Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 492-499

STRUCTURE-ACTIVITY RELATIONSHIP IN HEPARIN : A SYNTHETIC PENTASACCHARIDE WITH HIGH AFFINITY FOR ANTITHROMBIN III AND ELICITING HIGH ANTI-FACTOR Xa ACTIVITY

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Received September 12, 1983

SUMMARY : The structures of the tetrasaccharide (β-D-glucuronic acid)1→4(N-sulfate-3,6-di-O-sulfate-α-D-glucosamine)1→4(2-O-sulfate-α-L-iduronic acid)1→4(N-sulfate-6-O-sulfate-D-glucosamine) and of the pentasaccharide (N-sulfate-6-O-sulfate-α-D-glucosamine)1→4(β-D-glucuronic acid)1→4(N-sulfate-3,6-di-O-sulfate-α-D-glucosamine)1→4(2-O-sulfate-α-L-iduronic acid)1→4(N-sulfate-6-O-sulfate-D-glucosamine), both prepared for the first time, by chemical synthesis from D-glucose and D-glucosamine, have been confirmed by nuclear magnetic resonance. The synthetic tetrasaccharide neither binds to AT-III nor induces anti-factor Xa activity enhancement of this inhibitor. In contrast, the synthetic pentasaccharide strongly binds to AT-III (Ka : $7.10^6 M^{-1}$) forming an equimolar complex and also enhances the AT-III inhibitory activity towards factor Xa. These results confirm that the synthetic pentasaccharide with the above structure corresponds to the actual minimal sequence required in heparin for binding to AT-III.

Heparin inhibits a number of procoagulant proteases mainly by binding to antithrombin III(°) and enhancing the effects of this inhibitor (1). With respect to this inhibition, the blood coagulation proteases may be classified into two types : factor Xa type and factor IIa type (2). Whereas high molecular weight heparin species are able to interact with both types, a decrease in molecular weight is correlated with a decrease in activity towards the factor IIa type (3-6).

Our search for minimal molecular weight heparin fractions still retaining high anti-factor Xa potency in plasma led us to isolate from heparin a decasaccharide with high affinity for AT-III, the structure of which was analyzed by nuclear magnetic resonance (6). Two octasaccharides, subsequently obtained either by nitrous acid deamination (7) or cleavage by bacterial heparinase (8,9) also displayed similar AT-III affinity and

(°) Abbreviations : AT-III, antithrombin III ; anti-Xa, anti-factor Xa ;

0006-291X/83 $1.50

anti-Xa activity. A comparison between the structures of these two octasaccharides led us to the idea that the disaccharide sequence located at the non-reducing end was not essential (8), and prompted us to prepare, by exhaustive heparinase cleavage, a hexasaccharide which also proved to be active (10). Since it contained a modified (4,5-unsaturated) uronic acid at its non-reducing end, we hypothesized that, in heparin, the minimal sequence that binds to AT-III and elicits high anti-Xa activity was in fact contained in a pentasaccharide (10). A first experimental support for this hypothesis was obtained by other authors after enzymatic removal of non-essential residues in species with AT-III high affinity (11). However, until now, no pentasaccharide *per se* has been obtained in sufficient quantities for investigation.

Other studies on the hexasaccharidic common segment present in the two active octasaccharides have shown that some structural variability is compatible with affinity for AT-III. Thus the N-acetyl group on residue $A_{a/s}$ (Fig.1) can be replaced by an amino group without loss of affinity (11). These studies have also allowed the structural determination of major features. Selective enzymatic and chemical modifications have indicated that the three sulfate groups (in Fig.1, full circles) have a major role in affinity (11,12). However, in spite of its presence in all active molecular species (13,14), no definitive proof has been provided as regard to the essential role of the 3-O-sulfate group of residue A^{*}_{s} (in Fig.1, dotted circle).

We have designed a protocol mainly based on the chemical synthesis of heparin oligosaccharides, in order to rigorously test the hypothesis that a pentasaccharide represents the minimal AT-III binding sequence. Our first aim was to obtain a synthetic pentasaccharide fitting into the predicted structure of the minimal heparin binding site to AT-III and in addition presenting all the structural features reported as essential for this binding. For convenience we chose to prepare the tri-N-sulfated structural variant [as it occurs in beef-lung heparin (15)], this variant (Fig.2) also being sulfated on all the primary hydroxyl groups. For need of comparison we also decided to synthesize one of the two possible tetrasaccharides which are part of the pentasaccharide (Fig.3). This program represents the first approach to synthesize specifically substituted glycosaminoglycan sequences. Among other problems, it has required synthesizing α-linked-L-iduronate residues and introducing sulfate groups into the appropriate positions in the L-iduronic and D-glucosamine residues. Here we show that while the synthetic tetrasaccharide neither binds to AT-III nor induces anti-Xa activity, the synthetic pentasaccharide corresponds to the

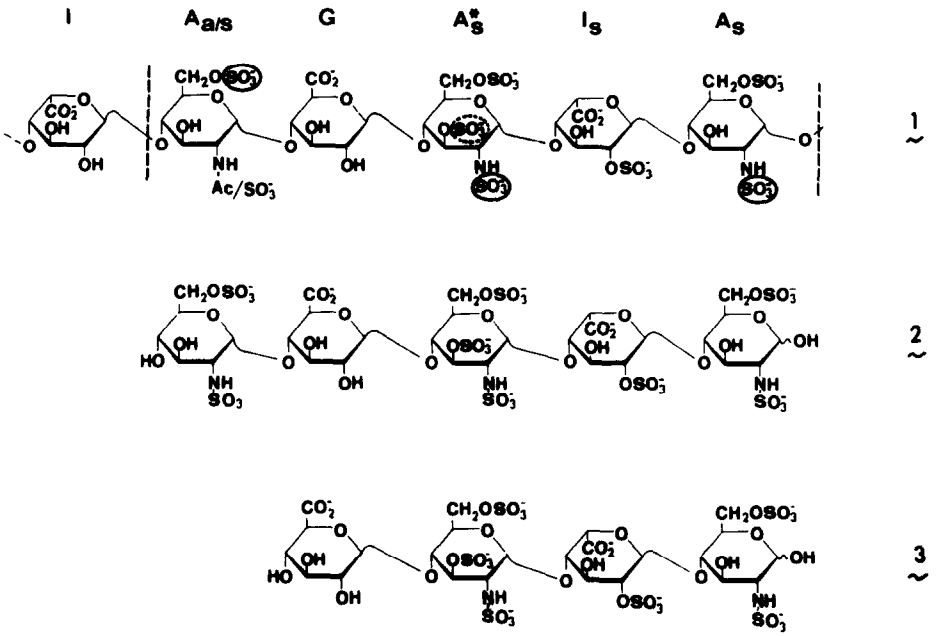

Figure 1 : HEPARIN SEQUENCES AND FRAGMENTS INVOLVED IN AT-III BINDING SEQUENCE

I : α-L-iduronic acid ; $A_{a/s}$: N-acetyl-6-O-sulfate-α-D-glucosamine (A_a) or N-sulfate-6-O-sulfate-α-D-glucosamine (A_s) ; G : β-D-glucuronic acid ; A^*_s : N-sulfate-3,6-di-O-sulfate-α-D-glucosamine ; I_s : 2-O-sulfate-α-L-iduronic acid ; A_s : N-sulfate-6-O-sulfate-α-D-glucosamine. 1 : hexasaccharide segment present in the two active octasaccharides obtained after depolymerization by nitrous acid or heparinase. The dotted lines encompass two structural variants ($A_{a/s}$) of the proposed pentasaccharide sequence responsible for the binding to AT-III. Full circles indicate proved essential sulfate groups. Dotted circle indicates a sulfate group that is always present but whose role has still not been proved essential. 2 : synthetic pentasaccharide (N-sulfate-6-O-sulfate-α-D-glucosamine)1→4(β-D-glucuronic acid)1→4(N-sulfate-3,6-di-O-sulfate-α-D-glucosamine)1→4(2-O-sulfate-α-L-iduronic acid)1→4(N-sulfate-6-O-sulfate-D-glucosamine) ; 3 : synthetic tetrasaccharide (β-D-glucuronic acid)1→4(N-sulfate-3,6-di-O-sulfate-α-D-glucosamine)1→4(2-O-sulfate-α-L-iduronic acid)1→4(N-sulfate-6-O-sulfate-D-glucosamine).

actual minimal sequence that is required in heparin species to bind AT-III and to induce anti-Xa activity.

MATERIALS AND METHODS

Synthetic tetrasaccharide and synthetic pentasaccharide. (In the text : the tetrasaccharide and the pentasaccharide). Their formula and molecular weight are respectively: $C_{24}H_{32}N_2O_{39}S_6Na_8$, 1349 daltons, and $C_{30}H_{41}N_3O_{49}S_8Na_{10}$, 1714 daltons. Their structures are shown in Fig.1. They were prepared from D-glucose and D-glucosamine by chemical synthesis following a methodology that allows the preparation of specifically substituted glycosaminoglycan fragments (16). This method permits the sulfation of hydroxyl groups as required.

Antithrombins. Human AT-III was obtained from Kabi A.B., Sweden. Bovine AT-III was prepared as described (17) ; purity was controlled by disc-electrophoresis.

NMR spectra. The proton-NMR spectra were obtained from solutions in D_2O (c = 1 % w/v) with a HX-270 Bruker spectrometer, in the Pulse-Fourier-Transform mode. Free induction decays were multiplied by Gaussian-to-Laurentian digital filter prior to transformation, for resolution enhancement. Chemi-

cal shifts are referred to internal sodium 3-Trimethylsilyl-Propionate-2,2,3,3-d_4.

AT-III binding experiments by gel filtration were adapted from (18) and performed with bovine AT-III on a 1 x 100 cm Sephadex G.50 in NaCl 0.15 M, Tris-HCl 0.01 M pH 7.5 buffer. AT-III was detected by its absorbance at 280 nm, and oligosaccharides by the borate carbazole method (19). The elution of AT-III, of the tetrasaccharide and of the pentasaccharide were measured. AT-III (0.1 µmole ; 6.5 mg) and the oligosaccharide to be tested (0.1 µmole) were mixed in the buffer (1 ml). After 15 minutes at room temperature, the solution was layered on top of the column.

Evaluation of the association constant of the pentasaccharide and AT-III. The effect of the pentasaccharide on the fluorescence enhancement of tryptophan residues in AT-III was measured (20) with a FICA 0.15 M ; excitation and emission wavelengths were respectively 278 and 335 nm. AT-III concentration was 1 µM. Determination of the association constant from the fluorescence enhancement data was done by Scatchard plot.

Activity determinations. Anti-Xa activity was measured by clotting assay (Yin et al.) (21) and by amidolytic assay (Teien and Lie) (22) using 0.02 M Tris-maleate buffer pH 7.5 instead of Tris-HCl, for plasma dilution. Anti-thrombin activity was measured by amidolytic assay (Larsen et al.) (23).

RESULTS

^{1}H-NMR characterization of the tetrasaccharide and of the pentasaccharide. Fig.2 shows the 270 MHz ^{1}H-NMR spectra of the tetrasaccharide and the pentasaccharide. Signals assignment correlates with a number of synthetic model compounds (16), and was confirmed by spin-decoupling. The spectra are in agreement with the structure of the two oligosaccharides.

AT-III binding experiments by gel filtration. 1) As illustrated in Fig.3, the elution volumes of AT-III, of the pentasaccharide and of the tetrasaccharide

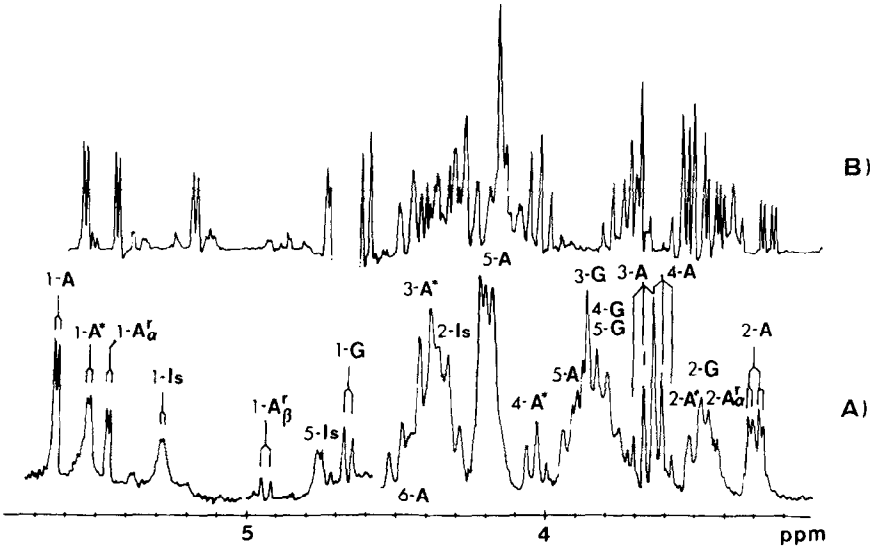

Figure 2 : ^{1}H-NMR SPECTRA OF THE SYNTHETIC PENTASACCHARIDE (A) AND OF THE SYNTHETIC TETRASACCHARIDE (B). These were recorded at 270 MHz and 35° C (c - 1 % w/v in D_2O). The spectrum A in the region 4.5-5.0 ppm was obtained at 60° C.

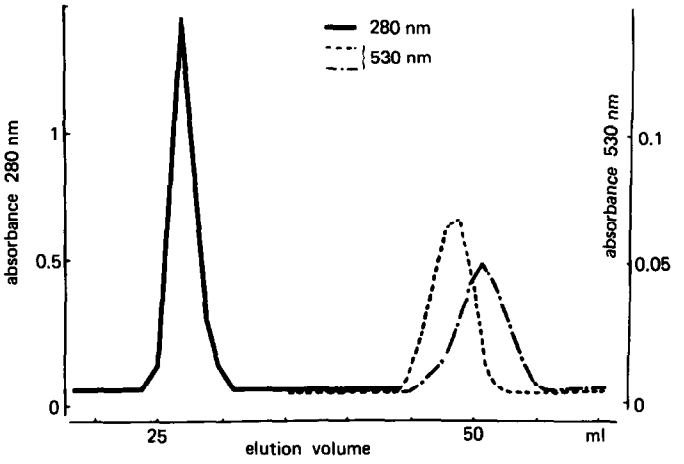

Figure 3 : STUDY OF THE AT-III BINDING PROPERTIES OF THE SYNTHETIC PENTASACCHARIDE AND OF THE SYNTHETIC TETRASACCHARIDE . A column of Sephadex G.50 (1 x 100 cm) was used, eluted with 0.15 M NaCl in Tris-HCl, 0.01 M, pH 7.5. When chromatographied individually, AT-III, pentasaccharide and tetrasaccharide showed peak A, B and C respectively. An equimolar mixture of AT-III and pentasaccharide yielded only peak A, which was in fact a complex AT-III/pentasaccharide. In contrast, an equimolar mixture of AT-III and tetrasaccharide gave the two peaks A and C. At higher ionic strength (2 M NaCl), the above complex was dissociated into peaks A and B.

are respectively 27 ml, 48 ml and 51 ml. 2) An equimolar mixture of AT-III and pentasaccharide prepared as described in Materials and Methods produces only one peak at 27 ml, and no peak at all at 48 ml. If the experiment is repeated with an excess of pentasaccharide (6.5 mg AT-III + 0.313 mg pentasaccharide), the excess is eluted at 48 ml. This indicates the formation of an equimolar complex and consequently the presence of only one binding site. 3) Fractions containing the AT-III/pentasaccharide complex have been collected, freeze-dried, dissolved in 1 ml 2 M NaCl buffer, and passed through the Sephadex column, previously equilibrated with the same buffer. The two peaks of AT-III and of the pentasaccharide were observed at their normal elution volumes (27 ml and 48 ml, respectively), indicating that the complex dissociates under these conditions. 4) An equimolar amount of AT-III and tetrasaccharide was treated as in 2). The tetrasaccharide was eluted entirely in the 51 ml peak, indicating a complete absence of binding to AT-III.

Evaluation of the association constant of the pentasaccharide and AT-III. Addition of the pentasaccharide to AT-III enhances the fluorescence of the tryptophan residues in the protein. In contrast, addition of the tetrasaccharide under the same conditions has no influence on the fluorescence. Fig.4 shows the Scatchard plot obtained for the AT-III pentasaccharide system. The association constant determined from the plot is $7.10^{6}M^{-1}$. The

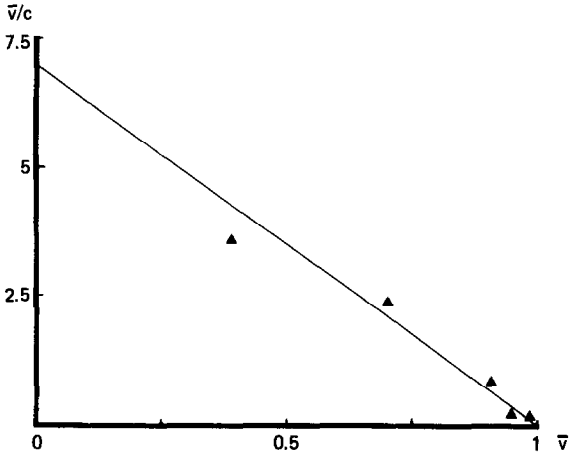

Figure 4 : SCATCHARD PLOT OF THE SYNTHETIC PENTASACCHARIDE BINDING TO ANTITHROMBIN III

The titration data was obtained at 25° C in 0.03 M NaCl, 0.2 M glycine, Tris-HCl, pH 7.4. Excitation wavelength 278 nm. Emission wavelength 335 nm. AT-III concentration was 1μmole. Each point represents the average of 5 determinations. $\bar{\nu}$: moles of bound pentasaccharide per mole of AT-III. C : concentration of unbound pentasaccharide μM.

curve runs accross the x-axis near the 1 value, proving that only one binding site exists in the complex.

Activity determinations. The results in Table 1 show that the pentasaccharide has a high anti-Xa activity, and no inhibiting activity on thrombin. The tetrasaccharide does not show any activity in either of the tests.

DISCUSSION

Our results show that chemical synthesis is a fruitful approach to the study of the structure/activity relationships in the field of specifically sulfated glycosaminoglycans. The fragments studied represent the sequences present in heparin and one of them, the pentasaccharide, exhibits the expected biophysical and biological properties. It is noteworthy that the complex between the pentasaccharide and AT-III has a high association constant : $7.10^{6}M^{-1}$, of the same order of magnitude as

TABLE 1
BIOLOGICAL ACTIVITY DETERMINATIONS

ASSAY	Pentasaccharide	Tetrasaccharide
Anti-Xa units/mg (clotting)	2,800	0
Anti-Xa units/mg (chromogenic)	4,000	0
Anti-Xa units/mg (chromogenic)	0	0

Anti-Xa activity was measured by clotting assay according to Yin et al. and by amidolytic assay according to Teien and Lie using 0.02 M Tris-maleate buffer pH 7.5 instead of Tris-HCl, for plasma dilution. Antithrombin activity was measured by amidolytic assay according to Larsen et al.

already reported for high-affinity heparin (20). In agreement with known characteristics of AT-III, gel filtration and fluorescence data indicate that this complex is equimolecular. Once formed, it can be isolated and handled as a new entity.

In order to assess the supposed influence of the pentasaccharide and of the tetrasaccharide on the function of AT-III in plasma, we have analyzed their potency in various coagulation tests.

The pentasaccharide displays a very high inhibiting activity with respect to factor Xa in both the clotting and the amidolytic assays. As expected, this confirms the high correlation between AT-III binding, with its subsequent activation, and factor Xa inhibition. As also expected, the pentasaccharide is definitively unable to activate AT-III in thrombin inhibition process. As a matter of fact, other segments of the heparin molecules are also required for this type of inhibition (24). That the two types of activity are totally unrelated is observed for the first time with a pentasacchairde *per se*.

The fact that the tetrasaccharide with its four residues identical with those of the pentasaccharide has no affinity for AT-III, and no anti-Xa activity highlights the specificity of the pentasaccharide/AT-III interaction. This confirms that the residue at the non-reducing end of the pentasaccharide, or at least its 6-O-sulfate group (11) is of major importance for this interaction.

One must however bear in mind that our results do not conclusively prove that all the structural features of the pentasaccharide are essential for binding to AT-III. Permissible permutations of this structure will be investigated by taking advantage of the flexibility of the synthetic approach for preparing other oligosaccharides with systematically different sequences and substitution patterns.

ACKNOWLEDGMENTS

The authors greatly acknowledge Ms B. CHUNG and Mr. J. ESPEJO for their expert technical assistance.

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