Carbohydrate Research, 185 (1989) 69–76
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

EXAMINATION, BY ^{1}H-N.M.R. SPECTROSCOPY, OF THE BINDING OF A SYNTHETIC, HIGH-AFFINITY HEPARIN PENTASACCHARIDE TO HUMAN ANTITHROMBIN III

PETER GETTINS*

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 (U.S.A.)

AND JEAN CHOAY,

Institut Choay, 46 avenue Théophile Gautier, F-75016 Paris (France)

(Received March 25th, 1988; accepted for publication, July 21st, 1988)

ABSTRACT

Binding of a synthetic, high-affinity heparin pentasaccharide and of intact heparin to both native and elastase-modified human antithrombin III have been examined by ^{1}H-n.m.r. spectroscopy. The pentasaccharide perturbs many protein resonances in the same way as does intact heparin. There are, however, differences that seem to arise both from fewer contacts in the heparin binding-site when the pentasaccharide binds and from dissimilar conformational changes in the protein. The resonance of the H-2 atom of the histidine, considered to be the N-terminal residue and to be located in the heparin binding-site, is strongly perturbed by heparin binding both to native and modified antithrombin. The pentasaccharide has little effect on this histidine in either protein. Resonances from two of the remaining four histidine units are sensitive to longer-range conformational changes, and show differences between binding of the two heparin species both in native and modified ATIII. It is concluded that the pentasaccharide only partly fills the heparin binding-site and does not produce a conformational change identical to that caused by intact heparin. This is particularly significant as regards the mechanism of action of heparin, because the synthetic pentasaccharide activates ATIII towards Factor Xa, but not towards thrombin.

INTRODUCTION

Heparin is a heterogeneous sulfated glycosaminoglycan that is used extensively as an anticoagulant. It binds to antithrombin III (ATIII) with high affinity, and accelerates, by up to 1000-fold, the rate at which ATIII inactivates thrombin and other activated serine proteases of the intrinsic pathway of the blood-clotting system[1]. Based on extensive examination of the binding properties of

*To whom correspondence should be addressed.

0008-6215/89/$ 03.50

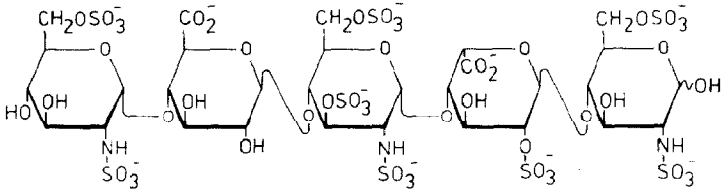

Fig. 1. Synthetic, high-affinity heparin pentasaccharide.

heparin fragments produced by a number of different degradative procedures, it was proposed that there is a minimum pentasaccharide sequence (see Fig. 1) necessary for high-affinity binding to[2] ATIII. This pentasaccharide was shown to contain the relatively uncommon 3-*O*-sulfate group on one of the D-glucosamine units that appears to be critical[2,3] in producing high-affinity binding to ATIII. The high-affinity pentasaccharide has been synthesized[4] and shown to possess, for the sulfated L-iduronate residue, an unusual conformation that may be due to the unusual 3-*O*-sulfated D-glucosamine residue adjacent to it[5].

^{1}H-N.m.r. studies of human ATIII have shown that heparin binding perturbs 10–20 aromatic proton resonances, as well as resonances at chemical shifts expected from lysine and arginine side-chains and several methyl groups[6]. The effects of heparin fragments of different length on the ^{1}H-n.m.r. spectrum of ATIII have been examined, and it was confirmed that most of the perturbations are produced by a fragment as small as an octasaccharide[6]. However, the n.m.r. studies were performed on fragments having a uniform size that were still chemically heterogeneous. We report here the ^{1}H-n.m.r.-spectral perturbations produced by binding the synthetic, high-affinity heparin pentasaccharide already described to both native human ATIII and elastase-modified ATIII.

Comparison of the difference spectra for pentasaccharide or intact-heparin binding to ATIII shows that the former perturbs many of the same resonances as does the latter, and in an identical way. There are, however, significant differences that are most unambiguously seen for the histidine resonances. The same pattern of common perturbations, but significant differences, is seen for binding of the pentasaccharide or intact heparin to elastase-modified ATIII.

EXPERIMENTAL

Antithrombin III. — ATIII was isolated from outdated human plasma by a modification[6] of the method of Thaler and Schmer[7]. The plasma was obtained from the Vanderbilt Hospital blood-bank. Elastase-modified ATIII was prepared by reaction for 20 min of ATIII with porcine intestinal elastase at a ratio of 400:1. The modified protein was separated on a heparin–Sepharose column by elution with a NaCl gradient. The structural and heparin-binding properties of thrombin-cleaved, human neutrophil elastase-cleaved, and porcine elastase-cleaved ATIIIs have been shown to be indistinguishable[8], despite small differences of a few amino acids in the

site of cleavage in the loop containing the reactive-site Arg^{393}–Ser^{394} bond[8,9]. Protein concentrations were determined spectrophotometrically, using $E^{1\%}_{280nm} = 0.65$ and a molecular weight of 58,000 for[10,11] native human ATIII. Björk and Fish[12] have shown an increase in extinction coefficient of 7.5% for thrombin-modified bovine ATIII. A similar percentage increase was assumed here, and $E^{1\%}_{280nm} = 0.70$ was used for proteolytically modified, human ATIII.

Heparin. — The methyl α-glycoside of the high-affinity pentasaccharide was prepared as previously described[4]. Intact heparin (porcine intestinal) was obtained from Sigma.

^{1}H-N.m.r. measurements. — ^{1}H-N.m.r. spectra were recorded at 400 MHz with a Bruker AM400 narrow-bore spectrometer equipped with a 5-mm, ^{1}H probe. Samples were dissolved in D_2O and residual water proton intensity was diminished by presaturation of the HOD resonance. 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. A pulse angle of 30° and a repetition rate of 0.85 s^{-1} were used. Values of pH* are reported as pH-meter readings uncorrected for deuterium isotope effects, and were measured in the n.m.r. tube by using an Ingold 3-mm diameter combination electrode. Chemical shifts are given relative to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate at 0 p.p.m.

RESULTS

Binding of heparin and pentasaccharide to native ATIII. — The aromatic region of the ^{1}H-n.m.r. spectrum of human AT III is shown in Fig. 2a. The

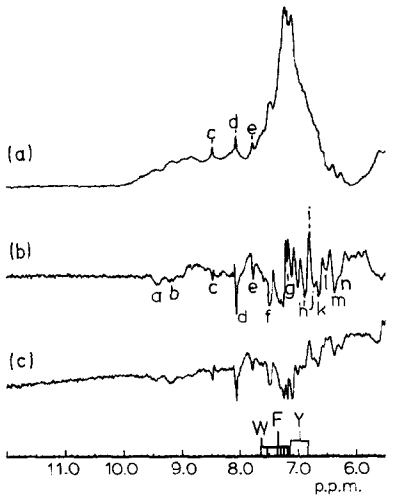

Fig. 2. The effects of binding heparin to native ATIII. (a) Aromatic region of ^{1}H-n.m.r. spectrum of native ATIII (0.40mM), 2000 scans, pH* 6.50. (b) Difference spectrum after addition of 1 equiv. high-affinity pentasaccharide. Vertical scale is twice that in (a). (c) Difference spectrum after adding intact heparin; added assuming at least one high-affinity binding site per 40 sugar residues. Vertical scale is twice that in (a).

resonances labelled c, d, and e arise from the H-2 atoms of three of the five histidine residues present in the protein. At this pH value, the resonances from the remaining two histidine residues are less clearly seen; they occur on each side of resonance d. The difference spectrum representing perturbations from binding of the synthetic pentasaccharide is shown in Fig. 2b. Negative peaks arise from resonance intensity occurring only in the spectrum of ATIII, and positive peaks from intensity present only in the spectrum of the complex. This difference spectrum shows a minimum of 20 perturbed resonances, of which only those that will subsequently be used in the discussion are labelled. Resonances a and b are broad, and occur in the region expected for amide protons. Their existence in D_2O solution indicated that they arise from stable hydrogen-bonded amides, probably in a region of secondary structure. Three clearly visible resonances (c, d, and e) are from the histidine H-2 atoms. Because their resonance positions can be identified in both the native and the complex spectra, the perturbation of each resonance by oligosaccharide can be determined (see Table I). These perturbations are small but readily and accurately measurable, as each histidine H-2 resonance is narrow, and well resolved from the underlying broad amide resonances. The differences in chemical shift are calculated from the peak positions in the original spectra, not from the difference spectra. Resonance g is identifiable as that of one of the histidine H-4 atoms. The rest of the perturbed resonances can only be identified as being from aromatic side-chains, without any attribution as to type. The "random coil" chemical shifts of tryptophan, phenylalanine, and tyrosine side-chain-proton resonances are indicated, although it is clear that, at least for resonances k, l, m, and n, there must be significant upfield shifts from these positions, whatever the nature of the side chain. In the high-field region of the difference spectrum (see Fig. 3b), there are at least 15 resonances perturbed by binding of the pentasaccharide. These include resonances at positions expected for Arg or Lys side chains (m) and leucine, isoleucine, or valine methyl groups (n, l, or r). In addition, there are several perturbed resonances to higher field that most probably arise from methyl groups in unusual environments; *e.g.*, shifted upfield by virtue of proximity to an aromatic side-chain.

TABLE I

CHANGES IN ^{1}H-CHEMICAL SHIFT FOR ATIII HISTIDINE H-2 RESONANCES AT 310K AND pH* 6.5

Sample	$\Delta\delta^a$ *for selected resonances (p.p.m.)*		
	c	*d*	*e*
Native ATIII + pentasaccharide	−0.008	+0.031	+0.018
Native ATIII + heparin	−0.017	+0.042	+0.091
Modified ATIII + pentasaccharide	+0.061	+0.009	+0.009
Modified ATIII + heparin	+0.037	+0.025	+0.091

[a]With respect to native ATIII. Errors in $\Delta\delta$ values are ±0.004 p.p.m.

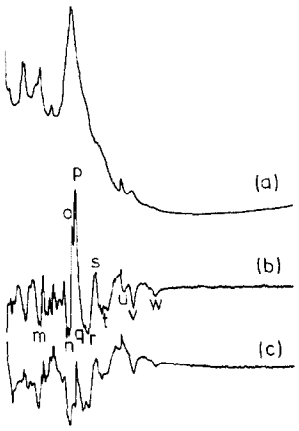

Fig. 3. The effects of binding heparin to native ATIII. (a) Upfield aliphatic region of ^{1}H-n.m.r. spectrum of native ATIII (0.40mM, pH* 6.5, 2000 scans). The vertical scale is one quarter that used in Fig. 2a. (b) Difference spectrum after addition of 1 equiv. of high-affinity pentasaccharide. Vertical scale is four times that in 3a. (c) Difference spectrum after adding intact heparin. Vertical scale is four times that in 3a.

The aromatic and aliphatic region difference-spectra from binding intact heparin to ATIII are shown in Figs. 2c and 3c, respectively. In the aromatic region, the three histidine H-2 resonances are again perturbed, but the magnitudes of the perturbations are different (see Table I), particularly for e, where the chemical shift changes by 0.091 p.p.m. compared with only 0.018 p.p.m. with the pentasaccharide. Difference peaks corresponding in position to f, j, k, m, and n of Fig. 2b. 2b occur in Fig. 2c. The remainder of the aromatic region of Fig. 2c is quite different in appearance. In the aliphatic region, there is much greater similarity between the two difference-spectra, particularly in the resonances in uncomplexed ATIII (negative peaks) that are perturbed. Thus, the features m, n, q, r, t, v, and w in Fig. 3b are very closely reproduced in Fig. 3c. The major differences are the absence, from Fig. 3c, of the group of sharp peaks between m and n, and of the intense peaks o and p.

Binding of heparin and pentasaccharide to elastase-modified ATIII. — Heparin binds to proteolytically modified ATIII, but with somewhat lessened affinity[12]. The aromatic-region difference-spectra from binding the pentasaccharide or intact heparin to modified ATIII are shown in Figs. 4b and 4c; it should be noted that the better s/n ratio for Fig. 4b is due to more scans being used for one of the initial spectra. The three histidine H-2 atoms can again be identified in the spectra of free and complexed ATIII. The changes in chemical shift upon heparin binding are summarized in Table I. The most striking difference between the effects of binding the synthetic pentasaccharide and intact heparin on the histidines is again for the resonance labelled e. Somewhat surprisingly, the chemical shift changes for binding to native or modified ATIII for a given heparin species are very similar for

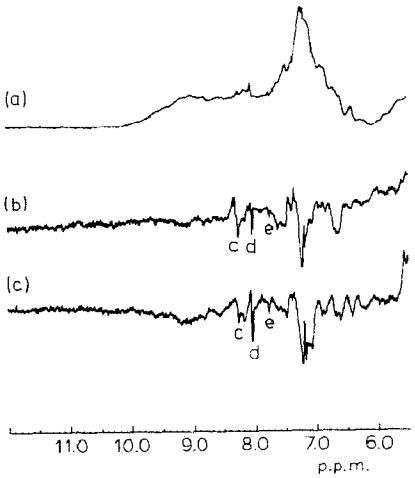

Fig. 4. The effects of binding heparin to elastase-modified ATIII. (a) Aromatic region of ^{1}H-n.m.r. spectrum of 0.32mM modified ATIII, pH* 6.50, 2000 scans. (b) Difference spectrum after addition of 1 equiv. of high-affinity pentasaccharide. Vertical scale is four times that in (a). (c) Difference spectrum after addition of intact heparin. Vertical scale is four times that in (a).

this histidine residue, but quite different for the other two histidine units. In the rest of the difference spectrum, the negative peak at 6.6 p.p.m. in Fig. 4b also appears in Fig. 4c, but otherwise there are no obvious similarities. In overall intensity of resonances perturbed, fewer are affected by the pentasaccharide than by intact heparin.

DISCUSSION

The five histidine residues in human ATIII occur at positions 1, 65, 120, 319, and 369 in the sequence. It has been shown that they are sensitive to changes produced by heparin binding[6], limited unfolding at low concentration of guanidine[13], and cleavage at, or close to, the reactive-site L-serine. There are also strong indications that histidine 1 is in the heparin-binding domain[6,13] and that its H-2 resonance is the one labelled e in Fig. 2. The chemical shift of resonance e is affected by almost the same amount by binding of intact heparin to either native or modified ATIII (see Table I). This change is also the largest perturbation to any of the histidines, and is in keeping with other indicators that this histidine unit is directly involved in heparin binding[6,13]. Upon binding the pentasaccharide to either native or modified ATIII, the same histidine unit is little affected. In contrast, resonances c and d show changes in chemical shift that differ much less for the pentasaccharide *versus* intact heparin, and are of significant, although different, magnitude, for native or modified ATIII. It seems that histidine units giving rise to resonances c and d reflect longer-range conformational changes produced by heparin binding. Because the starting conformations of native and modified ATIII are very different, it is not too surprising that heparin binding should produce changes of different magnitude, and even different sign, in the two protein forms.

In contrast, the perturbation of resonance e is not affected by whether the ATIII is in the native or cleaved form, but is very dependent on whether the bound heparin is a pentasaccharide or a much larger heparin species.

A comparison of the upfield aliphatic-region perturbations produced by binding pentasaccharide or intact heparin to native ATIII shows many more similarities than differences (see Fig. 3). In the aromatic region, however, there are some perturbations in common, but more differences. A qualitative difference is the sharper appearance of Fig. 2b than 2c. Ironically, this probably arises from perturbation of more resonances in 2c than in 2b, but with greater overlap and partial cancellation; this is always a problem with difference spectroscopy when peaks are perturbed by less than their linewidth; the resulting difference peaks then partially cancel one another. This is exacerbated when many resonances change position in different ways within a narrow frequency range, leading to fortuitous cancellations. Only a much higher-field spectrum could determine whether or not this occurs here. It is clear, however, that the two heparin species do not perturb histidine resonances c or d identically, either in intact or modified ATIII (see Table I). These differences presumably reflect dissimilar conformational changes produced by binding heparin pentasaccharide or intact heparin. This may correlate with the ability of heparin to activate ATIII against thrombin as well as Factor Xa, but of the pentasaccharide to activate ATIII only against[4] Factor Xa. In addition, fewer resonances may be directly perturbed by contacts in the heparin-binding region when the short pentasaccharide binds than when intact heparin binds. This seems more apparent on comparison of Figs. 4b and 4c than of Figs. 2b and 2c. In particular, it seems likely that the binding of classical heparin involves a chain longer than 5 sugar residues and includes histidine 1 near one extreme. The pentasaccharide does not fill the sugar site including histidine 1, and so it does not perturb it. In contrast, intact heparin does bind at this site, perturbs the histidine resonance by 0.091 p.p.m. at this pH, and raises the pKa of the histidine by 0.42 pH unit[6].

ACKNOWLEDGMENTS

Financial support from the National Institutes of Health through Grant HL32595 is gratefully acknowledged. We thank Brad Harten for preparation of antithrombin III.

REFERENCES

1 B. CASU, *Adv. Carbohydr. Chem. Biochem.*, 43 (1985) 51–134.
2 (a) J. CHOAY, J.-C. LORMEAU, M. PETITOU, P. SINA, AND J. FAREED, *Ann. N.Y. Acad. Sci.*, 370 (1981) 644–649; (b) L. THUNBERG, G. BÄCKSTRÖM, AND U. LINDAHL, *Carbohydr. Res.*, 100 (1982) 393–410.
3 (a) B. CASU, P. ORESTE, G. TORRI, G. ZOPETTI, J. CHOAY, J.-C. LORMEAU, M. PETITOU, AND P. SINA, *Biochem. J.*, 197 (1981) 509–609; (b) U. LINDAHL, G. BÄCKSTROM, L. THUNBERG, AND I. G. LEDER, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 6551–6555; (c) J. CHOAY, J.-C. LORMEAU, AND M. PETITOU, *Ann. Pharm. Fr.*, 39 (1981) 37–44.

4 M. PETITOU, P. DUCHAUSSOY, I. LEDERMAN, J. CHOAY, P. SINA, J.-C. JACQUINET, AND G. TORRI, *Carbohydr. Res.*, 147 (1986) 221–236.
5 G. TORRI, B. CASU, G. GATTI, M. PETITOU, J. CHOAY, J.-C. JACQUINET, AND P. SINA, *Biochem. Biophys. Res. Commun.*, 128 (1985) 134–140.
6 P. GETTINS, *Biochemistry*, 26 (1987) 1391–1398.
7 E. THALER AND G. SCHMER, *Br. J. Haematol.*, 31 (1975) 233–243.
8 P. GETTINS AND B. HARTEN, *Biochemistry*, 27 (1988) 3634–3639.
9 (a) S. J. OLSON, *J. Biol. Chem.*, 260 (1985) 10,153–10,160; (b) R. W. CARRELL AND M. C. OWEN, *Nature (London)*, 317 (1985) 730–732.
10 B. NORDENMEN, C. NYSTROM, AND I. BJÖRK, *Eur. J. Biochem.*, 78 (1977) 195–201.
11 T. CHANDRA, R. STACKHOUSE, V. J. KODO, AND S. L. C. WOO, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 1845–1848.
12 I. BJÖRK AND W. W. FISH, *J. Biol. Chem.*, 257 (1982) 9487–9493.
13 P. GETTINS AND E. W. WOOTEN, *Biochemistry*, 26 (1987) 4403–4408.