Antithrombin-binding Octasaccharides and Role of Extensions of the Active Pentasaccharide Sequence in the Specificity and Strength of Interaction

EVIDENCE FOR VERY HIGH AFFINITY INDUCED BY AN UNUSUAL GLUCURONIC ACID RESIDUE

The antithrombotic activity of low molecular weight heparins (LMWHs) is largely associated with the antithrombin (AT)-binding pentasaccharide sequence AGA*IA (GlcNNAc/NS,6S-GlcA-GlcNNS,3,6S-IdoUA2S-GlcNNS,6S). The location of the AGA*IA sequences along the LMWH chains is also expected to influence binding to AT. This study was aimed at investigating the role of the structure and molecular conformation of different disaccharide extensions on both sides of the AGA*IA sequence in modulating the affinity for AT. Four high purity octasaccharides isolated by size exclusion chromatography, high pressure liquid chromatography, and AT-affinity chromatography from the LMWH enoxaparin were selected for the study. All the four octasaccharides terminate at their nonreducing end with 4,5-unsaturated uronic acid residues (ΔU). In two octasaccharides, AGA*IA was elongated at the reducing end by units IdoUA2S-GlcNNS,6S (OCTA-1) or IdoUA-GlcNNAc,6S (OCTA-2). In the other two octasaccharides (OCTA-3 and OCTA-4), AGA*IA was elongated at the nonreducing side by units GlcNNS,6S-IdoUA and GlcNNS,6S-GlcA, respectively. Extensions increased the affinity for AT of octasaccharides with respect to pentasaccharide AGA*IA, as also confirmed by fluorescence titration. Two-dimensional NMR and docking studies clearly indicated that, although elongation of the AGA*IA sequence does not substantially modify the bond conformation of the AGA*IA segment, extensions promote additional contacts with the protein. It should be noted that, as not previously reported, the unusual GlcA residue that precedes the AGA*IA sequence in OCTA-4 induced an unexpected 1 order of magnitude increase in the affinity to AT with respect to its IdoUA-containing homolog OCTA-3. Such a residue was found to orientate its two hydroxyl groups at close distance to residues of the protein. Besides the well established ionic interactions, nonionic interactions may thus contribute to strengthen oligosaccharide-AT complexes.

Heparins and low molecular weight heparins (LMWHs) are the most common anticoagulant and antithrombotic drugs used in cardiovascular medicine (1). One of the primary mechanisms by which heparin species express their anticoagulant action is through binding antithrombin (AT). The specific heparin sequence involved in this binding corresponds to the pentasaccharide GlcNNAc/NS,6S-GlcA-GlcNNS,3,6S-IdoUA2S-GlcNNS,6S (AGA*IA) that, inducing a conformational change in the protein, activates AT and accelerates ~300–600-fold the inhibition of factor Xa (2, 3). Additional glucosamine and uronic acid residues, mainly constituted by GlcNNS,6S-IdoUA2S disaccharide repeating units, are thought not to significantly interact with AT and are not strictly required to achieve AT-mediated inhibition of factor Xa (4, 5). In fact, the synthetic pentasaccharide fondaparinux, consisting of the methyl glycoside of the AGA*IA variant where the first GlcN residue is N-sulfated, is in itself an effective antithrombotic drug (3, 6). LMWHs, successfully used as antithrombotic agents, are composed of numerous heparin fragments, a consistent proportion of which (about 20% of total) contains the AT-binding sequence AGA*IA, and endowed with high affinity for AT (7). Advances in analytical and separation methods have permitted the isolation and sequencing of a number of these “active” components of LMWHs and to assess that the AGA*IA sequences...
are located at different sites along the oligosaccharide chains (7, 8). The increasing interest in the development of “tailored” LMWHs and very low molecular weight heparins stimulates studies aimed at a better understanding at the molecular level the mechanisms of interaction between AT and AGA*IA-containing oligosaccharides. Earlier studies on tetrasaccharides sequences adjacent to the antithrombin-binding site have demonstrated two possible variants of AT-binding sequences, suggesting a possible role of the extensions of these sequences on binding to AT (9). Longer AT-binding sequences, such as decasaccharides, were also previously isolated (8). The influence of the position of the pentasaccharide sequence along the oligosaccharide chains together with the knowledge of the role of the residues prolonging the active sequence toward both its reducing and nonreducing side are among the major goals of current heparin research (10). Although the active pentasaccharide AGA*IA is taken as paradigm for a unique heparin sequence targeting a specific protein (i.e. AT) (3), different mechanisms have been proposed for its interaction with AT in terms of position and conformation of sugar residues. The possibility of a shift along the AT-D-helix for sequences longer than pentasaccharide was taken into consideration (11). Independent crystallographic and NMR studies on the structure of complexes of AT with AGA*IA and AGA*IA-containing oligosaccharides suggested that the position of the pentasaccharide in the protein binding region is unique (10, 12–15). These studies provided information on both the ring conformation of the monosaccharide residues and the geometry of the glycosidic linkages of the AT-bound pentasaccharide. In particular, it was shown that the 2-O-sulfated iduronic acid residue in the pentasaccharide, which in the free state in water solution is in equilibrium between two equienergetic conformations ($^1C_4\beta$ and $^3S_0$) (16), adopts the $^3S_0$ conformation when AGA*IA is bound to AT. Shifting toward this conformation, facilitated by the presence of the 2-OSO$_3^-$ group, enhances the contacts between the AGA*IA and basic amino acid residues in the AT binding region (15).

In this study, four high purity octasaccharides isolated by size exclusion and AT-affinity chromatography from the LMWH enoxaparin were selected. Like all fragments generated by $\beta$-elimination cleavage of heparin chains (7, 17), all four octasaccharides terminate at the nonreducing end with 4,5-unsaturated uronic acid residues (ΔU). In two octasaccharides, AGA*IA was found to be elongated toward the reducing end by the disaccharide units IdoUA$_{2S}$-GlcNNS$_{6S}$ (OCTA-1) and IdoUA-GlcN$_{4A,6S}$ (OCTA-2). In the other two octasaccharides (OCTA-3 and OCTA-4), AGA*IA was found to be elongated toward the nonreducing end by GlcN$_{NS,6S}$-IdoUA and GlcN$_{NS,6S}$-GlcA units, respectively. Earlier NMR studies on the interaction of OCTA-1 and OCTA-3 with AT suggested a possible role of both the reducing and nonreducing end extensions in favoring binding to the protein, and supported a specific binding between the pentasaccharide and the AT-binding site (10). In this work the interaction of OCTA-1 and OCTA-3 with AT was analyzed in greater detail, and the study was extended to the two novel octasaccharides (OCTA-2 and OCTA-4) described above. The structures of the four octasaccharides are shown in Fig. 1. Affinity chromatography on immobilized AT showed the following relative binding strength: OCTA-3 $<\ $OCTA-1 $<\ $OCTA-2 $<\ $OCTA-4. The highest affinity of OCTA-4 was confirmed also by fluorescence titration experiments. Furthermore, when this measurement is performed in 0.5 M NaCl (i.e. at the same ion strength used for the NMR studies; see “Experimental Procedures”), OCTA-4 was shown to bind AT with 1 order of magnitude higher affinity than its homolog OCTA-3. Saturation transfer difference (STD) experiments confirmed the specificity of the AGA*IA sequence for the AT binding. The conformational and AT binding properties of these octasaccharides were also investigated by NMR (transferred-NOESY) spectroscopy and docking simulations. The structural properties of the four octasaccharides have been correlated with the affinity to AT determined by affinity chromatography on a preparation that contains about 95% of active protein as judged by active site titration (18) and interpreted in terms of both ionic and nonionic interactions.

**EXPERIMENTAL PROCEDURES**

*Materials.—* Enoxaparin and fondaparinux were supplied by Sanofi-Aventis (Vitry sur Seine, France). Antithrombin was purchased from Biogenic (Perols, France).

*General Procedure for Octasaccharide Isolation, Purification, and Sequencing.—* Octasaccharides 1–4 (Fig. 1) were obtained by combining AT affinity chromatography and cetyltrimethylammonium-strong anion-exchange (CTA-SAX) chromatography on a semi-preparative scale, starting from octasaccharide gel permeation chromatography (GPC) fractions of enoxaparin. GPC of enoxaparin and the desalting conditions of the selected fractions were performed as described previously (19). The octasaccharide fraction was chromatographed on an AT-Sepharose column (40 $\times$ 5 cm) with a stepwise gradient of NaCl. The column was prepared by coupling human AT (1 g) to CNBr-activated Sepharose 4B (Sigma) according to Höök et al. (20). The low affinity portion was eluted from the column with a 0.25 M NaCl solution buffered at pH 7.4 with 1 mM Tris-HCl at 6 ml/min. The high affinity octasaccharide fractions were eluted with a step gradient of NaCl (range between 0.25 and 3 M NaCl, 1 mM Tris-HCl, pH 7.4). The NaCl gradient was monitored by conductivity measurements, and the octasaccharides in the eluants were detected by UV at 232 nm. Octasaccharides eluted in affinity fractions with conductivities between 30 and 85 mS/cm were gathered, desalted on Sephadex G-10, and used as starting material for the purification of OCTA-3. Octasaccharides eluted between 85 and 115 mS/cm were used for the purification of OCTA-1. Octasaccharides eluted between 115 and 150 mS/cm were gathered and used to purify OCTA-2. Fractions eluted for conductivities over 145 mS/cm were used after desalting to purify OCTA-4. The final purifications of all the octasaccharides were achieved using CTA-SAX chromatography. CTA-SAX semi-preparative columns (25 $\times$ 5 cm or 25 $\times$ 2.2 cm) were prepared as described in Ref. 19 and filled with Hypersil BDS C18 (5 μm particle size). Mobile phases for oligosaccharide separation were aqueous sodium methanesulfonate (Interchim) at concentrations varying between 0 and 2.5 M. The pH was adjusted to 2.5 by addition of diluted methanesulfonic acid. Separations were achieved at 40°C. Salt concentration in the mobile phase was increased.
Heparin Octasaccharides-AT Interaction

linearly from 0 to 2.5 M over 60 min. Flow rate was 40 ml/min for 25 \times 3$-cm columns, and UV detection at 234 nm was used. Collected fractions were neutralized and desalted on Sephadex G-10 after a preliminary treatment on Mega Bondelut C18 cartridges (Varian). Sequencing of the octasaccharides was performed by a combination of controlled and exhaustive cleavage with heparitinases and HPLC analysis of fragments as reported previously (19).

Separation of the Octasaccharide High Affinity Fractions and Fractionation with ion-exchange Chromatography—An octasaccharide mixture, obtained by solubilizing a mixture of about 500 \mu g of each crude octasaccharide, was analyzed by CTA-SAX to obtain the chromatographic $T = 0$ prior to the affinity experiment (data not shown). The mixture of the four octasaccharides was injected on an AT-Sepharose column (40 \times 1.6 cm). The low affinity portion was eluted from the column with a 62.5 mM NaCl solution buffered at pH 7.4 with 1 mM Tris-HCl, and the high affinity octasaccharide fraction was eluted with a step gradient of NaCl (in a range between 0.21 and 2 M) over a period of 60 min. Flow rate was 40 ml/min for 25 \times 3$-cm columns, and UV detection at 234 nm was used. After exchanging the samples three times, samples were dissolved in 2H2O (99.9%) and freeze-dried to remove residual water. After exchanging the samples three times, samples were dissolved in 0.2 ml of 10 mM phosphate buffer (0.5 M NaCl, pH 7.4) with 3 mM EDTA in 2H2O (99.996%). For the binding studies, samples were prepared by dissolving 1 mg of AT and 150–250 \mu g of each octasaccharide sample in the phosphate buffer so as to reach a 1:3.5 AT/octasaccharide molar ratio (10). In OCTA-1 some spectra were needed to be repeated with a smaller AT/octasaccharide ratio (1:5), to reduce the signal overlapping affecting the quantitative analysis.

Proton spectra were recorded with presaturation of the residual water signal, with a recycle delay of 12 s and 256 scans. Bidimensional double-quantum filter correlation spectroscopy (DQF-COSY) and two-dimensional TOCSY spectra were acquired using 32 scans per series of 2 K \times 512 W data points with zero filling in F1 (4 K \times 2 K), and a shifted ($\pi/3$) squared cosine function was applied prior to Fourier transformation. All two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) and two-dimensional transferred NOESY experiments were performed in a similar way. A total of 48 scans was collected for each free-induction decay (matrix 2048 \times 512 points) and data were zero-filled to 4 K \times 2 K points before Fourier transformation. Mixing times of 100, 200, and 300 ms were used.

One-dimensional STD Experiments—Samples were prepared dissolving octasaccharides in the same buffer described previously. Between 0.4 and 0.5 mg of each sample was dissolved in a 3.4–4.2 \times 10^{-6} mM protein solution reaching a ligand/AT ratio of 50:1. The AGA1/AT sample was prepared dissolving 0.5 mg of pentasaccharide in a 8.9 \times 10^{-6} mM protein solution, maintaining the same ligand/AT ratio.

The pulse sequence used for the monodimensional STD NMR experiments includes a 30-ms spin-lock pulse to eliminate the broad resonances of the protein. A train of 40 Gaussian-shaped pulses of 50 ms each was applied to produce selective saturation. Because H5 of IdoUA residues shifts very close to water, signal solvent suppression was not included in the pulse sequence. The on-resonance irradiation was performed at the low field protein resonances (4.7 ppm), whereas the

where $[\text{AT}]_0$ and $[\text{ols}]_0$ stand for the AT and oligosaccharide initial concentration, respectively. $\Delta f$ and $\Delta f_{\text{max}}$ are the absolute change of fluorescence intensity ($f - f_0$) for a given oligosaccharide concentration and that of the maximum fluorescence intensity change ($f - f_0$)$_{\text{max}}$, respectively. $n$ is the binding stoichiometry (the ratio of the concentration of the high affinity species present in the reaction mixture versus the total concentration of the studied product). Here the value of this parameter was set to 1 because the analyzed solutions were considered to contain a single product, each molecule of which is capable of binding to one molecule of AT. Nonlinear regression analysis was done with the GraFit software (Erithacus Software).

$NMR$ Spectra—All one- and two-dimensional NMR spectra were measured at 35 °C, at 600 MHz with a Bruker Avance 600 spectrometer equipped with a high sensitivity 5-mm TCI cryo-probe. To reduce the water humping, particularly strong in cryoprobes, 3-mm NMR tubes were used instead 5-mm tubes reducing the volume from 0.6 to 0.2 ml. For proton detection, between 150 and 250 \mu g of octasaccharide samples were dissolved in 2H2O (99.9%) and freeze-dried to remove residual water. After exchanging the samples three times, samples were dissolved in 0.2 ml of 10 mM phosphate buffer (0.5 M NaCl, pH 7.4) with 3 mM EDTA in 2H2O (99.996%). For the binding studies, samples were prepared by dissolving 1 mg of AT and 150–250 \mu g of each octasaccharide sample in the phosphate buffer so as to reach a 1:3.5 AT/octasaccharide molar ratio (10). In OCTA-1 some spectra were needed to be repeated with a smaller AT/octasaccharide ratio (1:5), to reduce the signal overlapping affecting the quantitative analysis.

Proton spectra were recorded with presaturation of the residual water signal, with a recycle delay of 12 s and 256 scans. Bidimensional double-quantum filter correlation spectroscopy (DQF-COSY) and two-dimensional TOCSY spectra were acquired using 32 scans per series of 2 K \times 512 W data points with zero filling in F1 (4 K \times 2 K), and a shifted ($\pi/3$) squared cosine function was applied prior to Fourier transformation. All two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) and two-dimensional transferred NOESY experiments were performed in a similar way. A total of 48 scans was collected for each free-induction decay (matrix 2048 \times 512 points) and data were zero-filled to 4 K \times 2 K points before Fourier transformation. Mixing times of 100, 200, and 300 ms were used.

One-dimensional STD Experiments—Samples were prepared dissolving octasaccharides in the same buffer described previously. Between 0.4 and 0.5 mg of each sample was dissolved in a 3.4–4.2 \times 10^{-6} mM protein solution reaching a ligand/AT ratio of 50:1. The AGA1/AT sample was prepared dissolving 0.5 mg of pentasaccharide in a 8.9 \times 10^{-6} mM protein solution, maintaining the same ligand/AT ratio.

The pulse sequence used for the monodimensional STD NMR experiments includes a 30-ms spin-lock pulse to eliminate the broad resonances of the protein. A train of 40 Gaussian-shaped pulses of 50 ms each was applied to produce selective saturation. Because H5 of IdoUA residues shifts very close to water, signal solvent suppression was not included in the pulse sequence. The on-resonance irradiation was performed at the low field protein resonances (4.7 ppm), whereas the
off-resonance control irradiation was performed at 24 ppm. The STD spectrum was obtained by phase cycling subtraction of the on-resonance and off-resonance data acquired in interleaved mode. The number of scans and dummy scans were 2048 and 16, respectively.

**Computational Studies on Otsaccharide-AT Complexes**—Flexible docking calculations of octasaccharides 1–4 onto AT were performed by AutoDock 3.0 program (26), following the procedure described for rigid docking (10). Each simulation was performed using 30 genetic algorithm runs and 3000 generations for each run. Eight torsions were allowed to move (see “Results”). Resulting ensembles of 30 conformations were then clustered using a root mean square deviation tolerance of 0.5 Å.

Theoretical tr-NOEs were computed on selected octasaccharide models by the CORCEMA program (27). All AT protons within 15 Å from ligands were included in the calculation, as possibly interacting with ligand protons. For each octasaccharide, the experimental $K_d$ value measured in a 0.5 m NaCl solution was used for CORCEMA simulation to compare theoretical tr-NOEs with experimental ones at the same buffer solution. $k_{off}$ values were then estimated to obtain the best agreement with experimental NOEs ($k_{off}$ OCTA-1, OCTA-2, and OCTA-3, 8 s$^{-1}$; $k_{off}$ OCTA-4, 6 s$^{-1}$).

A ligand/protein ratio of 3.5:1 was set for OCTA-2, -3, and -4, and a ratio of 5:1 was set for OCTA-1. The correlation time of AT was considered isotropic and estimated from published data ($\tau_{AT}$, 46 ns) (15), whereas an average correlation time value characteristic of oligosaccharides having similar structure was used for octasaccharides ($\tau_{octasaccharide}$, 0.9 ns) (28).

The fitting between experimental and theoretical NOEs was evaluated by computing $R$ factors according with the Equation 2:

$$R = \left( \sum I_{ij}^{\text{exp}}(\tau_{\text{mix}}) - I_{ij}^{\text{calc}}(\tau_{\text{mix}}) \right)^2 / \sum I_{ij}^{\text{exp}}(\tau_{\text{mix}})^2 \right)^{1/2}$$

(Eq. 2)

where $I_{ij}^{\text{exp}}$ indicates the experimental cross-peak intensities; $I_{ij}^{\text{calc}}$ indicates the calculated cross-peak intensities, and $\tau_{\text{mix}}$ indicates the mixing times.

**RESULTS**

**Isolation and Structural Characterization of Octasaccharides**—OCTA-1 and OCTA-3 were isolated and characterized as described previously (10). OCTA-2 and OCTA-4 were similarly obtained, by combining AT affinity and CTA-SAX chromatography, starting from the octasaccharide GPC fraction of enoxaparin, as described under “Experimental Procedures.” The structures of the four octasaccharides are reported in Fig. 1. Originally, the structures of the OCTA-2 and -4 were determined by sequencing methodology involving a controlled digestion of the sample with heparinase followed by HPLC analysis according to the procedure described by Mourier and Viskov (19) (data not shown). Briefly, OCTA-2 was sequenced by the chromatographic profile of anomers, peculiar of N-acetylated reducing end (19).

The substantial increase of the intrinsic protein fluorescence ($33.3\%$) that resulted from complex formation was used as the signal. $K_d$ was determined from the experimental data sets (oligosaccharide concentration: fluorescence intensity pairs) by a least square fit based on Equation 1 (data not shown). The value of $K_d$ and its standard error for each AT-oligosaccharide complex and for the various NaCl concentrations are summarized in Table 2. The binding of fondaparinux to AT was investigated in the presence of 0.1, 0.25, and 0.5 m NaCl yielding $K_d$ values of...
Heparin Octasaccharides-AT Interaction

0.024, 0.242, and 4.75 μM, respectively, a salt concentration dependence substantially in agreement with the data reported by Olson et al. (22). In the presence of 0.5 M NaCl (the salt concentration used in the NMR studies), OCTA-1, OCTA-2, and OCTA-3 bound the serpin with a moderately higher affinity than fondaparinux (Table 2), whereas OCTA-4 bound AT with a 20-fold lower $K_d$ (0.24 μM). This latter interaction was also investigated in the presence of 0.1 and 0.25 M NaCl. We found $K_d$ values of 1.5 and 13.0 nM, respectively, here again 2 values lower by more than 1 order of magnitude than those measured for the binding of fondaparinux to AT (Table 2).

The plots of log $K_d$ versus log[Na$^+$] shown in Fig. 3 illustrate the Na$^+$ concentration dependence of OCTA-4 and fondaparinux-AT interactions. Assuming that fondaparinux and OCTA-4 behave as polyelectrolytes in solution, we analyzed the effect of Na$^+$ concentration on their binding to AT according to the theory of macromolecule-polyelectrolyte interactions. This approach, used by Record et al. (29) to investigate ligand nucleic acid interaction in the presence of monovalent ions, was also used by others to investigate the effect of the salt concentration on the binding properties of heparin or pentasaccharide to antithrombin (22, 30, 31), thrombin (32), fibroblast growth factor (33), or peptide (34). Complex formation between a polyelectrolyte with bound counterions (Na$^+$) such as an oligosaccharide (fondaparinux or OCTA-4) and a protein (AT) is accompanied by an entropically favorable release of Na$^+$ from the oligosaccharide chain by cationic residues located within the binding site of the protein. Although this process (the polyelectrolyte effect (29)) accounts for the ionic component of the global interaction of AT with the oligosaccharide, its nonionic component results from hydrogen bonding and/or hydrophobic interactions. For these systems the whole interaction is described by Equation 3, which relates the observed equilibrium dissociation constant $K_d$ to the Na$^+$ concentration (29),

$$\log K_d = \log K_{d_{\text{non}}} + Z \psi \log [\text{Na}^+]$$  \hspace{1cm} (Eq. 3)

where $K_{d_{\text{non}}}$ is the equilibrium dissociation constant characterizing the nonionic component at 1 M NaCl; $Z$ is the number of ionic interactions (or ion pairs) formed between oligosaccharide and AT, and $\psi$ is the fraction of Na$^+$ counterion bound to oligosaccharide per unit of charge. $\psi$, a parameter related to the axial charge density of the polyelectrolyte, was determined to be 0.8 for heparin (30, 32). Equation 3 predicts a linear depend-
ence of log $K_d$ on log [Na$^+$], and $Z$ can thus be derived from the slope of the plot, whereas log $K_{dn-i}$ represents its intercept with the y axis. From the theoretical straight lines generated by linear regression using the data of Fig. 3, we found that between 4 and 5 ionic interactions are involved in the binding to AT of OCTA-4 ($Z = 4.7 \pm 0.6$). A similar result was found for fondaparinux, the reference product ($Z = 4.5 \pm 0.6$), in agreement with the literature (22). From the y intercepts of the plots, we found that the nonionic contribution to the binding of fondaparinux to AT is characterized by $K_{dn-i} = 42.6 \mu M$, in reasonable agreement with published data (31). In contrast, $K_{dn-i} = 1.8 \mu M$ for the interaction of OCTA-4 with AT, a 22-fold lower value than for fondaparinux, indicates that the nonionic contributions to the protein binding are significantly enhanced in the case of OCTA-4. Due to $K_d$ values for the complex of OCTA-4 with AT = 1.5 nM at 0.1 M NaCl and pH 7.4, we calculated from the ratio log $K_{dn-i}$/log $K_d$ that the nonionic interactions account for about 65% of the total free energy of binding in these conditions.

**Molecular Conformation of the Oligosaccharides**—As reported previously for OCTA-1 and OCTA-3 (10), the conformation of OCTA-2 and OCTA-4 in buffer solution was determined by analysis of $^3J_{H-H}$ (three-bond proton-proton coupling constants) and NOEs. $^3J_{H-H}$ couplings measured by one-dimensional $^1H$ spectra (Table 1) indicated that all glucosamine and glucuronic acid residues were present in aqueous solution in the $^4C_1$ conformation. In contrast, the conformations of the unsaturated terminal uronic acid residues ($U$ and $U_2S$) were influenced by 2-O-sulfation. Their measured $^3J_{H-H}$ values are consistent with a preferred $^2H_1$ half-chair conformation for $U$ residue of OCTA-2 and a preferred $^1H_2$ half-chair conformation for $U_2S$ residue of OCTA-4 (35), similarly to what was observed for OCTA-1 and OCTA-3, respectively (10).

$^3J_{H-H}$ couplings of the IdoUA$_{2S}$ within the pentasaccharide (I) indicate an equilibrium between $^1C_4$ and $^2S_O$ forms in both octasaccharides. Measurement of $^3J_{H-H}$ couplings for I$^*$ residue of OCTA-2 was not possible because of the large line width of its H1 signal and the overlap of the H5 signal with the $\beta$-forms of the reducing N-acetylated glucosamine.

Because $^1C_4$ and $^2S_O$ conformations exhibit distinct H5-H2 distances (4.0 and 2.4 Å, respectively) (16), the corresponding NOEs can be considered as a marker for the $^2S_O$ conformation. Particularly, the ratio between H5 and H4 (showing the same distance in both $^1C_4$ and $^2S_O$ geometries) and H5-H2 NOEs can
Heparin Octasaccharides-AT Interaction

**TABLE 1**
600-MHz proton chemical shifts of OCTA-2 (top) and OCTA-4 (bottom) residues

Proton spectra were measured at $T = 35^\circ C$ in 10 mM phosphate buffer, pH 7.4, and 0.5 M NaCl. Standard errors, $^{1}H \pm 0.001$ ppm. Three bond proton-proton coupling constants ($^{3}J_{HH}$) are indicated in parenthesis. Standard error on all couplings $\pm 0.1$ Hz. Residues bearing to AGA*IA sequence are in boldface. ND, not detectable $^{3}J_{HH}$.

| Oligosaccharide | [NaCl] | $K_d$ |
|----------------|--------|-------|
| OCTA-1         | 0.5    | $3.10 \pm 0.12 \times 10^{-6}$ |
| OCTA-2         | 0.5    | $3.30 \pm 0.11 \times 10^{-6}$ |
| OCTA-3         | 0.5    | $2.74 \pm 0.09 \times 10^{-6}$ |
| OCTA-4         | 0.5    | $1.50 \pm 0.14 \times 10^{-6}$ |
| Fondaparinux   | 0.25   | $1.30 \pm 0.20 \times 10^{-6}$ |
|                | 0.5    | $2.40 \pm 0.11 \times 10^{-6}$ |

$^{a}$ Referred to a $^{2}$-anomer.

$^{b}$ The standard error is $^{1}H \pm 0.01$ ppm.

**TABLE 2**
Equilibrium dissociation constant for the various oligosaccharide-AT interactions

| Oligosaccharide | [NaCl] | $K_d$ |
|----------------|--------|-------|
| OCTA-1         | 0.5    | $3.10 \pm 0.12 \times 10^{-6}$ |
| OCTA-2         | 0.5    | $3.30 \pm 0.11 \times 10^{-6}$ |
| OCTA-3         | 0.5    | $2.74 \pm 0.09 \times 10^{-6}$ |
| OCTA-4         | 0.5    | $1.50 \pm 0.14 \times 10^{-6}$ |
| Fondaparinux   | 0.25   | $1.30 \pm 0.20 \times 10^{-6}$ |
|                | 0.5    | $2.40 \pm 0.11 \times 10^{-6}$ |

$^{a}$ Referred to a $^{2}$-anomer.

$^{b}$ The standard error is $^{1}H \pm 0.01$ ppm.

be related to the relative percentage of the two conformers. An H5-H2 NOE having smaller intensity than its corresponding H5-H4 NOE was measured for the I moiety in both OCTA-2 and OCTA-4 (Table 3), indicating that this residue is present in solution in equilibrium between the $^{1}C_{b}$ and $^{3}S_{C}$ conformations. On the contrary, no H5-H2 correlation was detected for I' in OCTA-2, confirming that this moiety is present in solution in a pure $^{1}C_{b}$ form. Iduronic acid residue regions of the two-dimensional NOESY spectra of OCTA-2 and OCTA-4 are shown in supplemental Fig. S5.

**STD Experiments**—To identify the ligand epitope binding, one-dimensional STD experiments (36, 37) were performed on both the pentasaccharide fondaparinux and the four octasaccharides in the presence of AT (about 50-fold excess of the ligand). The STD spectra in comparison with their corresponding reference spectra are shown in Figs. 4 and 5. The STD signals of AGA*IA residues are the most intense ones, whereas signals of residues belonging to the reducing and nonreducing end extensions are weaker or almost disappear from the spectra. Because H2 of the trisulfated glucosamine (GlcNNS,3,6S) residue is not affected by signal overlapping in all the spectra, it was chosen as reference peak. The STD intensity of anomic and H2 signals of AGA*IA ranges from 80 to 120% in all octasaccharides. The relative STD intensity of each AGA*IA signal remains constant independently from the structure of the octasaccharides (e.g. it is 90–100% for both H1 of I and H2 of G) (Fig. 6), as expected when their pentasaccharide sequence is located in the same position within the AT-binding site.

Relatively intense signals (40–60%) were observed also for H1 and H4 of the unsaturated uronate residue in the STD spectrum of the OCTA-1, indicating that this residue is close to the binding region. On the contrary, both H1 and H2 of A* show weak STD signals, indicating a larger distance of this residue...
from AT. Moreover, the H1 signal of I’ is almost absent in the STD spectrum, whereas the H5 signal of the same residue is relatively intense, suggesting that this proton is oriented toward the protein surface. Because signals of the α- and β-forms of the reducing N-acetylglycosamine of OCTA-2 overlap with the

| TABLE 3 |
| H5-H2 and H5-H4 NOE magnitudes (%) of iduronic acid residues of OCTA-2 and OCTA-4, in their free state, measured at 35 °C in phosphate buffer 10 mM, pH 7.4, and 0.5 M NaCl at three mixing time values |
| Mixing | OCTA-2 | OCTA-4 |
| ms | | |
| H5-H2 I | 100 | 1.0 | 1.5 |
| | 200 | 2.5 | 1.7 |
| | 300 | 3.6 | 3.4 |
| H5-H4 I | 100 | 3.0 | 2.8 |
| | 200 | 5.4 | 5.2 |
| | 300 | 8.4 | 7.6 |
| H5-H2 I’ | 100 | 0 | |
| | 200 | 0 | |
| | 300 | 0 | |
| H5-H4 I’ | 100 | 1.8 | |
| | 200 | 2.6 | |
| | 300 | 4.3 | |

Conformations of AT-bound Octasaccharides—Similarly to what was observed for OCTA-1 and OCTA-3 (10), the 1H NMR spectra of OCTA-2 and OCTA-4 in their complexes with AT, compared with the corresponding ones in the free state (supplemental Figs. S1 and S2), show small shifts of the proton res-
onances and increased line width, arising from the higher correlation times induced by protein binding. This indicates an interaction between the octasaccharides and AT in an equilibrium regulated by intermediate dynamic exchange. The evidence of the intermolecular interaction was supported by the increased NOE magnitudes induced by AT (supplemental Tables S2–S5). Notably, only a weaker increment is detectable in OCTA-1 because the quantitative analysis of its transferred-NOESY spectra needed to be performed using a smaller protein/ligand ratio (see “Experimental Procedures”).

Iduronic acid conformations were investigated by analyzing intra-residue tr-NOE effects (supplemental Fig. S3) because the increased line width of bound spectra does not allow measurement of $^3J_{H-H}$. A significant enhancement of $H_5$-$H_2/H_5$-$H_4$ NOE ratio of the I residue was observed for both OCTA-2 and OCTA-4. This indicates that the conformation of such moiety is driven toward the $2S_0$ form by the presence of AT, as observed in all AGA*IA-containing oligosaccharides so far described (10, 12, 15). In contrast, the I” residue of OCTA-2 does not show an H5-H2 tr-NOE signal, indicating that I” maintains its $1C_4$ conformation in the presence of AT. In OCTA-4, G’ maintains its $4C_1$ conformation as confirmed by its $^3J_{H_1-H_2}$ that assumes the same value measured in the free state (8 Hz).

**FIGURE 5.** Partial $^1H$ NMR spectra of the complex OCTA-3/AT and OCTA-4/AT (molar ratio 50:1) at 35 °C and 600 MHz. a, STD; b, reference $^1H$ spectrum. i indicates impurity. Spectra were recorded as indicated in Fig. 4 legend.

**FIGURE 6.** Relative intensities of STD proton signals, normalized to that of the corresponding H2 of residue A* of sequence AGA*IA showing that the pentasaccharide protons receive the largest amount of saturation transfer. Asterisks indicate signals overlapping in the corresponding proton spectrum.
Computational Studies on Octasaccharide-AT Complexes—
The earlier conformational studies on OCTA-1 and OCTA-3 in their complex with AT performed by rigid docking simulations were refined in the present work by performing flexible runs and extended to OCTA-2 and OCTA-4. Ring conformations were set as indicated previously (10). Eight selected glycosidic torsions in each octasaccharide were allowed to move freely. On the basis of STD results, glycosidic linkage geometries of residues inside the AGA*IA sequence were taken as invariable in all the analyzed ligands and they were kept fixed during docking simulations. Protein side chains of amino acid residues involved in binding with AGA*IA extensions are shown (blue tubes). Polar hydrogens of ligand are omitted.

FIGURE 7. Models of OCTA-3/AT (a) and OCTA-4/AT (b) complexes obtained by flexible docking simulations, superimposed to x-ray pentasaccharide (10) (black wires). δ and φ torsions of ΔU2S A', A'1'/G', I'/G'-A, and A-G glycosidic linkages were allowed to move freely during docking simulations. Protein side chains of amino acid residues involved in binding with AGA*IA extensions are shown (blue tubes). Polar hydrogens of ligand are omitted.

Also allowed to move, because its geometry is expected to affect the conformation of the extension.

At least 8 of 30 AT-binding structures with AGA*IA in the same position found in the pentasaccharide-AT x-ray complex (12) were calculated by the docking simulation of each octasaccharide. From all the simulated docking oligosaccharide/protein ensembles, the structure that was able to better fit the experimental STD data was selected. In all analyzed ligands, the models maintaining AGA*IA in its original position show essentially the same distances between protons of the AGA*IA sequence and AT residues. In contrast, ligand-protein distances involving proton residues of both reducing and nonreducing end extensions are, on average, larger and different among the four octasaccharides (supplemental Table S6). These findings are in good agreement with experimental data, indicating that STD effects involving AGA*IA residues have similar magnitudes in all the analyzed octasaccharides, whereas smaller STD magnitudes were detected on proton signals of residues corresponding to both reducing and nonreducing end extensions.

In both OCTA-3 and OCTA-4, from all the simulated docking oligosaccharide/protein ensembles, two main clusters of structures can be identified. Models belonging to the first cluster show I'/G'-A residues with their 2-OH and 3-OH groups oriented toward the AT surface (Fig. 7). In models of the second cluster, the orientation of these groups is completely reversed by a drastic change of I'/G'-A glycosidic linkage, driving the carboxylic group toward the AT surface (supplemental Fig. S4). Consequently, in the latter cluster, the orientation of I'/G'-A residues is also reversed.

Comparison with STD data indicates that models of the first cluster are the only ones able to interpret experimental data. Indeed, such models account for interactions between AT and H2 and H1 of both G'/I' and the A' residues, whereas in models of the second cluster these protons are far from the AT surface. The nonreducing end extension residues of OCTA-3 and OCTA-4 models belonging to the first cluster show I'/G'-A residues with their 2-OH and 3-OH groups oriented toward the AT surface (Fig. 7). In models of the second cluster, the orientation of these groups is completely reversed by a drastic change of I'/G'-A glycosidic linkage, driving the carboxylic group toward the AT surface (supplemental Fig. S4). Consequently, in the latter cluster, the orientation of I'/G'-A residues is also reversed. Comparison with STD data indicates that models of the first cluster are the only ones able to interpret experimental data. Indeed, such models account for interactions between AT and H2 and H1 of both G'/I' and the A' residues, whereas in models of the second cluster these protons are far from the AT surface.
residue and such amino acids. Short distances between hydroxyl groups of G/H11032/I/H11032 moieties and Lys-125, Lys-129, and Arg-132 can also be detected, suggesting that nonionic interactions involving these groups could occur in both octasaccharides (Table 4). Notably, the two octasaccharides differ only in the epimerization and conformation of the uronic acid preceding the AGA*IA sequence, which is that of G/glucuronic acid in OCTA-4 and I/iduronic acid in OCTA-3. The selected model of OCTA-3, having I' in skew-boat conformation, shows a slight increment of the distance from the protein surface of OH-2 and OH-3 of I', as well as of H2 of A1' (Table 4 and supplemental Table S6).

The docking output models both OCTA-1 and OCTA-2, maintaining AGA*IA in its original position (Fig. 8) shows distances between H1 and H5 atom of I' moieties and the Glu-113 residue (about 7.0 Å) (supplemental Table S6) accounting for the small STD effects experimentally detected on signals of these protons. Moreover, shorter distances between H1 and H4 atoms of the ΔU moiety and the AT surface were measured in these models and found to be in agreement with the observed STD effects. Both octasaccharides show OH-2 and OH-3 of the ΔU moiety and the AT surface were measured in these models and found to be in agreement with the observed STD effects. Both octasaccharides show OH-2 and OH-3 of the ΔU residue close to Lys-125 and Arg-132, indicating possible non-ionic interactions between these hydroxyl groups and AT (Table 4).

Theoretical tr-NOEs were computed on octasaccharide models by the CORCEMA program, evaluating full relaxation and exchange matrix. Following the procedure previously adopted for the AGA*IA pentasaccharide (15), we evaluated the agreement between theoretical and experimental tr-NOEs by calculating $R$ factors. To evaluate the ability of our models to interpret the position of the ligand in the heparin-binding site of AT regardless of ligand internal conformation, tr-NOEs of H1-H2 protons cross-relaxing within glucosamine residues were first analyzed. Models in Figs. 7 and 8 showed a good fitting between theoretical and experimental intra-residue tr-NOEs ($R$ factors ranged from 0.05 to 0.30; see Table 5). H1-H2 tr-NOEs were then also computed on a model of OCTA-1 moved up by a disaccharide unit from the normal AT-binding site (supplemental Fig. S5). This type of shifted structures was also found using rigid docking simulation of OCTA-1, and it corresponds to one possible docking to AT described for an heptasaccharide containing AGA*IA at its nonreducing end (11). However, tr-NOEs com-

### TABLE 4

Selected ligand-protein distances (in Å) measured on models of OCTA-1, -2, -3 and -4/AT complexes obtained by flexible docking simulations (models are shown in Figs. 7 and 8).

| Ligand atom | AT residue | OCTA-1 | OCTA-2 | Ligand atom | AT residue | OCTA-3 | OCTA-4 |
|-------------|------------|--------|--------|-------------|------------|--------|--------|
| OH2AU       | Lys-125    | 5.6    | 5.7    | OH2' /G'    | Lys-125    | 5.7    | 5.1    |
| Arg-129     | Arg-129    | 5.7    | 4.4    | Arg-132     | Arg-129   | 4.1    | 3.5    |
| Arg-132     | Arg-132    | 4.2    | 4.3    | Arg-132     | Arg-132   | 4.2    | 3.3    |
| OH3AU       | Arg-132    | 5.2    | 5.4    | OH3' /G'    | Arg-132   | 5.2    | 5.4    |
| OH3 I'      | Glu-113    | 5.2    | 5.4    |              |            |        |        |

**FIGURE 8.** Models of OCTA-1/AT (a) and OCTA-2/AT (b) complexes obtained by flexible docking simulations, superimposed to x-ray pentasaccharide (10) (black wires). $\phi$ and $\psi$ torsions of ΔU-A, I-A, A-I', and I"-A" glycosidic linkages were allowed to move freely during docking simulations. Protein side chains of amino acid residues involved in binding with AGA*IA extensions are shown (blue tubes). Polar hydrogens of ligand are omitted.

---

**FIGURE 8.** Models of OCTA-1/AT (a) and OCTA-2/AT (b) complexes obtained by flexible docking simulations, superimposed to x-ray pentasaccharide (10) (black wires). $\phi$ and $\psi$ torsions of ΔU-A, I-A, A-I', and I"-A" glycosidic linkages were allowed to move freely during docking simulations. Protein side chains of amino acid residues involved in binding with AGA*IA extensions are shown (blue tubes). Polar hydrogens of ligand are omitted.
on the “true” octasaccharide geometry (Table 6). Therefore, the models so far obtained provide useful indications that the CORCEMA program can discriminate among differently shifted ligands and that, in all the analyzed octasaccharides, the models maintaining the AGA*IA sequence in its original placement best fit the experimental data.

Inter-residue tr-NOEs between protons cross-relaxing across the glycosidic bonds were also analyzed. Models in Figs. 7 and 8 give relatively good factors ranging from 0.10 to 0.62 (supplemental Tables S2–S5). Such models thus need to be refined by further calculation, to reach a complete satisfactory agreement with the experimental data. However, because small variations of φ and ψ glycosidic torsions give rise to strong variations of inter-glycosidic proton distances (and consequently in theoretical tr-NOE magnitudes), no dramatic change in octasaccharide geometry is expected to occur during the optimization. Therefore, the models so far obtained provide useful indications on the “true” octasaccharide geometry (Table 6).

**DISCUSSION**

In this work, the structure of two novel AT-binding octasaccharides (OCTA-2 and OCTA-4) isolated from enoxaparin have been characterized by sequence analysis and NMR spectroscopy. Their AT binding properties and molecular conformations in the absence and presence of AT have been compared with those of previously described OCTA-1 and OCTA-3 (10). In OCTA-4, the AGA*IA sequence is preceded by a glucuronic acid (G) residue instead of iduronic acid that normally occurs in most common heparins and low molecular weight heparins (5).

Fluorescence titrations performed in 0.5 M NaCl indicated that at this salt concentration the affinity to AT of octasaccharides 1–4 is characterized by equilibrium dissociation constants 2–20-fold lower than those measured for the pentasaccharide-AT complex, OCTA-4 forming the tightest complex with the serpin-Kd = 0.24 μM. Affinity chromatography on immobilized AT of an octasaccharide mixture indicated the following order of elution: OCTA-3 < OCTA-1 < OCTA-2 < OCTA-4. These results confirmed that both reducing and non-reducing end AGA*IA extensions contribute to binding to AT. Moreover, they confirmed that the different affinity of AGA*IA-containing oligomers depends on the structure of these extensions.

Despite the strong evidence accumulated on the specificity of heparin-AT binding, (3, 10, 15), recent studies suggested other possible assemblies between the negatively charged heparin chains and AT (38, 39). In these latter studies, the possibility has been considered that sulfated residues in heparin sequences, different from that of the AT-binding site, may activate AT through nonspecific interactions. The STD analysis carried out in this study, and for the first time applied to glycosaminoglycan-protein complexes, indicates that all the octasaccharide-AT complexes show the whole pattern of contacts identified in the pentasaccharide-AT complex, further supporting the specificity of binding. This study also suggests that, to a lesser extent, additional contacts involving reducing and non-reducing extensions of the AGA*IA sequence contribute to the binding. In fact, STD experiments indicate that the sequence AGA*IA lies closer to the AT-binding site than its reducing or nonreducing end extensions. All analyzed compounds showed either comparable STD effects on AGA*IA signals. tr-NOEs experiments indicated also that the bound conformation of the AGA*IA sequences in the four octasaccharides

### Table 5

| Heparin Octasaccharides-AT Interaction | Experimental | Calculated | R |
|--------------------------------------|-------------|-----------|---|
| **OCTA-1**                           |            |           |   |
| ANAc 5.4                             | 9.9        | 14.8      | 6.0 |
| A 7.0                                | 11.3       | 17.8      | 5.5 |
| A* 7.9                               | 10.3       | 14.4      | 7.6 |
|                         |            |           |   |
| **OCTA-2**                           |            |           |   |
| ANAc 7.6                             | 8.8        | 14.5      | 7.4 |
| A 5.8                                | 11.4       | 16.3      | 7.1 |
| A* 2.2                               | 6.8        | 10.7      | 6.2 |
|                         |            |           |   |
| **OCTA-3**                           |            |           |   |
| ANAc 5.8                             | 11.9       | 18.3      | 9.3 |
| A 6.5                                | 12.9       | 18.3      | 7.1 |
| A* 6.2                               | 11.8       | 18.2      | 6.0 |
|                         |            |           |   |
| **OCTA-4**                           |            |           |   |
| ANAc 8.1                             | 12.9       | 18.4      | 8.6 |
| A 5.1                                | 10.6       | 15.0      | 7.2 |
| A* 4.5                               | 8.3        | 12.8      | 4.9 |
|                         |            |           |   |
| **OCTA-1 shifted**                   |            |           |   |
| ANAc 5.4                             | 9.9        | 14.8      | 4.2 |
| A 7.0                                | 11.3       | 17.8      | 3.8 |
| A* 7.9                               | 10.3       | 14.4      | 3.9 |
|                         |            |           |   |
was essentially the same as that assumed in the pentasaccharide, including the $^2S_o$ skew-boat conformation for the I residue. tr-NOE analysis also indicated that in the presence of AT both the 2-O-sulfated residue I in OCTA-1 (10) and the non-sulfated I residue of OCTA-2 adopt the $^1C_4$ conformation. These findings suggest that the $^1C_4$ conformation of the idurionate residue located immediately after the AGA*IA sequence could enhance the AT affinity of AGA*IA containing oligosaccharides, either by optimizing contacts between AGA*IA and AT or promoting additional contacts involving the AGA*IA reducing extension. On the other hand, the I residue of OCTA-3, despite lacking in 2-O-sulfation, is known to be driven to the $^2S_o$ form by the presence of AT (10), whereas G of OCTA-4 maintains its $^5C_1$ conformation. We can thus speculate that the $^3C_1$ and $^2S_o$ conformations of the GlcA and IdoUA residues, respectively, preceding the AGA*IA sequence could enhance the AT affinity of AGA*IA-containing oligosaccharides, whereas the $^1C_4$ chair form does not optimize contacts with the protein.

Model outputs from flexible docking simulations, with the AGA*IA sequence maintaining the same position adopted in pentasaccharide/AT structures, can interpret both STD and tr-NOE data. In such models, short distances were found between uronic acid OH groups of both reducing and nonreducing end AGA*IA extensions and AT, accounting for the occurrence of nonionic interaction that could contribute to enhance the affinity of the octasaccharide with respect to the pentasaccharide (Table 3).

The higher nonionic contribution to AT affinity of OCTA-4 ($K_{on,i} = 1.8$ μM), with respect to the pentasaccharide ($K_{on,i} = 42.6$ μM) (Fig. 3), can be attributed to the stronger nonionic interactions between AT and OH-2 and OH-3 groups of G’ residue. Besides nonionic contacts with Lys-125 and Arg-129, the proposed model (Fig. 7) shows particularly short distances between these hydroxyl groups and Arg-132 (Table 4).

These observations support the idea put forward in a recent study (31), indicating that nonionic interactions can play important roles in the binding of charged saccharides to proteins and further emphasize the importance of characterizing these binding components. The presence of iduronic acid instead of glucuronic acid at the nonreducing extension of OCTA-3 reduces the affinity by 1 order of magnitude. The conformational flexibility of iduronic acid may affect the binding efficacy of the octasaccharide both in terms of entropic and enthalpic contributions and by increasing intermolecular distances between I’ hydroxyl groups and AT as proposed in our model. Such hypothesis needs to be confirmed by further studies using isothermal titration calorimetry and designed AT mutants.

The occurrence of a GlcA residue near AGA*IA in OCTA-4 deserves a special comment. In fact, IdoUA residues are the prevalent uronic acids in this position near the active site of heparin for AT (3). Because relatively higher amounts of 2-O-sulfated GlcA were found in enoxaparin as compared with

![Table 6: $\phi, \psi$ dihedral angles at the glycosidic linkages measured on models of OCTA-1, -2, -3 and -4/AT complexes obtained by flexible docking simulations (models are shown in Figs. 7 and 8). Glycosidic linkages within AGA*IA sequence are highlighted in bold boxes.](image)

|                | A-G     | G-A*    | A*-I   | I-A     | A-I"   | I"-A"  |
|----------------|---------|---------|--------|---------|--------|--------|
| OCTA-1         | -47, -31| 43, 9   | -47, -36| 52, 4   | -39, -25| 36, -13|
| OCTA-2         | -57, -45| 46, 7   | -46, -38| 48, 4   | -38, -32| 43, 6  |
| OCTA-3         | -4, 35  | -50, -44| 49, 2  | -45, -29| 43, 2  | -58, -52| 51, 23|
| OCTA-4         | -51, -39| 52, 4   | -50, -44| 44, 2   | -60, -54| 60, 18|

was essentially the same as that assumed in the pentasaccharide, including the $^2S_o$ skew-boat conformation for the I residue. tr-NOE analysis also indicated that in the presence of AT both the 2-O-sulfated residue I in OCTA-1 (10) and the non-sulfated I residue of OCTA-2 adopt the $^1C_4$ conformation. These findings suggest that the $^1C_4$ conformation of the idurionate residue located immediately after the AGA*IA sequence could enhance the AT affinity of AGA*IA containing oligosaccharides, either by optimizing contacts between AGA*IA and AT or promoting additional contacts involving the AGA*IA reducing extension. On the other hand, the I residue of OCTA-3, despite lacking in 2-O-sulfation, is known to be driven to the $^2S_o$ form by the presence of AT (10), whereas G of OCTA-4 maintains its $^5C_1$ conformation. We can thus speculate that the $^3C_1$ and $^2S_o$ conformations of the GlcA and IdoUA residues, respectively, preceding the AGA*IA sequence could enhance the AT affinity of AGA*IA-containing oligosaccharides, whereas the $^1C_4$ chair form does not optimize contacts with the protein.

Model outputs from flexible docking simulations, with the AGA*IA sequence maintaining the same position adopted in pentasaccharide/AT structures, can interpret both STD and tr-NOE data. In such models, short distances were found between uronic acid OH groups of both reducing and nonreducing end AGA*IA extensions and AT, accounting for the occurrence of nonionic interaction that could contribute to enhance the affinity of the octasaccharide with respect to the pentasaccharide (Table 3).

The higher nonionic contribution to AT affinity of OCTA-4 ($K_{on,i} = 1.8$ μM), with respect to the pentasaccharide ($K_{on,i} = 42.6$ μM) (Fig. 3), can be attributed to the stronger nonionic interactions between AT and OH-2 and OH-3 groups of G’ residue. Besides nonionic contacts with Lys-125 and Arg-129, the proposed model (Fig. 7) shows particularly short distances between these hydroxyl groups and Arg-132 (Table 4).

These observations support the idea put forward in a recent study (31), indicating that nonionic interactions can play important roles in the binding of charged saccharides to proteins and further emphasize the importance of characterizing these binding components. The presence of iduronic acid instead of glucuronic acid at the nonreducing extension of OCTA-3 reduces the affinity by 1 order of magnitude. The conformational flexibility of iduronic acid may affect the binding efficacy of the octasaccharide both in terms of entropic and enthalpic contributions and by increasing intermolecular distances between I’ hydroxyl groups and AT as proposed in our model. Such hypothesis needs to be confirmed by further studies using isothermal titration calorimetry and designed AT mutants.

The occurrence of a GlcA residue near AGA*IA in OCTA-4 deserves a special comment. In fact, IdoUA residues are the prevalent uronic acids in this position near the active site of heparin for AT (3). Because relatively higher amounts of 2-O-sulfated GlcA were found in enoxaparin as compared with
unfractionated heparin (40), the present finding suggests that GlcA could be generated by C5-epimerization of IdoUA residues under the basic conditions used for the preparation of enoxaparin. Studies are underway to validate this hypothesis. The extension role of the active pentasaccharide sequence on AT binding properties of heparin oligosaccharides has to be considered in the design of a new generation of tailored low and very low molecular weight heparins. The type of depolymerization process applied to complex heparin chains may generate many structural variants that influence the AT binding properties and regulate the interaction with several other proteins as well.

Acknowledgment—We thank Dr. Davide Vecchietti (G. Ronzoni Institute, Milan, Italy) for the fruitful collaboration in protein purification and sample preparations.

REFERENCES

1. Hoppensteadt, D., Iqbal, O., and Fareed, J. (2005) in Chemistry and Biology of Heparin and Heparan Sulfate (Garg, H. G., Linhardt, R. J., and Hales, C. A., eds) pp. 583–606, Elsevier Ltd., Oxford, UK
2. Thunberg, L., Bäckström, G., and Lindahl, U. (1982) Carbohydr. Res. 100, 393–410
3. Petitou, M., and van Boeckel, C. A. A. (2004) Angew. Chem. Int. Ed. 43, 3118–3133
4. Casu, B. (2005) in Chemistry and Biology of Heparin and Heparan Sulphate (Garg, H. G., Linhardt, R. J., and Hales, C. A., eds) pp. 1–28, Elsevier Ltd., Oxford, UK
5. Casu, B., and Lindahl, U. (2001) Adv. Carbohydr. Chem. Biochem. 57, 159–206
6. de Kort, M., Buijsman, R. C., and van Boeckel, C. A. (2005) Drug Discov. Today 10, 769–779
7. Linhardt, R. J., and Gunay, N. S. (1999) Semin. Thromb. Hemostasis 25, 5–16
8. Toida, T., Hileman, R. E., Smith, A. E., Vlahova, P. I., and Linhardt, R. J. (1996) J. Biol. Chem. 271, 32040–32047
9. Loganathan, D., Wang, H. M., Mallis, L. M., and Linhardt, R. J. (1990) Biochemistry 29, 4362–4368
10. Guerrini, M., Guglieri, S., Beccati, D., Torri, G., Viskov, C., and Mourier, P. (2006) Biochem. J. 399, 191–198
11. Belz, K. J., Dafforn, T. R., Petitou, M., Carrell, R. W., and Huntington, J. A. (2000) J. Biol. Chem. 275, 8733–8741
12. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14683–14688
13. Johnson, D. J., Li, W., Adams, T. E., and Huntington, J. A. (2006) EMBO J. 25, 2029–2037
14. Li, W., Johnson, D. J., Esmen, C. T., and Huntington, J. A. (2004) Nat. Struct. Mol. Biol. 11, 857–862
15. Hricovini, M., Guerrini, M., Bisio, A., Torri, G., Petitou, M., and Casu, B. (2001) Biochem. J. 359, 265–272
16. Ragazzi, M., Ferro, D. M., and Provasoli, M. (1986) J. Comput. Chem. 7, 105–112
17. Casu, B., and Torri, G. (1999) Semin. Thromb. Hemostasis 25, 17–26
18. Boudier, C., and Bieth, J. G. (2001) Biochemistry 40, 9962–9967
19. Mourier, P. A. J., and Viskov, C. (2004) Anal. Biochem. 332, 299–313
20. Höök, M., Björk, I., Hopwood, J., and Lindahl, U. (1976) FEBS Lett. 66, 90–93
21. Olson, S., and Shore, J. (1981) J. Biol. Chem. 256, 11065–11072
22. Olson, S., Björk, I., Sheffer, R., Craig, P., Shore, J., and Choay, J. (1992) J. Biol. Chem. 267, 12528–12538
23. Huntington, J. A., Olson, S. T., Fan, B., and Gettins, P. G. W. (1996) Biochemistry 35, 8495–8503
24. Desai, U. R., Petitou, M., Björk, I., and Olson, S. T. (1998) J. Biol. Chem. 273, 7478–7487
25. Lin, P., Sinha, U., and Betz, A. (2001) Biochim. Biophys. Acta 1526, 105–113
26. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R., and Olson, A. J. (1998) J. Comput. Chem. 19, 1639–1662
27. Moseley, H. N. B., Curtto, E. V., and Krishna, N. R. (1995) J. Magn. Reson. 108, 243–261
28. Angulo, J., Hricovini, M., Gairi, M., Guerrini, M., de Paz, J. L., Ojeda, R., Martin-Lomas, M., and Nieto, P. M. (2005) Glycobiology 15, 1008–1015
29. Record, M. T., Jr., Lohman, M. L., and De Haseth, P. (1976) J. Mol. Biol. 107, 145–158
30. Olson, S. T., and Björk, I. (1991) J. Biol. Chem. 266, 6353–6364
31. Hjelm, R., and Schedin-Weiss, S. (2007) Biochemistry 46, 3378–3384
32. Olson, S. T., Halvorson, H. R., and Björk, I. (1991) J. Biol. Chem. 266, 6342–6352
33. Thompson, L. D., Pantoliano, M. W., and Springer, B. A. (1994) Biochemistry 33, 3831–3840
34. Hileman, R. E., Jennings, R. N., and Linhardt, R. J. (1998) Biochemistry 37, 15231–15237
35. Ragazzi, M., Ferro, D. R., Provasoli, A., Pumilia, P., Cassinari, A., Torri, G., Guerrini, M., Casu, B., Nader, H. B., and Dietrich, C. P. (1993) J. Carbohydr. Chem. 12, 523–535
36. Mayer, M., and Meyer, B. (1999) Angew. Chem. Int. Ed. 38, 1784–1788
37. Mayer, M., and Meyer, B. (2001) J. Am. Chem. Soc. 123, 6108–6117
38. Seyrek, E., Dubin, P. L., and Henriksen, J. (2007) Biopolymers 86, 249–259
39. Verli, H., and Guimarães, J. A. (1997) J. Mol. Graph. Model. 15, 18–36
40. Guerrini, M., Guglieri, S., Naggi, A., Sasisekharan, R., and Torri, G. (2007) Semin. Thromb. Hemostasis 33, 478–487