Conformation of heparin pentasaccharide bound to antithrombin III

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The interaction, in aqueous solution, of the synthetic pentasaccharide AGA*IA_M (GlcN,6-SO₃α1-4GlcAβ1-4GlcN,3,6-SO₃α1-4IdoA,2-SO₃α1-4GlcN,6-SO₃αOMe; where GlcN,6-SO₃ is 2-deoxy-2-sulphamino-α-D-glucopyranosyl 6-sulphate, IdoA is L-iduronic acid and IdoA2-SO₃ is L-iduronic acid 2-sulphate), which exactly reproduces the structure of the specific binding sequence of heparin and heparan sulphate for antithrombin III, has been studied by NMR. In the presence of antithrombin there were marked changes in the chemical shifts and nuclear Overhauser effects (NOEs), compared with the free state. On the basis of the optimized geometry of the pentasaccharide the transferred NOEs were interpreted with full relaxation and conformational

exchange matrix analysis. An analysis of the three-dimensional structures of the pentasaccharide in the free state, and in the complex, revealed the binding to be accompanied by dihedral angle variation at the A–G and I–A_M (where G, I, A and A_M are β-D-glucuronic acid, 2-O-sulphated α-L-iduronic acid, N,6-O-sulphated α-D-glucosamine and the α-methyl-glycoside of A respectively) glycosidic linkages. Evidence is also provided that the protein drives the conformation of the 2-O-sulphated iduronic acid residue towards the skewed ²S₀ form.

Key words: NMR spectroscopy, protein–carbohydrate interaction, solution structure.

INTRODUCTION

Heparin, a complex glycosaminoglycan, interacts with a number of proteins involved in a variety of biological processes, and one particular interaction, that with the plasma protein antithrombin III (AT), prevents blood coagulation [1,2]. Due to the biological importance of this interaction, considerable effort has been made to understand the process at the molecular level. The structure of heparin is largely made up by relatively long sequences (IA)_n, where I and A are 2-O-sulphated α-L-iduronic acid and N,6-O-sulphated α-D-glucosamine respectively. However, most of the anti-coagulant activity of heparin is associated with the unique pentasaccharide sequence AGA*IA, where G is β-D-glucuronic acid and A* a 3-O-sulphated (usually also 6-O-sulphated) N-sulphated glucosamine [2,3]. This sequence, which is contained in only about one-third of the polysaccharide chains constituting mammalian heparins, has been synthesized as pentasaccharide AGA*IA_M (where A_M is the α-methyl-glycoside of A) [4]. Extensive structure–activity studies of this core pentasaccharide, as well as of a number of its variants, have led to a deeper insight into the structural features required for high affinity for AT and for the associated anti-coagulant properties [5].

For a detailed understanding of the interaction of glycosaminoglycans with AT at the molecular level it is necessary to determine both the ring conformation of the monosaccharide units and the torsion angles at the glycosidic linkages of the saccharide structure in the complex. Furthermore, additional information on the mechanism of intermolecular interaction can be obtained by comparing the three-dimensional (3D) structure of the ligand molecule in the free state and in the complex. Both the conformation [6] and the dynamics [7] of the heparin pentasaccharide AGA*IA_M were determined by computational and NMR methods in aqueous solution, and to interpret the experimental data it was necessary to consider a rather complex conformer equilibrium. Particularly interesting was the analysis of the conformation of the L-iduronic acid (IdoA) residue, which

has long been a matter of controversy [8,9]. The exclusive presence of either of the two chair forms of the pyranose ring, ¹C₄ and ⁴C₁, did not allow interpretation of the kinetics of dermatan sulphate periodate oxidation [8]. Extensive NMR and molecular-mechanics studies have shown that the inter-proton coupling constants for IdoA and L-iduronic acid 2-sulphate (IdoA2-SO₃) in heparin-related oligosaccharides can be interpreted in terms of a dynamic equilibrium between the chair ¹C₄ and the skew-boat ²S₀ forms, the population of individual conformers depending on the structure of neighbouring units and sulphation of the IdoA residue [9–12]. For example, in heparin the ratio of the ¹C₄ and ²S₀ conformers of the 2-O-sulphated IdoA residue is about 60:40, and in pentasaccharide AGA*IA_M this ratio is more or less reversed, i.e. ≈ 40:60 in aqueous solution [12].

The possibility of different equi-energetic conformations of IdoA2-SO₃ led to speculation on the possible role(s) of the conformational flexibility of this residue during interaction with proteins. Such an extra degree of local freedom at the level of the component residues of oligo- and polysaccharide chains is not common in saccharide structures, where flexibility is usually associated only with rotational freedom at the glycosidic linkages, and can contribute to a more favourable search for, and best fit to, the binding sites on a protein receptor [13]. The crystal structure of the complex of a heparin hexasaccharide with basic fibroblast growth factor, where one of the two IdoA2-SO₃ residues of the hexasaccharide is in the ¹C₄ conformation and the other in the ²S₀ [14], provided conclusive support to this concept.

If the 3D structure of the heparin pentasaccharide AGA*IA_M (Figure 1A) was known in the *bound form*, the proper approaches could be undertaken to synthesize tailor-made ligands with higher affinity than natural heparin or heparin-derived oligosaccharides. In principle, the actual conformation of the molecule in the bound state could be obtained from NMR studies in solution and from analysis of X-ray diffraction patterns in the solid state. NMR spectroscopy relies mainly on the analysis of coupling constants and/or nuclear Overhauser effects (NOEs),

Abbreviations used: 3D, three-dimensional; AT, antithrombin III; IdoA, L-iduronic acid; IdoA2-SO₃, L-iduronic acid 2-sulphate; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; I, 2-O-sulphated α-L-iduronic acid; A, N,6-O-sulphated α-D-glucosamine; G, β-D-glucuronic acid; A*, N,3,6-O-sulphated α-D-glucosamine; A_M, α-methyl-glycoside of A.

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Figure 1 Structure of heparin pentasaccharide

(A) Synthetic pentasaccharide AGA*IA_M corresponding to the binding site of heparin for AT. ϕ and φ are the dihedral angles at the glycosidic linkages, which are defined as $\phi = (\text{H-1})-(\text{C-1})-(\text{O-1})-(\text{C-4})$ and $\varphi = (\text{H-4})-(\text{C-4})-(\text{O-1})-(\text{C-1})$. The twisted-boat 2S_0 form is more abundant (about 60%) in AGA*IA_M than the chair form. In polymer heparin, the ratio of both forms is approximately reversed, with the 1C_4 chair form prevailing at about 60%. (B) Conformational forms of the IdoA2-SO₃ residue present in glycosaminoglycans and distances between hydrogens 15 and 12.

as performed for a number of heparin oligosaccharides in aqueous solution [6,10,15]. In the presence of interacting proteins, experiments for obtaining these NMR parameters are made difficult by the molecular rigidity associated with protein binding. In the first approach of NMR-binding studies with heparin oligosaccharides, we started with the synthetic tetrasaccharide AGAI_M (where the terminal I was non-sulphated and M stands for methyl glycoside). Analysis of chemical shifts, scalar coupling constants and transferred NOEs indicated that considerable changes in the torsion angles between the A and G residues occur in this tetrasaccharide during complexation with AT, and that the conformation of the non-sulphated IdoA residue is driven towards the 1C_4 form [16]. A recent study of co-crystals of AT and a synthetic pentasaccharide, differing from AGA*IA_M by additional sulphate and methyl groups, provided evidence of conformational changes in the heparin-binding site, and showed that the skew-boat form of the 2-O-sulphated IdoA residue was adopted in the bound state [17]. In the present work, for the very first time by NMR spectroscopy, the 3D structure of the active heparin pentasaccharide in the presence of AT in solution was studied. The analysis was based on two-dimensional NOE spectroscopy (NOESY) of the free AGA*IA_M (in buffer) and transferred NOESY spectra of the complex of AGA*IA_M with AT. The NMR-derived structure of this core pentasaccharide in the bound state is discussed and compared with that in the free state and the AGA*IA_M analogue studied in the solid state.

EXPERIMENTAL

NMR experiments

For the study of the free ligand, 1.5 mg of AGA*IA_M was dissolved in 0.5 ml of 10 mM phosphate buffer/0.5 M NaCl,

pH 7.4 (in 99.99% $^2\text{H}_2\text{O}$). AT (3.06 mg) and 1.2 mg of AGA*IA_M (1:15, AT/AGA*IA_M) were dissolved in the same buffer for the binding studies. Relatively high concentration of NaCl was used to increase the K_d value to achieve fast exchange conditions between pentasaccharide and AT for transferred NOESY experiments. The ^1H -NMR spectra were recorded at 500 MHz on a Bruker AMX 500 spectrometer, equipped with a 5 mm inverse broad-band probe with a shielded z-gradient at 10 °C and 30 °C respectively. Two-dimensional transferred NOESY spectra were performed in a similar way to two-dimensional NOESY in the case of free ligand using standard pulse sequence at both 10 °C and 30 °C. The lower temperature was used to avoid the resonance overlap of the H²HO signal with 15 and G1 to also measure NOEs for these protons. The spectral width was 4000 Hz, and a short (20 ms) spin-lock pulse was performed after the $\pi/2$ excitation pulse. A total of 32 transients were collected for each free-induction decay and data (matrix 1024 × 512 points) were zero-filled to 2048 × 2048 points before Fourier transformation.

Computational procedures

The structure of the pentasaccharide was obtained by Monte Carlo conformational search using the MacroModel program version 5.0 [18]. This geometry was further optimized using molecular mechanics MM2 force field in the binding site, considering 35 amino acids including those with possible dipole interactions with the protons in AGA*IA_M in the bound state (Arg-13, Arg-46, Arg-47, Arg-129, Lys-11, Lys-114, Lys-125 and Asn-45) [17,19]. The geometry of the receptor site was kept fixed. The ϕ and φ dihedral angles in AGA*IA_M [defined as $\phi = (\text{H-1})-(\text{C-1})-(\text{O-1})-(\text{C-4})$ and $\varphi = (\text{H-4})-(\text{C-4})-(\text{O-1})-(\text{C-1})$]

were allowed to vary, as were the inter-atomic distances between the sulphates, carboxylates and amino acids. Theoretical NOEs were computed by the complete relaxation and conformational exchange matrix (CORCEMA) program [20] using the optimized geometry of AGA*IA_M and considering the presence of eight amino acids in the binding site, namely Arg-13, Arg-46, Arg-47, Arg-129, Lys-11, Lys-114, Lys-125 and Asn-45. The values of the dissociation constant (K_d) and the off-rate (k_{off}) used in the calculations were based on published data [21]. The correlation time of AT ($\tau_{antithrombin}$) was considered as isotropic and estimated from the previous data [16]. However, the values of K_d , k_{off} and the AT correlation time varied during the optimization. The R factors were computed according to the following formula:

$$R = \sum [I_{ij}^{exp}(\tau_{mix}) - I_{ij}^{calc}(\tau_{mix})]^2 / \sum [I_{ij}^{exp}(\tau_{mix})]^2$$

where I_{ij}^{exp} are the experimental cross-peak intensities, I_{ij}^{calc} are the calculated cross-peak intensities and τ_{mix} are the mixing times. Other details on experimental and computational procedures have already been described [16].

RESULTS AND DISCUSSION

The ¹H-NMR spectra of pentasaccharide AGA*IA_M in buffer and in the presence of AT at 10 °C are shown in Figure 2. The influence of the protein on the structure of the ligand molecule can be monitored qualitatively from the changes in chemical shift and linewidth. Both upfield and downfield protein-induced

shifts are seen in the spectra. The anomeric G1 proton resonance shifted upfield (−0.003 p.p.m.), whereas A1* and I1 shifted downfield (0.008 p.p.m. and 0.005 p.p.m., respectively). The chemical shift of the remaining two anomeric signals (A1 and A_M1) remained unchanged (Figures 2C and 2D). Variations in other proton shieldings were also monitored, e.g. I5 and A2* (0.006 p.p.m. and −0.004 p.p.m., respectively), although the differences in the ring protons were less detectable owing to the complexity of the ¹H-NMR spectra in the presence of AT. Furthermore, the linewidths increased as a consequence of the presence of AT. As the NMR spectra were collected in buffer with the addition of 0.5 M NaCl ('free state') the 1.5–2.0 Hz increase in linewidth for measurable signals reflected the influence of AT on these NMR parameters.

Parts of two-dimensional NOESY (free state) and transferred NOESY (complex) of AGA*IA_M spectra are shown in Figure 3. Since, in both cases, the cross-relaxation rates differ considerably there is great variation in the cross-peak intensities of the spectra. The numerical NOE values, collected at various mixing times, are listed in Table 1 (at 30 °C) and in Table 2 (at 10 °C). The structure of the pentasaccharide AGA*IA_M molecule was obtained from a Monte Carlo conformational search. The geometry of the lowest four energy minima was further optimized within the AT binding site and the transferred NOEs were subsequently computed with the program CORCEMA, evaluating full relaxation and exchange matrix. Table 3 shows the structures that had the glycosidic linkage conformations that were the best fits to the experimental data. The conformations of the monosaccharide residues were in the ⁴C₁ forms, except for the IdoA residue which was in the ²S₀ conformation. Calculated transferred NOEs for protons cross-relaxing within the residues (e.g. A1–A2, A1*–A2*, I1–I3) agreed well with the experimental data, where the computed R factors ranged from 0.2 to 0.26 (Tables 1 and 2). The only larger R factor was obtained for NOEs between A_M1 and A_M2 protons at the non-reducing end glucosamine residue. The latter discrepancy between theory and experiment was caused by the derived inter-atomic A_M1–A_M2 distance. The computed distance (2.4 Å; Table 1) is shorter than in other residues (typically 2.45–2.55 Å) and resulted in considerably higher theoretical NOEs.

The conformation of the IdoA2-SO₃ residue has been a subject of interest due to the variation in its 3D structure in different heparin-derived oligosaccharides [6,10,12,22–24]. An earlier NMR analysis of a heparin tetrasaccharide in solution showed that the more abundant conformer (IdoA was non-sulphated in this compound) in solution (¹C₄, 75%) is also present in the complex with AT [16]. A similar observation was made recently when the ²S₀ form was found in the pentasaccharide analogue in the crystal state [17]. On the other hand, the crystal structure of a heparin hexasaccharide complexed to the basic fibroblast growth factor has shown that the IdoA2-SO₃ residue adopts multiple conformations in the presence of protein: one residue was in the ¹C₄ form, the other in the skew ²S₀ conformation [14]. In the present study heparin pentasaccharide, both the ¹C₄ and ²S₀ conformers in the IdoA2-SO₃ residue are nearly equi-energetic (the skew form being slightly more abundant, about 60%) in solution. The structure of this residue in the bound form was obtained from the inter-proton distances within the unit. In the ¹C₄ conformation, protons I1, I2, I3 and I4 are equatorial and I5 is axial. In the twisted-boat ²S₀ conformation, I1 and I4 have an isoclinal position that results in considerably different inter-proton distances within the residue (Figure 1B). The most significant variation is between I5 and I2, where the distance is shorter in the ²S₀ form (0.25 nm) than in the chair form (0.4 nm). Similarly, the values of the three-bond proton–proton coupling

Figure 2 NMR spectra in the free state and in complex

¹H-NMR spectra (500 MHz) of pentasaccharide AGA*IA_M in 10 mM phosphate buffer (with 0.5 M NaCl, pH 7.4) at 10 °C (A) and in complex with AT (AGA*IA_M/AT, 15:1) (B). Anomeric resonances in the free state (C) and in the presence of AT (D) are shown.

Figure 3 Two-dimensional NOESY spectra

Parts of the two-dimensional NOESY spectrum of AGA*IA_M in buffer (10 mM phosphate buffer/0.5 M NaCl, pH 7.4; **A**) and the transferred NOESY spectrum of the AGA*IA_M-AT complex (AGA*IA_M/AT, 15:1) at 10 °C recorded with a 200 ms mixing time. (**B**).

constants ($^3J_{\text{H-H}}$) also change due to the different values of the dihedral angles among protons. This evidence was confirmed by $^3J_{\text{H-H}}$ where the values of $^3J_{\text{I1-I2}}$ and $^3J_{\text{I4-I5}}$ were 3.6 Hz and 3.1 Hz in the free state, respectively. Both these magnitudes agree well with a relatively high (about 60 %) population of the 2S_0 form [12]. Since the values of $^3J_{\text{H-H}}$ could not be measured in the presence of AT owing to the larger linewidths, the analysis was based on NOE data measured at 10 °C. The magnitudes of experimental I5-I2 NOEs (Table 2) were comparable with NOEs between protons I5 and I4, indicating comparable distances between the I5-I4 and I5-I2 protons. As mentioned, such a geometrical arrangement occurs in the twisted-boat form of the IdoA2-SO₃ residue. Computed transferred NOEs, using the geometry of the twisted-boat form with $r_{\text{I5-I2}} = 2.55$ Å and $r_{\text{I5-I4}} = 2.37$ Å, gave theoretical values in a good agreement with those found experimentally (Table 2). Transferred NOEs, com-

puted with the 1C_4 chair form of the IdoA2-SO₃ residue for comparison purposes, gave considerably smaller I5-I2 NOE values (4.6 % at 200 ms mixing time), the consequence of a large inter-atomic distance (3.99 Å) between both protons. The present data thus show that the IdoA2-SO₃ residue adopts the 2S_0 conformation in the heparin pentasaccharide-AT complex.

The conformation at the glycosidic linkages was determined from the NOEs of protons cross-relaxing across the glycosidic bonds. Most of the computed transferred NOEs were found to correspond well with the experimental data (Tables 1 and 2). The computed ϕ and φ values found for the AGA*IA_M structure in the complex indicate that the geometry for two linkages changed during the binding process, compared with the free state (Table 3). Furthermore we noted considerable differences in the ϕ_1 and φ_1 dihedral angles for the A-G linkage in the complex ($\phi_1 = -56^\circ$ and $\varphi_1 = -60^\circ$), compared with the solution structure

Table 1 Experimental and computed transferred NOEs at 30 °C

Selected NOEs in the complex with AT for four mixing times. The computed values were obtained using the geometry with ϕ_1 and ϕ_1 of -56° and -60° , ϕ_2 and ϕ_2 of 40° and 12° , ϕ_3 and ϕ_3 of -32° and -43° , and ϕ_4 and ϕ_4 of 45° and 16° , and assuming the twisted-boat 2S_0 conformation of the IdoA residue. The presence of eight amino acids in the binding site (Arg-13, -46, -47 and -129, Lys-11, -114 and -125, and Asn 45) was evaluated in full relaxation and exchange matrix and $K_d = 4 \times 10^{-4}$ M, $k_{\text{off}} = 15 \text{ s}^{-1}$, $\tau_{\text{ligand}} = 0.45 \text{ ns}$ and $\tau_{\text{antithrombin}} = 46 \text{ ns}$. The computed inter-proton distances (r , distances in Å) are shown. The R factor is also given.

		Experimental				Calculated					
		50 ms	100 ms	200 ms	300 ms	50 ms	100 ms	200 ms	300 ms	r (Å)	R
A1	A2	-4.4	-9.4	-18.2	-28.8	-7.9	-12.5	-21.3	-30.4	2.48	0.26
	G3									2.69	
	G4	-5.3	-10.1	-20.2	-33.4	-7.1	-11.5	-20.4	-30.0	3.12	
A1*	A2*	-4.6	-9.8	-19.6	-28.5	-6.2	-9.2	-14.7	-20.1	2.51	0.23
	I3									2.91	
	I4	-6.4	-13.8	-31.6	-48.4	-8.4	-12.7	-21.2	-29.9	2.53	
I1	I3	-2.3	-4.8	-9.0	-18.2	-2.0	-3.6	-7.3	-11.8	2.93	0.2
	A _M 4	-4.6	-8.6	-21.2	-30.2	-3.8	-5.4	-8.8	-12.1	2.47	
	A _M 5	-	-1.6	-6.5	-10.3	-	-2.8	-5.0	-7.4	3.74	
A _M 1	A _M 2	-3.8	-7.5	-12.9	-19.1	-7.1	-12.2	-19.8	-27.3	2.4	0.58

Table 2 Experimental and computed transferred NOEs at 10 °C

Selected NOEs in heparin pentasaccharide in complex with AT for three mixing times. Details were as for Table 1, except that $K_d = 2 \times 10^{-4}$ M, $k_{\text{off}} = 7 \text{ s}^{-1}$, $\tau_{\text{ligand}} = 0.55 \text{ ns}$ and $\tau_{\text{antithrombin}} = 56 \text{ ns}$.

		Experimental			Calculated			r (Å)	R
		100 ms	200 ms	300 ms	100 ms	200 ms	300 ms		
I5	I2	-7.5	-16.1	-24.3	-10.3	-16.0	-21.0	2.55	0.19
	I4	-10.7	-19.8	-29.5	-12.3	-17.1	-21.8	2.37	
G1	A4*	-8.2	-18.7	-25.0	-8.7	-12.1	-15.2	2.26	0.37
	A6*	-4.5	-14.5	-19.4	-7.6	-10.5	-13.8	2.49	

($\phi_1 = -34^\circ$ and $\phi_1 = -27^\circ$). Transferred NOEs based on the solution structure ($\phi_1 = -34^\circ$ and $\phi_1 = -27^\circ$) could not interpret the experiments since the inter-atomic A1-G4 distance is shorter ($r_{\text{A1-G4}} = 2.41 \text{ Å}$) than that ($r_{\text{A1-G4}} = 3.13 \text{ Å}$) with ϕ_1 and ϕ_1 (-56° and -60°). Similarly, differences were also found (Table 3) between the present NMR-derived AGA*IA_M structure and that of its analogue co-crystallized with AT [17]. The geometry of the latter analogue is not discussed explicitly and was retrieved from published crystal co-ordinates [17]. The main differences were found between the ϕ_1 and ϕ_1 dihedral angles (a change of about 25° for the A-G linkage for both ϕ_1 and ϕ_1) and the ϕ_3 and ϕ_3

dihedral angles (a change of about 40° for ϕ_3 and 10° for ϕ_3) for the A*-I linkage. The computed NOE data, using the modified analogue crystal data geometry at the glycosidic linkages, again gave the stronger NOEs due to the shorter inter-atomic distance between A1 and G4 ($r_{\text{A1-G4}} = 2.42 \text{ Å}$) and, at the same time, smaller A1*(I3+I4) NOEs due to the longer distances between A1* and both I3 and I4 ($r_{\text{A1*-I4}} = 2.88 \text{ Å}$ and $r_{\text{A1*-I3}} = 2.96 \text{ Å}$) than those in AGA*IA_M ($r_{\text{A1*-I4}} = 2.53 \text{ Å}$ and $r_{\text{A1*-I3}} = 2.91 \text{ Å}$). Thus the changes observed in the glycosidic linkage conformation between the A-G and A*-I linkages express the differences in the structure of AGA*IA_M and its analogue in the bound state.

The higher R factor was obtained for the inter-residue I1-A_M4 NOE. The conformation with $\phi_4 = 45^\circ$ and $\phi_4 = 16^\circ$ was determined, in the present analysis, to be the energetically most favourable. This geometry is comparable with the conformation found in the molecular modelling study ($\phi_4 = 43^\circ$ and $\phi_4 = 6^\circ$) [6] and with the crystal data of the synthetic analogue ($\phi_4 = 44^\circ$ and $\phi_4 = 16^\circ$) [17]. Obviously, none of the geometry could interpret the experimental data satisfactorily, due to the similarity in the geometry. The observed discrepancy therefore suggests that ϕ_4 and ϕ_4 , and, consequently, the I1-A_M4 distance, are slightly different in the complex, compared with AGA*IA_M in free solution as well as in the solid-state structure of the AGA*IA_M analogue. Similar ϕ_2 , ϕ_2 and ϕ_3 , ϕ_3 dihedral angle values, corresponding to the free and bound states AGA*IA_M structure, suggest that the conformation at the glycosidic linkages between the G-A* and A*-I residues is comparable in the free

Table 3 Geometry at the glycosidic linkages

The ϕ and ϕ dihedral angles at the glycosidic linkages in the pentasaccharide AGA*IA_M exactly reproducing the structure of the specific binding sequence of heparin and heparan sulphate for AT in the AT-AGA*IA_M complex are shown, as derived from NMR solution data and molecular modelling. The ϕ and ϕ values are also listed for AGA*IA_M in the free state [6] for comparison. The ϕ and ϕ dihedral angles retrieved from the crystal structure for the AGA*IA_M analogue (bearing extra sulphate and methyl groups) [17] are also shown.

	ϕ_1, ϕ_1	ϕ_2, ϕ_2	ϕ_3, ϕ_3	ϕ_4, ϕ_4
AT-AGA*IA _M complex	-56, -60	40, 12	-32, -43	45, 16
AGA*IA _M free state	-34, -27	47, 6	-35, -42	43, 6
Pentasaccharide analogue (crystal structure)*				
Latent-antithrombin	-31, -35	41, 1	-71, -33	44, 16
Inhibitory-antithrombin	-31, -22	35, 8	-66, -35	48, 5

* Corresponds to two different forms complexed to latent-antithrombin and inhibitory-antithrombin.

Figure 4 Conformation of the heparin pentasaccharide in complex with AT

Structure of AGA*IA_M in the AT binding site. The corresponding dihedral angles at the glycosidic linkages are shown in Table 3. The IdoA2-SO₃ residue is in the twisted-boat ²S₀ conformation, the remaining residues are in the ⁴C₁ chair conformation. AT amino acids: Arg, red; Asn, orange; Lys, pink; Asp, blue; Ile, grey; Cys, yellow; Ala, white; Pro, green.

state and in the presence of AT. As the computed NOEs vary with glycosidic linkage geometry, our data suggest that the variation in conformation occurs at the A–G and I–A_M linkages in AGA*IA_M during its binding to AT. The transferred NOE data further indicate that the addition of other functional groups into the core pentasaccharide structure may also result in different stereochemistry at the glycosidic linkages in the modified analogue–AT complex with respect to the AGA*IA_M–AT complex.

An analysis of the pentasaccharide structure in the complex with AT suggests, on the basis of NMR experimental data interpreted with molecular modelling, that the AGA*IA_M structure in the complex differs from that in free solution. The NMR-derived 3D structure agrees with not only the transferred NOEs but is also in agreement with the observed non-bonded interactions between positively charged AT amino acids in the binding site and the negatively charged sulphates and carboxylates in the pentasaccharide [25–27]. For example, the computed distance

between the nitrogen atom in the NH₂ group in Arg-129 and the sulphur atom in the 6-SO₃[−] group in A is 4.3 Å, corresponding well with the expected strong interaction [25] between these two groups. Similarly, spatial closeness was observed for the NH₂ group of Lys-125 and both the 6-SO₃[−] group in A and the carboxyl group in G. The computed distances were 3.5 Å (the distance between the NH₂ group in Lys-125 and oxygen in the C=O group in G) and 3.8 Å (the distance between the NH₂ group and sulphur in the 6-SO₃[−] group in A; the distance in the solid-state of the modified analogue is 4.4 Å). Strong non-bonded interaction can also be expected between the N-SO₃[−] group in the A* residue and Arg-13 as well as Lys-114 due to short inter-atomic distances between the sulphate and both amino acids.

The lack of any dipolar interaction between the 2-O-sulphate group and the amino acid in the receptor, as observed in the present modelling and NMR data, and the crystal state [17],

indicates that no basic groups in the binding site are within reach of the 2-O-sulphate group of IdoA2-SO₃ (Figure 4). Thus this group is not directly involved in the binding process. The 2-O-sulphate group therefore appears to be the main driving force affecting the conformational equilibrium in IdoA2-SO₃ towards the skew form, enhancing the electrostatic interaction between the carboxylate group and the binding-site amino acids, Arg-47 and Lys-114. The computed distance between the carboxylate oxygen and the basic NH in Arg-47 is 4.5 Å, the distance between the carboxylate oxygen and the NH₂ group in Lys-114 is 3.0 Å. A similar situation appears to be the case for the structure of the A_M residue and the amino acids in the binding site. There is strong interaction between the N-sulphate group of this residue with Arg-46, Arg-47 and Lys-114. The computed distance between the NH group in Arg-46 and sulphur in the N-SO₃⁻ group is 4.2 Å, the same distance as between the NH group in Arg-47 and the N-SO₃⁻ group (the corresponding distances in the crystal structure of the synthetic analogue are 3.9 Å and 4.7 Å, respectively). It thus appears that the influence of the IdoA residue conformation and the I-A_M glycosidic linkage geometry plays a crucial role in achieving the complementary 3D structure of the reducing end of the heparin pentasaccharide (Figure 4). This 3D structure is not obtained when IdoA adopts the ¹C₄ chair form in the complex, where both the axial orientation of the C-O linkage (anomeric carbon in IdoA2-SO₃) and the different ϕ₄ and φ₄ dihedral angles cause an unfavourable stereochemical arrangement. Consequently, the kink observed in the crystal data ([17], and M. Hricovini, G. Venkataraman, M. Guerrini, A. Bisio, G. Torri, M. Petitou, R. Raman, S. Ernst, V. Sasisekharan, B. Casu and R. Sasisekharan, unpublished work) is therefore the result of the stereochemical arrangement of AGA*IA_M where IdoA2-SO₃ adopts the ²S₀ form in the complex.

Further evidence of the role played by the 2-O-sulphate group and ring conformation in the binding properties with AT has been found in the interaction of structurally modified heparin-like pentasaccharides. The very high biological activity of a heparin pentasaccharide, bearing an extra sulphate group at the C-3 position of the glucosamine residue, at the reducing end [28] was speculated to be the consequence of a high population of the skew ²S₀ conformer (about 90%) of the IdoA2-SO₃ residue in aqueous solution. On the contrary, the conformationally restricted derivative with the ¹C₄ form showed very low biological activity, leading to the conclusion that this chair form is most likely not active [29,30]. Furthermore, a pentasaccharide lacking the 2-O-sulphate group in the IdoA2-SO₃ residue showed only about 50% of the biological activity of the pentasaccharide used in this study. The conformational equilibrium of the IdoA2-SO₃ residue is considerably shifted towards the chair form (75%) for the non-sulphated derivative (M. Hricovini, G. Venkataraman, M. Guerrini, A. Bisio, G. Torri, M. Petitou, R. Raman, S. Ernst, V. Sasisekharan, B. Casu and R. Sasisekharan, unpublished work). As already mentioned, the 2-O-sulphate group in the IdoA2-SO₃ residue has no direct electrostatic interaction with any residue in the binding site. Thus its main role towards the achievement of the appropriate stereochemical arrangement is to affect the conformational equilibrium of the IdoA2-SO₃ residue.

Conclusions

The first NMR experimental data on the heparin pentasaccharide-AT complex in solution confirm that both the conformational equilibrium in the IdoA2-SO₃ residue and the conformation of the glycosidic linkages play important roles in inducing biological activity in heparin oligosaccharides.

The presence of the 2-O-sulphate group and the structure of the neighbouring residues (mainly the presence of the 3-O-sulphate group in the A* residue and the 6-O-sulphate group in the A_M residue) seem to be the major driving forces that result in different conformational equilibria in the IdoA2-SO₃ residue. This very fine equilibrium is perturbed by the presence of AT, leading to the stabilization of one of the conformers in the IdoA2-SO₃ residue present in solution and changes at the glycosidic linkages. Consequently, in various heparin oligosaccharides, a different orientation of the charged groups in different conformers, also associated with the conformation of the glycosidic linkage, leads to different affinities to AT. In the case of the heparin pentasaccharide-AT complex, the IdoA2-SO₃ residue adopts the twisted-boat ²S₀ conformation. The conformation at the glycosidic linkages differs from that in aqueous solution during the binding process. This 3D structure of AGA*IA_M appears to best fit the binding site of AT to enhance weak electrostatic interaction between the negatively charged sulphates and carboxylates and the AT amino acids involved in binding, mainly Arg-46, Arg-47, Arg-129, Lys-11, Lys-114 and Lys-125. This knowledge may help in the synthesis of ligands [31,32] with an appropriate structure to ensure high affinity to AT.

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