances assigned to tryptophan 49 (peaks f and j), as well as those for another tryptophan (peak q) (Crews et al., 1992) and a phenylalanine or tyrosine residue (peak r)[1] are perturbed by the binding of intact heparin. These same resonances are also perturbed upon binding of the high-affinity synthetic pentasaccharide, as shown in the difference spectrum in Figure 2e [taken from Crews et al., (1992)]. Although this spectrum was collected at 500 MHz on a sample of antithrombin at pH 7.4, the similarities in the difference spectra compared to intact heparin at a lower magnetic field indicate that minimal cancellation of overlapping perturbed resonances is occurring in the spectra collected at 400 MHz. This is an important point, since it means that quantitation of the number of protons perturbed by heparin binding should not give a greatly underestimated value.

Binding of intact heparin also perturbs methyl proton signals, which resonate in the upfield region (−0.6 to 1.1 ppm) of the antithrombin ^{1}H spectrum (Figure 3a). Several of these resonances are shifted further upfield than the majority of the methyl proton resonances, reflecting an unusual local magnetic environment (Gross & Kalbitzer, 1988) (typically due to proximity to aromatic amino acid side chains). Binding of intact heparin to antithrombin perturbs between 20 and 30 methyl proton resonances (Figure 3b). In the difference spectrum, the perturbed peaks in the far upfield region (peaks w–z) represent individual or small clusters of resonances. The lack of peaks of positive intensity in this region of the upfield difference spectrum indicates that these resonances have been shifted downfield, out of their unusual environments, by heparin binding. In contrast to the changes seen in the farthest-upfield individual methyl resonances, changes in the heavily populated region of methyl resonances (peaks g–r) arise from perturbation of many overlapping resonances. Despite the error inherent in the subtraction of large numbers of proton resonances from one another to generate the difference spectra, perturbations seen in this region of the spectrum are highly reproducible. As seen for the downfield region, the perturbations induced in the upfield region of the antithrombin spectrum upon intact heparin binding are similar to results published previously (Gettins & Wooten, 1987; Gettins & Choay, 1989).

Binding of Octasaccharides 1 and 2. Perturbations upon binding of octasaccharide-1 and octasaccharide-2 are shown for the downfield region (Figure 2c,d) and upfield region (Figure 3c,d) of the antithrombin ^{1}H NMR spectrum. The resonance of positive intensity at 6.0 ppm in the downfield region of the difference spectrum in Figure 2c,d arises from the H(4) proton of the $\Delta^{4,5}$-unsaturated residue of the octasaccharide fragment. Other heparin resonances occur much further upfield and are unlikely to have been shifted downfield into this region of the spectrum by binding to antithrombin. These difference spectra are very similar to that generated by both intact heparin (Figures 2b and 3b) and the high affinity pentasaccharide (Figures 2e and 3e), indicating that the two octasaccharides perturb the antithrombin conformation in a manner similar to intact heparin. However, neither octasaccharide is able to catalytically enhance the rate of antithrombin-mediated inhibition of factor Xa to the same extent as these highly active species (Table I), which suggests that the conformational change upon heparin binding, as measured by NMR spectroscopy, is not sufficient for antithrombin activation.

In the downfield region of the ^{1}H NMR spectrum of bound antithrombin, histidine peak c titrates further downfield upon addition of equivalent amounts of octasaccharide-1 (0.175 ppm) compared to octasaccharide-2 (0.037 ppm) (Table II). This peak is in slow exchange upon binding of intact heparin, reappearing 0.111 ppm downfield in the antithrombin–heparin complex. The three protons in peak d titrate substantially downfield for both octasaccharide-1 (0.060 ppm) and octasaccharide-2 (0.026 ppm) binding, compared to a perturbation of 0.017 ppm downfield when intact heparin is bound. The fifth histidine C(2) resonance, peak e, is shifted 0.010 ppm upfield by intact heparin. However, octasaccharide-1 and octasaccharide-2 shift this resonance 0.004 and 0.010 ppm downfield, respectively. These perturbations are not due to changes in pH of the protein sample upon addition of heparin but rather reflect differences in protein conformation caused by octasaccharide binding. All five histidine residues are affected by octasaccharide binding, indicating that these fragments are able to induce global changes in the antithrombin conformation.

Other comparisons between octasaccharide-1, octasaccharide-2, and intact heparin binding can only be made in more general terms, since the resonances in regions other than the histidine C(2) region of the antithrombin spectrum are broader and overlap to a greater extent. The same amide peaks are perturbed upon octasaccharide binding as when intact heparin binds. Peaks f and j, which contain resonances from tryptophan 49, as well as several other unassigned peaks (g, h, i, m, p, and s), all of which appear in the intact heparin difference spectrum, are perturbed by both octasaccharides. Perturbation of those same specific aromatic and amide res-

onances as seen with intact heparin demonstrates that these fragments are binding in the primary heparin-binding site. One difference in the difference spectra which is seen between these low-activity fragments and high-activity heparin is that neither octasaccharide induces the resonance of positive intensity labeled "peak n" in the difference spectra of intact heparin (Figure 2b) and of high-affinity pentasaccharide (Figure 2e). Either this peak is not perturbed by octasaccharide binding or the perturbed resonance appears in a different location in the octasaccharide-bound spectrum.

In the upfield region of the spectrum, the difference spectra for octasaccharide-1 (Figure 3b) and octasaccharide-2 (Figure 3c) binding are also very similar to that seen upon binding of intact heparin or high-affinity pentasaccharide. The octasaccharide-induced perturbations are noticeably sharper in the more densely populated regions of the spectrum compared to that for intact heparin, probably due to fewer perturbed protons and fewer cancellations of overlapping peaks. The resonances farthest upfield, peaks u–z, are perturbed upon octasaccharide binding, as are several resonances (peaks g–r) in the region more typical of methyl protons. As with intact heparin, the paucity of resonances having positive intensity in the farthest upfield region of the difference spectra suggests that the conformational changes which occur upon octasaccharide binding remove these protons from their unusual shielded environment shifting the resonances downfield by at least 1 ppm.

The striking number of similarities among the intact heparin and octasaccharide difference spectra (both in the upfield and downfield regions) indicate that the majority of the perturbations induced upon heparin binding are not due to any specific structural feature of the heparin species but rather result from occupation of the primary heparin-binding site by a minimum of five heparin residues. This suggests that the conformational change represented by these common perturbations to the ^{1}H NMR spectrum is not sufficient for activation of antithrombin activity. The only perturbation unique to the binding of catalytically active heparin is seen for aromatic peak n; this resonance is perturbed upon binding of both intact heparin and the high-affinity pentasaccharide (Figure 2b,e). Thus, perturbation of this specific though presently unassigned aromatic resonance appears to correlate with catalytic enhancement of antithrombin activity and possibly the presence of a 3-*O*-sulfated glucosamine residue in the heparin structure. However, without knowing which resonance in the unbound antithrombin spectrum is perturbed to produce this peak, the possibility that this resonance is still affected but to a different extent by the relatively inactive octasaccharide fragments cannot be excluded. Preliminary results on changes in antithrombin fluorescence upon heparin fragment binding indicate that both of the octasaccharides result in a 12–15% enhancement of protein fluorescence (data not shown), similar to that seen for the binding of low-affinity heparin (Nordenman et al., 1978). The absence of the typical 40% enhancement of protein fluorescence seen upon high-affinity heparin binding suggests that further conformational changes affecting the two buried tryptophan residues (Olson & Shore, 1982) are necessary for catalytic activation by heparin. These perturbations, which must propagate from the primary heparin-binding site upon heparin binding, are not induced upon binding of the two octasaccharides examined here.

Comparison of Series 2 Fragment Binding Effects. Figures 4 and 5 show perturbations in the downfield and upfield regions of the antithrombin spectrum, respectively, upon binding of

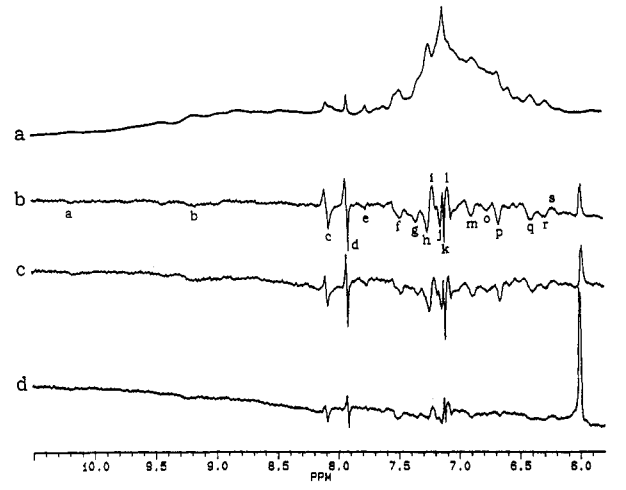

FIGURE 4: Downfield region of the ^{1}H spectrum of antithrombin, showing perturbations upon binding of the series 2 oligosaccharides: a, ^{1}H spectrum of antithrombin from 5.8 to 10.5 ppm; b, difference spectrum upon saturation with octasaccharide-2; c, difference spectrum upon saturation with hexasaccharide-2; d, difference spectrum upon binding tetrasaccharide-2 (at 50% saturation).

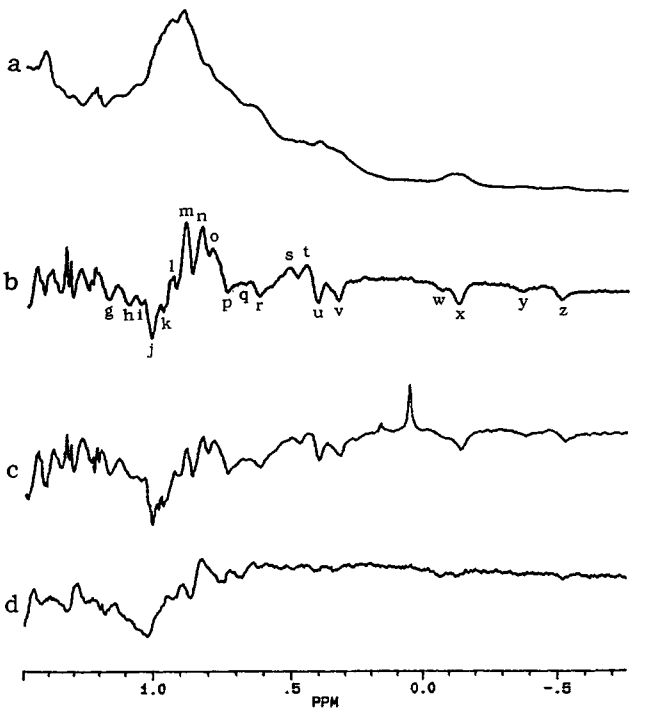

FIGURE 5: Upfield region of the ^{1}H spectrum of antithrombin, showing perturbations upon binding of the series 2 oligosaccharides: a, ^{1}H spectrum of antithrombin between −0.75 and 1.5 ppm; b, difference spectrum upon saturation with octasaccharide-2; c, difference spectrum upon saturation with hexasaccharide-2; d, difference spectrum upon binding tetrasaccharide-2 (at 50% saturation).

the series 2 heparin fragments (Figure 1). The resonance of positive intensity at 6.0 ppm in the difference spectra in Figure 4 (and likewise for the series 1 difference spectra in Figure 6) arises from the $\Delta^{4,5}$ proton of the unsaturated residue in each of the heparin fragments. Due to the weaker binding of the tetrasaccharide-2 fragment, saturation could not be reached with the quantities of this heparin fragment available. A 6-fold molar excess of tetrasaccharide-2 to antithrombin was the final point in the titration, resulting in approximately 50.0% saturation of the antithrombin molecule.

Perturbations in the downfield region caused by octasaccharide-2 (Figure 4b) and hexasaccharide-2 (Figure 4c)

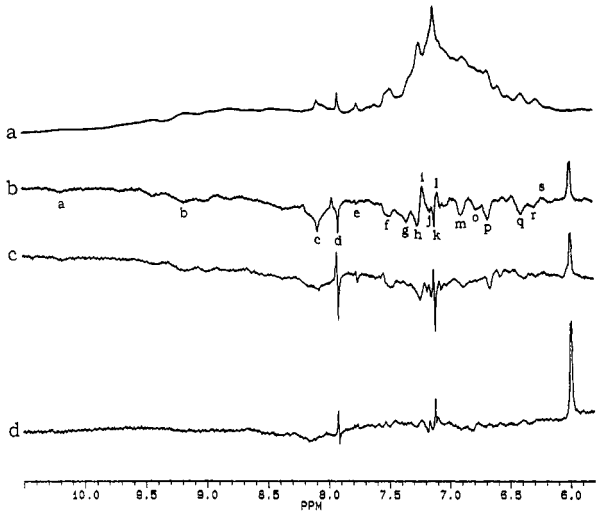

FIGURE 6: Downfield region of the ^1H spectrum of antithrombin, showing perturbations upon binding of the series 1 oligosaccharides: a, ^1H spectrum of antithrombin from 5.8 to 10.5 ppm; b, difference spectrum upon saturation with octasaccharide-1; c, difference spectrum upon saturation with hexasaccharide-1; d, difference spectrum upon binding tetrasaccharide-1 (at 38% saturation).

binding are very similar, while tetrasaccharide-2 binding (Figure 4d) has a lesser effect despite the greater quantity of heparin fragment added. Only a few of the nonexchanged amide protons (peaks a and b) are perturbed by tetrasaccharide-2 binding. In the histidine C(2) region, all three histidine peaks (c, d, and e) are perturbed by the three series 2 fragments; however, the perturbation of peak e by tetrasaccharide-2 is smaller, and the shift is downfield instead of upfield. In the region of the aromatic resonances, the tryptophan 49 resonances (peaks f and j) are perturbed by all three heparin fragments. Of the other resonances perturbed by both octasaccharide-2 and hexasaccharide-2, only peaks i, k [from a histidine C(4)], and s are perturbed by the binding of tetrasaccharide-2. None of the aromatic residues having unusual upfield chemical shifts are affected by this fragment. That tetrasaccharide-2 perturbs some of the same resonances as intact heparin and the larger series 2 fragments indicates that this fragment also binds specifically in the primary heparin-binding site. However, the difference in degree of perturbation suggests that this fragment does not have the same effect upon protein structure when bound, even when allowance is made for the lower degree of binding site occupancy.

Perturbations induced in the upfield region of the antithrombin ^1H spectrum upon octasaccharide-2 and hexasaccharide-2 binding are not as similar between these two fragments as they are in the aromatic region of the spectrum, suggesting that the changes seen in the methyl proton region are more sensitive to fragment length. The greater number of total protons perturbed as well as the differences due to fragment length suggest that many of these perturbations result from interactions in the vicinity of the heparin-binding region. Both octasaccharide-2 and hexasaccharide-2 perturb the farthest upfield methyl proton resonances u–z, while tetrasaccharide-2 has little effect on these resonances. Perturbations to the main region of methyl proton resonances (0.9–1.1 ppm) differ among all three of the series 2 fragments, especially regarding the intensity of the heparin-bound antithrombin peaks m, n, and o and the unbound antithrombin peaks j and k.

Comparison of Series 1 Fragment Binding Effects. Figures 6 and 7 show perturbations in the downfield and upfield regions of the antithrombin ^1H spectrum, respectively, upon binding

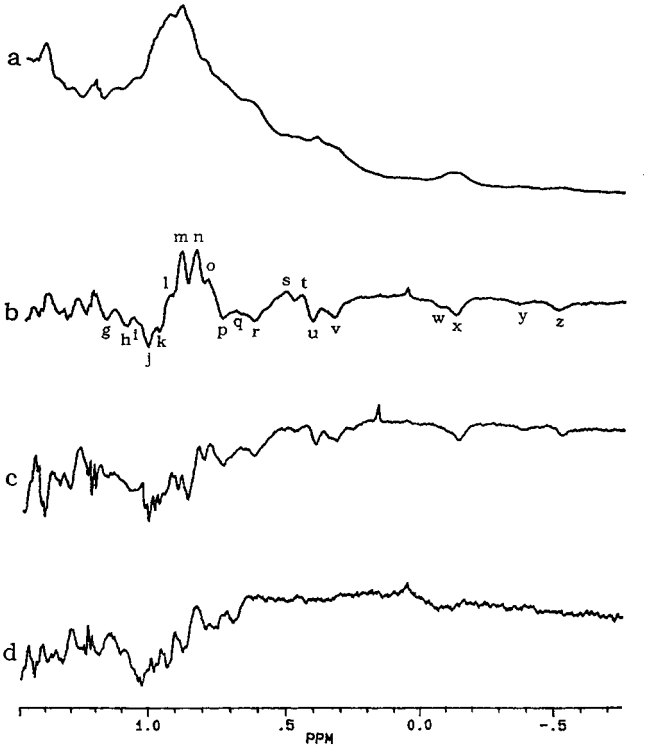

FIGURE 7: Upfield region of the ^1H spectrum of antithrombin, showing perturbations upon binding of the series 1 oligosaccharides: a, ^1H spectrum of antithrombin between –0.75 and 1.5 ppm; b, difference spectrum upon saturation with octasaccharide-1; c, difference spectrum upon saturation with hexasaccharide-1; d, difference spectrum upon binding tetrasaccharide-1 (at 38% saturation).

of the glucuronate-containing heparin fragments. The series 1 heparin fragments differ from their series 2 counterparts by the substitution of an unsulfated glucuronate residue for the iduronate-2-*O*-sulfate closest to the reducing end of the fragment. As with the series 2 tetrasaccharide, saturation could not be reached with the quantities of tetrasaccharide-1 available. A 6-fold molar excess of tetrasaccharide-1 to antithrombin was the final point in the titration, resulting in approximately 38% saturation of the antithrombin molecule. Fewer resonances are perturbed in the downfield region of the antithrombin difference spectrum (Figure 6) with decreasing fragment length. Only those amide resonances in the vicinity of peak b are perturbed by all three fragments; the amide peak a is not perturbed by tetrasaccharide-1 binding. Histidine peak d is perturbed upon binding of all three series 1 heparin fragments (Table II). In contrast, histidine peak c is not perturbed by either hexasaccharide-1 or tetrasaccharide-1, while it is perturbed by the series 2 hexasaccharide. Similarly, peak e is slightly perturbed by octasaccharide-1 and hexasaccharide-1, but not by tetrasaccharide-1. These data suggest that the glucuronate-containing fragments are either positioned differently in the primary heparin-binding site or that three iduronate-containing disaccharide units are necessary to perturb the antithrombin conformation enough to affect histidine peak c. Perturbations in the aromatic region of the antithrombin spectrum are simplified upon binding of the shorter series 1 fragments, as compared to series 2. While octasaccharide-1 perturbs similar aromatic resonances in this region as does octasaccharide-2 and intact heparin, hexasaccharide-1 perturbs peaks f (Trp-49), h, j (Trp-49), k, m, and p. Tetrasaccharide-1 perturbs only peak k [from a histidine C(4) proton] to any measurable extent, though more perturbations may be apparent if the molecule were saturated with the heparin fragment.

In the upfield region of the spectrum, the effects of fragment length on perturbation of methyl protons are also seen, although the differences between spectra are not as dramatic as seen in the downfield region, due to the larger number of protons involved. The methyl resonances farthest upfield (peaks u–z) are perturbed by binding of both octasaccharide-1 and hexasaccharide-1, but not by tetrasaccharide-1. As with the series 2 tetrasaccharide, tetrasaccharide-1 is not able to perturb the overall conformation of antithrombin to the same extent as the larger heparin fragments. This may be due in part to the tetrasaccharide fragments having only three normal heparin residues; the nonreducing end residue is a $\Delta^{4,5}$-unsaturated uronic acid produced by heparinase cleavage.

Comparison of Binding Effects for Hexasaccharide-1 versus High-Affinity Pentasaccharide. Of the heparin fragments examined, hexasaccharide-1 is most comparable to the structure of the original synthetic pentasaccharide having high affinity for antithrombin (Figure 1d), with three notable structural differences. The pentasaccharide does not contain the unsaturated uronic acid residue generated by heparinase cleavage at the nonreducing end of the hexasaccharide. However, Atha et al. (1984) have shown that, at least for an octasaccharide, the nonreducing end iduronic acid does not contribute to the energy of binding to antithrombin. In accordance with this finding, no change is noted in the unsaturated sugar's H(4) proton resonance upon binding to antithrombin for any of the fragments examined. Secondly, the glucuronic acid in hexasaccharide-1 is located penultimate to the reducing end of the oligosaccharide, while it is penultimate to the nonreducing end in the pentasaccharide. Most importantly, the hexasaccharide-1 fragment does not contain the 3-*O*-sulfate group shown to be necessary for high-affinity binding and enhancement of antithrombin inhibitory activity (Walenga et al., 1988). Hexasaccharide-1 does not perturb the antithrombin spectrum to the same extent as the pentasaccharide: histidine peak c is not perturbed, many of the aromatic resonances are not affected, and fewer methyl protons are perturbed upon hexasaccharide binding. However, both of the labeled amide protons (peaks a and b) and the far-upfield methyl protons (peaks u–z) are perturbed, indicating that hexasaccharide-1 binds in the primary heparin-binding site and perturbs the conformation of antithrombin in this region to some extent. Most of the differences in perturbations between these two heparin species cannot be due to loss of the 3-*O*-sulfate group, since octasaccharides 1 and 2 both perturb the antithrombin spectrum in a manner similar to intact heparin or pentasaccharide, yet neither fragment contains the 3-*O*-sulfate moiety. Nor can all of the perturbations seen be due to direct contact between heparin residues and antithrombin, since the pentasaccharide and the octasaccharide fragments (containing three extra sugar residues) produce very similar difference spectra upon binding. Instead, the difference in position of the glucuronic acid within hexasaccharide-1 may position the fragment within the heparin-binding region such that it is unable to induce the same local conformational changes as seen with the high-affinity pentasaccharide. Some of the same perturbations are made, as seen for histidine peak d, tryptophan 49 peaks f and j, and aromatic peaks h and p. However, the changes in the local magnetic field of the antithrombin protons, which lead to perturbation of histidine peak c and aromatic peaks g, i, l, q, and r, are not induced by hexasaccharide-1 binding.

In conclusion, the perturbations in the antithrombin ^{1}H NMR spectrum upon binding of defined low molecular weight heparin fragments indicate that the majority of the changes induced in the antithrombin conformation are common to binding of both active and inactive heparin species. Octasaccharide-1, octasaccharide-2, and hexasaccharide-2 perturb the ^{1}H spectrum in a manner similar to intact heparin and high-affinity pentasaccharide, despite the absence of the high-affinity structural motif. The smaller heparin fragments perturb many of the same specific resonances affected by the binding of intact heparin, indicating that these fragments are binding in the primary heparin-binding site. However, one particular resonance in the aromatic region of the ^{1}H spectrum is perturbed specifically by high-affinity heparin species, demonstrating that further minor changes are occurring between antithrombin and the high-affinity heparin species as a result of binding. Because the majority of the resonances perturbed are common to both active and inactive heparin species, these perturbations are probably localized to the vicinity of the heparin-binding site. Any additional structural changes resulting in catalytic activation do not appear to involve further large-scale changes in protein structure, since there are few differences between the ^{1}H NMR difference spectra of antithrombin upon binding of active heparin (either pentasaccharide or the intact species) as compared to the inactive heparin fragments. The inability of the fragments examined to activate antithrombin-mediated anti-factor Xa activity to the same extent as the high-affinity heparin species indicates that the majority of the conformational changes induced by heparin binding are not adequate for activation of antithrombin.

Acknowledgments

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Primary Structure and Function of Novel *O*-Glycosylated Hirudins from the Leech *Hirudinaria manillensis*

Verena Steiner,*,‡ René Knecht,‡ K. Olaf Börnsen,‡ Ernst Gassmann,‡ Stuart R. Stone,§ Fritz Raschdorf,‡ Jean-Marc Schlaeppi,‡ and Reinhard Maschler∥

Ciba-Geigy Ltd., CH-4002 Basel, Switzerland, Friedrich Miescher Institut, CH-4002 Basel, Switzerland, and GEN Therapeutica OHG, D-2903 Bad Zwischenahn, FRG

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ABSTRACT: Hirudin from the leech *Hirudo medicinalis* is a most powerful anticoagulant, and many isoforms have been described. In the present work, the primary structure of two hirudins from the leech *Hirudinaria manillensis* has been elucidated. The antithrombotic activity is similar to that of *H. medicinalis* hirudins although the sequence identity is below 60%. Surprisingly, the hirudins were found to be glycosylated at one site. Sugar analysis after methanolysis yielded fucose, galactose, and *N*-acetylgalactosamine. These results combined with data from matrix-assisted laser desorption ionization mass spectrometry, plasma desorption mass spectrometry, capillary zone electrophoresis, and lectin-binding tests indicate that the sequence is Fuc-Galβ1-3GalNAc-(*O*-threonine). This structure shows an interesting similarity to human blood group H determinants.

Hirudin, a 65 amino acid peptide produced in the salivary glands of the medicinal leech, is a most potent inhibitor of blood coagulation. It binds specifically and tightly to thrombin [K_I = 22 fM] (Stone & Hofsteenge, 1986), the last regulatory enzyme of the blood clotting cascade, and thus has a high therapeutic potential for treatment of various thrombotic diseases. To date, the amino acid sequences of about 20 isohirudins are known (Dodt et al., 1984, 1986; Scharf et al., 1989; Tripier, 1988) as well as a sequence predicted from cDNA (Harvey et al., 1986). No glycosylation has been found so far, the only posttranslational modification being sulfation at Tyr^{63}. The synthesis of a gene encoding hirudin and its expression in *Escherichia coli* or *Saccharomyces cerevisiae* has been reported by different groups (Bergmann et al., 1986; Loison et al., 1988). The three-dimensional structure of natural and recombinant hirudin has been elucidated by ^{1}H NMR spectroscopy (Clore et al., 1987; Haruyama & Wüthrich, 1989; Folkers et al., 1989), and, recently, the crystal structure of the thrombin-hirudin complex has been reported by two groups (Grütter et al., 1990; Rydel et al., 1990). These structures indicate that hirudin inhibits thrombin by a novel mechanism which does not utilize binding to the primary specificity pocket of the latter.

Apart from hirudins isolated from the leech *Hirudo medicinalis*, no complete hirudin structure from another leech has been described to date although knowledge of conserved regions could be of interest for the design of new thrombin inhibitors. For our study, we chose the leech *Hirudinaria manillensis*, called "buffalo leech", which is reported to be more specialized for mammalian parasitism than *H. medicinalis* (Sawyer, 1986). *H. manillensis* is prevalent in southeast Asia and like *H. medicinalis* belongs to the Hirudidae family (Mann, 1962). We report the complete amino acid sequence of two *O*-glycosylated hirudins (designated P6 and P18) from *H. manillensis*, the characterization of their carbohydrate moiety, and their inhibitory activity.

Materials and Methods

Isolation and Purification of P6 and P18. Hirudin P6 and P18 were isolated and purified to homogeneity as described (Steiner et al., 1990).

Amino Acid Analysis. Approximately 1.5 μg of each reduced and *S*-pyridylethylated isoform was hydrolyzed with 6

* To whom correspondence should be addressed.
‡ Ciba-Geigy Ltd.
§ Friedrich Miescher Institut.
∥ GEN Therapeutica OHG.

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