Heparin Dodecasaccharide Containing Two Antithrombin-binding Pentasaccharides

STRUCTURAL FEATURES AND BIOLOGICAL PROPERTIES

Background: Heparin is a linear sulfated polysaccharide used clinically as an anticoagulant.

Results: A heparin dodecasaccharide, containing two contiguous antithrombin-binding sequences, has been described and characterized for the first time.

Conclusion: The dodecasaccharide binds antithrombin in two different molecular assemblies enhancing the probability of the binding and the affinity.

Significance: The discovery of this dodecasaccharide improves the knowledge of heparin structure.

The antithrombin (AT) binding properties of heparin and low molecular weight heparins are strongly associated to the presence of the pentasaccharide sequence AGA*IA (ANAc,6S-GlcUA-\(\beta\)-1,4)-linked to a \(\alpha\)-L-iduronic acid residue. By using the highly chemoselective deprotonization to prepare new ultra low molecular weight heparin and coupling it with the original separation techniques, it was possible to isolate a polysaccharide with a biosynthetically unexpected structure and excellent antithrombotic properties. It consisted of a dodecasaccharide containing an unsaturated uronate unit at the nonreducing end and two contiguous AT-binding sequences separated by a nonsulfated iduronate residue. This novel oligosaccharide was characterized by NMR spectroscopy, and its binding with AT was determined by fluorescence titration, NMR, and LC-MS. The dodecasaccharide displayed a significantly increased anti-FXa activity compared with those of the pentasaccharide, fondaparinux, and low molecular weight heparin enoxaparin.

Heparin is a linear sulfated polysaccharide belonging to the glycosaminoglycan family and has been used clinically as an anticoagulant for many years, and its worldwide consumption has increased to 100 tons per year (1). Over the last few decades, heparin was shown to be involved in many other biological processes through interaction with a large number of proteins (2). One of the main molecular bases for its anticoagulant activity was elucidated about 30 years ago, when a specific pentasaccharide sequence within heparin chains was identified as responsible for binding and activating antithrombin (3). Other mechanisms such as tissue factor pathway inhibitor release also contribute to the anticoagulant properties of heparin (4).

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The abbreviations used are: A, N,3,6-O-trisulfated glucosamine; A\(_{NS,6S}\), \(\alpha\)-O-6-O-N-sulfoglucosamine; A\(_{NS,6S,4S}\), \(\alpha\)-O-6-O-N-sulfoglucosamine, I\(_{1,2}\), \(\alpha\)-L-2-O-sulfated iduronic acid; AGA*IA, pentasaccharide sequence of A\(_{NS,6S}\) GlcUA-ANAc,6S-IdoUA\(_{2S}\)A\(_{NS,6S}\). N-acetylated 6-O-sulfated glucosamine; IdoUA, \(\alpha\)-iduronic acid; AT, antithrombin; LMWH, low molecular weight heparin; anti-FXa, anti-Factor-Xa; ULMWH, ultra low molecular weight heparins; \(\Delta\), 4,5-unsaturated uronic acid; STD, saturation transfer difference; CTA-SAX, cetyltrimethylammonium strong anion exchange; ESI, electrospray ionization; SEC, size exclusion chromatography.
Heparin Dodecasaccharide Containing Two AT-binding Sites

In agreement with the method of depolymerization used, the AT-binding oligosaccharides were isolated from LMWHs, such as enoxaparin, using a combination of orthogonal separation techniques, including gel permeation chromatography, HPLC, and AT affinity chromatography (13).

These studies demonstrated that the AGA*IA sequence can be flanked by both sulfated and undersulfated disaccharides and that these elongating sequences can have an active role in binding AT (14, 15). Notably, the presence of glucuronic acid instead of iduronic acid before the pentasaccharide moiety increases the affinity to AT by 1 order of magnitude, whereas the presence of 1,6-anhydrohexosamine at the reducing side of the pentasaccharide significantly reduces the affinity (15, 16).

Recently, an octasaccharide having a pentasaccharide sequence at the nonreducing end with two 3-O-sulfated glucosamine residues has been characterized (17). Fluorescence titration experiments indicated that the AT affinity of this octasaccharide was characterized by an equilibrium dissociation constant 20-fold lower than that measured for the AGA*IA-AT complex.

The type of depolymerization process applied to complex heparin chains may thus generate many structural variants, including cleavage within the pentasaccharide sequence, that strongly influence the AT binding properties. A depolymerization procedure able to preserve the AT-binding sequences is the main condition to achieve an ideal and cost-effective LMWH. Because of their lower molecular size compared with their parent heparins, LMWHs have improved bioavailability and higher anti-Factor-Xa (anti-FXa) activity than AT-mediated anti-Factor-IIa (anti-FIIa), with decreased hemorrhagic risk during prolonged treatment (18).

Particularly, the highly selective depolymerization of heparin by the phosphazene base used for preparation of the ULMWH semuloparin protects the native antithrombin-binding sequence from cleavage. This was demonstrated by the anti-FXa activity of semuloparin, which, in contrast to other LMWHs, remains substantially comparable with that of unfractionated heparin despite a 6-fold size reduction of the polysaccharide chain (19, 20).

In this work, a novel dodecasaccharide containing two contiguous AT-binding sequences was isolated from semuloparin. In agreement with the method of depolymerization used, the dodecasaccharide terminates at the nonreducing end with a 4,5-unsaturated uronic acid residue (ΔU). The two AGA*IA moieties, both with the first glucosamine N-acetylated and 6-O-sulfated, are separated by a nonsulfated iduronic acid residue. The structure of the dodecasaccharide was unambiguously defined by controlled enzymatic digestion and NMR spectroscopy.

Mapping of the binding region involved in the interaction with AT was determined by saturation transfer difference (STD) experiments. The conformational and AT binding properties of this dodecasaccharide were also investigated by NMR spectroscopy (NOESY and transferred-NOESY experiments). The presence of two different 1:1 AT-dodecasaccharide complexes was supported by NMR and modeling results and finally confirmed by LC-MS spectrometry. The dodecasaccharide showed significantly higher affinity to AT as well as an increased anti-FXa activity when compared with the pentasaccharide fondaparinux and LMWH enoxaparin.

EXPERIMENTAL PROCEDURES

Materials—Semuloparin was supplied by Sanofi (Vitry sur Seine, France). Antithrombin was purchased from Biogenic (Perols, France). Semuloparin is the result of a chemoselective depolymerization of heparin macromolecules by 2-tert-butylimino-2-diethylamino-1,3-dimethyl-1H-pyrido-1,2,3-diazaphosphorine (phosphazene base BEMP). The reaction principle is based on a β-eliminative cleavage of heparin through its activated benzyl ester derivative. The hemi-synthetic pathway is summarized in the following steps: 1) transallylation of sodium heparin with benzenthionium salts; 2) esterification of heparin benzenthionium salts by benzyl chloride; 3) transallylation of the heparin benzyl ester by benzenthionium salts; 4) depolymerization of the heparin benzyl ester, benzenthionium salt, by BEMP; 5) saponification of benzyl esters; and 6) purification to finally obtain the product.

This procedure yields ULMWH products with an average molecular mass of ∼2400 Da, anti-FXa of ∼160 units/mg, and anti-Factor IIa of ∼2 units/mg (21). All experimental details regarding the ULMWH preparation are reported in example 2 of United States Patent 8,003,623 B2 (22).

Procedure for Dodecasaccharide Isolation and Purification—Semuloparin is fractionated by gel permeation chromatography on a column packed with BioGel P30 (200 × 5 cm) (2 g per injection). The dodecasaccharide fraction was collected and then desalted on a column packed with Sephadex G-10 (100 × 7 cm). In a second step, 200 mg of the original fraction (5 g) was injected onto an AT affinity column (30 × 5 cm) using a step gradient of NaCl concentration (0.7, 1.15, 1.6, 2.05, 2.65, and 3 M NaCl) in 1 M Tris-HCl, pH 7.4. The fractions eluted for conductivities between 80 and 130 millisiemens/cm, which are enriched in dodecasaccharide, are pooled (Fig. 2). These fractions were desalted and purified on semi-preparative CTA-SAX columns (250 × 22 mm), and each desalted fraction was tested by analytical AS11 or CTA-SAX. After pH neutralization, the collected fractions were passed through a Mega Bond Elute C18 cartridge (Varian) before the final purification on a semi-preparative AS11 column (Dionex). The fractions containing the pure dodecasaccharide were pooled, desalted on Sephadex G-10, and freeze-dried.

Enzymatic Sequencing of the Dodecasaccharide—The sequencing of the dodecasaccharide was performed by a combination of controlled and exhaustive cleavage with heparin lyases and HPLC analysis of fragments. To obtain a unique form of the glucosamine at the reducing end, a preliminary reducing step was performed before sequencing experiments. Briefly, 15 μl of a 150 g/liter NaBH₄ solution in 100 mM sodium acetate was added to 1 ml of a 1–2 g/liter aqueous solution of the dode-
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Antithrombin Affinity—The interaction with antithrombin of the dodecasaccharide and of fondaparinux, as a reference compound, was studied by the fluorimetric titration method, according to the method previously described (15).

Anti-FXa Activity in Plasma or in a Buffer Medium—The anti-FXa activity of the dodecasaccharide was determined with the 10 ACL 7000 automated instrument for measuring coagulation (Instrumentation Laboratory), using the Heparin® kit (Instrumentation Laboratory), which contains antithrombin, Factor Xa, and the chromogenic substrate S-2765. The measurements were carried out according to the manufacturer’s recommendations. The second low molecular weight heparin international standard (National Institute for Biological Standards and Control, London, UK, code 01/608) was used to construct the calibration curve. Enoxaparin (Clexane®) and fondaparinux (Arixtra®) were used as internal standard compounds.

The test samples or the second LMWH international standard were first diluted in standard human plasma (Instrumentation Laboratory) or in a buffer (0.05 M Tris-HCl, 0.154 M NaCl, pH 7.4). Test solutions containing samples in plasma or in buffered medium were then diluted 1:20 with a buffered solution containing antithrombin and placed in duplicate in dedicated sites of the apparatus. Factor Xa reagent and chromogenic substrate were introduced into reservoirs provided for this purpose in the ACL 7000 instrument. Anti-FXa activity test was carried out according to the “Heparin” program integrated into the user interface (software) of ACL 7000. During the assay, 50 μL of the sample diluted in buffer were mixed with 50 μL of Factor Xa reagent. After an incubation time of 60 s at 37 °C, 50 μL of chromogenic substrate (1.1 mM) was added, and changes in 405 nm absorption were measured as a function of time. Anti-FXa activity of tested samples was determined using a calibration curve constructed with the second LMWH international standard (code 01/608).

LC-MS—Size exclusion chromatographic separations were performed with a liquid chromatography system ( Dionex Ultimate 3000) equipped with a quaternary pump, an eluent degasser, an auto-sampler, and a variable wavelength detector. A sample volume of 25 μL was injected on to the Superdex 75 10/300 GL column (300 × 10 mm; 13 μm average particle size). For sample concentrations, an AT solution at 2.0 mg/mL was run to verify separation profiles and retention times. Protein/oligosaccharide solutions were prepared at 1:1 molar ratio containing AT at 2 mg/mL and an equimolar amount of the added oligosaccharide in CH3COONH4 (100 mM). After 30 min of incubation at room temperature, the separation was run using an isocratically 100 mM CH3COONH4/CH3CN 97:3 (v/v) at 0.1 mL/min and monitored at 280 nm.

Mass spectrometric analyses were made on an ESI-TOF instrument (microTOFQ Bruker Daltonics), operating both in positive mode, in the expanded mass range from 1000 to 6000 m/z (normal conditions), and in negative mode, in the mass range from 200 to 2000 m/z (denaturing conditions). In normal conditions, a fraction (33 μL/min) of total eluent flow was delivered by a splitter in the ionization source of the ESI-TOF mass spectrometer and the acquisition in positive polarity over the higher mass range allowing detection of high molecular weight proteins and binding studies. (Instrumental parameters were as...
follows: capillary \(-4500\) V; nebulizer 1.0 bar; dry gas 7 liters/min; dry temperature 200 °C; mass range 1000–6000 m/z).

In the first experiment, denaturing conditions used a solution of 5% HCOOH in 50% CH₃CN introduced at 10 μl/min in the ESI source from a syringe pump (located between the UV detector and ESI ionization), with the aim of reducing noncovalent interactions. In the next experiments, denaturing conditions were increased using 25% HCOOH in 100% CH₃CN introduced at 10 μl/min in the ESI source. In that case, the acquisition in negative mode allows detection of the previously interacting oligosaccharide, and it was then removed from the noncovalent protein-oligosaccharide complex by applying denaturing conditions. (Instrumental parameters were as follows: capillary +3200 V; nebulizer 1.0 bar; Dry gas 7 liters/min; dry temperature 200 °C; mass range 200–2000 m/z).

**Model Complex Building**—The molecular editing was done with the Maestro 9.1 graphical interface. Two models of the 1:1 AT-dodecasaccharide complex, with the nonreducing and reducing AGA*IA framed by a nonsulfated IdoUA residue and terminating at the reducing end with a 4,5-unsaturated (ΔU) residue (structure b of Fig. 1) were built fitting the corresponding AT-binding site. The basic element of the complex between AT and AGA*IA pentasaccharide was isolated by molecular editing from the ternary complex AT-T-Hep (antithrombin-thrombin-heparin) included in the Protein Data Bank code 1TB6. Both AT-dodecasaccharide complexes were created with the conformation of unsaturated uronate and IdoUA residues in \(^2\)H₁ and \(^1\)C₄, respectively, in accord with a previous study (15). The conformation of the \(^1\)G₅ residue of the interacting AGA*IA sequence was in \(^2\)S₀ conformation as found in previous studies (14–17), whereas that of the noninteracting AGA*IA \(^1\)I₂₅ was maintained in \(^1\)C₄, according to the prevalent conformation assumed when AGA*IA is in free solution. The torsional angles between ΔU-A\(_{\text{NS,65}}\)^⁻¹ and I-A\(_{\text{NS,65}}\)^⁻² of the AT-interacting pentasaccharide are in agreement with the previous conformational analysis of the AT-octasaccharide complexes (octasaccharide 2 and 3 of Ref. 15). The structure of the two complexes, AT/ΔU-AGA*IA\(^1\)-IAGA*IA\(^2\) and ΔU-AGA*IA\(^1\)-I-AGA*IA\(^2\)/AT, was optimized by energy minimization applying the bmin procedure included in Macromodel/Maestro 9.1. Amber* force field (all atom force field) was used to represent the potential energy for both the AT and the sugar. The nonbonded interactions (electrostatic and dispersive) were treated using a continuum with a dielectric constant value of 80.0, whereas their cutoff was set to 20.0 and 8.0 Å, respectively. The hydrogen bond cutoff was defined separately as 4.0 Å. The minimization procedure stopped until the default gradient threshold of 0.05 kJ Å⁻¹ mol⁻¹ was reached.

The hypothetical AT₂-dodecasaccharide complex geometry was built starting from two complex structures, AT/ΔU-AGA*IA\(^1\) and AT/AGA*IA\(^2\), that are linked together by an IdoUA residue in \(^1\)C₄ conformation, forming the two glycosidic bonds A\(_{\text{NS,65}}\)^⁻¹-I and I-A\(_{\text{NS,65}}\)^⁻². To avoid molecular interpenetration of the AT molecules, the two AGA*IA-binding sites and the corresponding sulfate groups were oppositely oriented through proper rotation of A\(_{\text{NS,65}}\)^⁻¹-I and I-A\(_{\text{NS,65}}\)^⁻² torsional angle pairs φ/φ. After that, energy minimization, using the setting parameters previously defined, was performed.

**RESULTS**

**Isolation and Characterization of the Dodecasaccharide**—The dodecasaccharide (Fig. 1b) was isolated from semuloparin, using the procedure described under “Experimental Procedures.” The isolated dodecasaccharide fraction, representing ~6–11% of the product, was injected onto an AT affinity column. The high affinity fraction was analyzed by HPLC (column UPLC Waters Acquity BEH C18) (Fig. 2). The fraction, eluted at conductivities between 80 and 130 millisiemens/cm (enriched...
in the dodecasaccharide), was collected and desalted on a column packed with Sephadex G-10 (100 × 7 cm). The dodecasaccharide was then purified by CTA-SAX chromatography. By applying enzymatic sequencing methodology to the dodecasaccharide analysis, its key structural determinants were obtained such as building block composition and their respective order within the polysaccharide (Fig. 3). This first structural analysis was confirmed subsequently with full assignment of NMR spectra. Proton and carbon resonances of each residue were assigned using standard COSY, TOCSY, and HSQC pulse sequences (Tables 1 and 2). The sequence-specific resonance assignments have been made by identifying inter-residue H1-H4 NOE connectivity in NOESY spectra. Briefly, the $\text{ANAc,6S}^1$ was assigned through H4-H1 correlation with $\text{U}$, whereas $\text{ANS}^1$ and $\text{ANS, red}$ were assigned for the H1-H4 correlation with IdoUA and lack of the interglycosidic correlation, respectively (Fig. 4). The H1-H4 correlation between $\text{I}_2^2$ and $\text{ANAc,6S}$ allowed assignment of $\text{A}^2$ and, subsequently, $\text{A}^1$. It was more difficult to
assign G1 and G2, whose resonances are superimposed; only the small difference of H4 chemical shifts, being observable from the NOEs with H1 of ANAc,6S1 and ANAc,6S2 residues, allowed these residues to be assigned.

**Antithrombin Affinity**—The equilibrium dissociation constant, $K_{d}$, for the interaction between AT and dodecasaccharide or fondaparinux was measured by fluorescence titration (Table 3).

**TABLE 1**
Proton chemical shifts (in ppm) and $^{3}J_{HH}$ coupling constants (in Hz) of dodecasaccharide residues measured at 900 MHz, 306 K in phosphate buffer 10 mM, pH 7.4, and 0.5 M NaCl

| AGA*IA sequence 1 | AGA*IA sequence 2 |
|-------------------|-------------------|
| ΔU                | $^{3}J_{HH}$      | G1  | A1  | I2S1 | A3,6S1 | IdoUA | A1,6S2 | G2  | A2  | I2S2 | A3,6S2 |
| H1  | 5.216   | 5.461   | 4.658 | 5.530 | 5.254 | 5.390       | 5.062   | 5.437   | 4.658 | 5.549 | 5.250 | 5.496 (4.759)* |
| H2  | 3.875   | 4.007   | 3.437 | 3.504 | 4.380 | 3.308       | 3.840   | 3.982   | 3.433 | 3.505 | 4.372 | 3.316 (3.109)* |
| H3  | 4.290   | 3.854   | 3.749 | 4.427 | 4.230 | 3.710       | 4.171   | 3.827   | 3.753 | 4.431 | 4.226 | 3.751 |
| H4  | 5.878   | 3.911   | 3.855 | 4.019 | 4.182 | 3.834       | 4.122   | 3.782   | 3.852 | 4.019 | 4.196 | 3.830 |
| H5  | 4.267   | 3.818   | 4.194 | 4.857 | 4.019 | 4.843       | 4.070   | 4.358   | 4.198 | 4.822 | 4.169 |
| H6a | 4.493   | 4.536   | 4.49  | 4.387 | 4.536 | 4.473       | 4.316   | 4.435   |
| H6b | 4.246   | 4.316   | 4.25  | 4.265 | 4.316 | 4.345       |

* a indicates chemical shift of the monosaccharide beta configuration.
* ND means not determined.

**TABLE 2**
Carbon chemical shifts (in ppm) of dodecasaccharide measured at 900 MHz, 306 K in phosphate buffer 10 mM, pH 7.4, and 0.5 M NaCl

| AGA*IA sequence 1 | AGA*IA sequence 2 |
|-------------------|-------------------|
| ΔU                | $^{3}J_{HH}$      | C1  | C2  | C3  | C4  | C5  | C6  |
| H1  | 103.84  | 99.75  | 103.94 | 99.11 | 102.25 | 99.89 | 104.87 | 99.89 | 103.94 | 99.11 | 102.25 | 94.03 |
| H2  | 73.25   | 56.32  | 76.34 | 59.50 | 79.27 | 56.74 | 71.41  | 56.74 | 76.34 | 59.50 | 79.80 | 60.80 |
| H3  | 69.65   | 72.13  | 79.24 | 79.15 | 72.76 | 72.52 | 70.52  | 72.43 | 79.24 | 79.15 | 72.85 | 72.43 |
| H4  | 110.55  | 80.87  | 79.40 | 75.79 | 78.65 | 80.03 | 77.50  | 80.03 | 79.40 | 75.79 | 78.79 | 80.03 |
| H5  | 69.12   | 68.85  | 68.8  | 68.8  | 68.8  | 68.8  | 69.12  | 68.8  | 69.12  | 68.8  | 69.12  | 69.68 |

**TABLE 3**
Dissociation constants ($K_{d}$) in $\mu$m

| [NaCl] | 0.1 M | 0.25 M | 0.5 M |
|--------|-------|--------|-------|
| Dodecasaccharide | 0.0099 | 0.086 | 1.23 |
| Fondaparinux | 0.0207 | 0.279 | 3.47 |

**FIGURE 4.** Superimposition of 900 MHz TOCSY (black) and NOESY (red) spectra of the dodecasaccharide. H1 I2S1 – H4 ANAc,6S1 and H1 I2S2 – H4 ANAc,6S2 inter-glycosidic NOEs are magnified in the top right side of the figure.
The $K_d$ value was obtained by monitoring the enhancement of intrinsic fluorescence of the serpin upon its reaction with increasing concentrations of each products, using the procedure previously described (15). Binding of the dodecasaccharide to AT was investigated in the presence of 0.1, 0.25, and 0.5 M NaCl yielding $K_d$ values of 0.0099, 0.086, and 1.23 $\mu$M, respectively. The $K_d$ values obtained for fondaparinux were very similar to those previously published (14).

Depending on the salt concentration, the dodecasaccharide had 2.1 to 3.2 times higher affinity for AT than fondaparinux. These results are surprising in view of current knowledge. Indeed, only a single affinity site would have been expected to bind with the antithrombin, and therefore the dodecasaccharide should have exhibited the same affinity as fondaparinux. However, our data imply that either the two affinity sites bind to AT or that just one site binds, and the second site reinforces the interaction.

**Anti-Xa Activity**—Results indicate a comparable anti-FXa activity on weight basis between the dodecasaccharide and the pentasaccharide fondaparinux (Table 4). However, on a molar basis, which is more representative of the phenomenon taking place at the molecular level, the dodecasaccharide is about twice as active as the fondaparinux reference. Interestingly, this is in agreement with the dodecasaccharide/fondaparinux molar mass ratio (respective molecular masses of fondaparinux and of dodecasaccharide in the form of sodium salts, 1728 and 3666 g/mol).

**Interaction Study between AT and Dodecasaccharide**—The comparison between free dodecasaccharide and the dodecasaccharide-AT complex proton spectra is shown in Fig. 5. Both high and low field protein-induced shifts were observed in the NMR spectra. Together with the increased linewidth, arising from the higher correlation time induced by the protein binding, the observed shifts indicate interaction between the dodecasaccharide and AT. Notably, the anomic signal of A*1 and H5 of I2S1 residues belonging to the nonreducing pentasaccharide show the larger shifts and line width increase, indicating stronger involvement in the binding of the corresponding residues. Evidence of the intermolecular interaction was also supported by the increased NOE magnitudes induced by the protein (Table 5). Particular attention is drawn to the H5-H2 NOE of the iduronate residues. This NOE contact is the marker of the $^{2}S_{0}$ conformation, since it cannot take place in the pure $^{1}C_{4}$ conformer (23). The ratio between H5-H2/H5-H4 NOE magnitudes in the absence of AT is around 0.4 for all iduronate moieties, compatible with an equilibrium between $^{1}C_{4}$ and $^{2}S_{0}$ conformation. In the presence of AT, the H5-H2/H5-H4 NOE ratio never reaches a value close to 1.0, typical of the pure $^{2}S_{0}$ conformation. This is in contrast to what was observed for the pentasaccharide (24) and other AGA*IA-containing oligosaccharides so far described, where the AT-binding surface forces I2S to assume the $^{2}S_{0}$ conformation (14–17). Assuming that the dodecasaccharide interacts with AT with a single pentasaccha-

**TABLE 4**

| Human plasma | Tris buffer |
|--------------|-------------|
| Anti-FXa activities in values by mass | IU/mg·IU/µmol | IU/mg·IU/µmol |
| Dodecasaccharide | 986.5–3618 | 890.5–3265 |
| Fondaparinux | 868.3–1500 | 856.5–1480 |
| Enoxaparin | 125.0 | 103.0 |

**FIGURE 5.** Anomeric region of the 900 MHz proton spectrum of the dodecasaccharide in the absence (a) and presence of AT (b). Shifts induced by protein of A*1, A*2, and I5S1 are shown.
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The inter-glycosidic NOE magnitude ratios between 5. However, the simultaneous binding of dodecasaccharide with two AT molecules cannot, at least in principle, be excluded. The inter-glycosidic NOE magnitude ratios between H1 \(\text{ANAc,6S} \rightarrow\) H1-4 H1-H6 (ratio 1) and H1-4 H1-H6 \(\text{ANAc,6S} \rightarrow\) H1-4 H1-H6 \(\text{ANAc,6S} \rightarrow\) (ratio 2) in the absence and presence of AT does not change significantly, indicating that these geometries are not influenced by the protein binding (Table 5). Similar results have been obtained in previous studies on octasaccharides having sequences comparable with the reducing and nonreducing parts of the dodecasaccharide (Fig. 1, a and c) and showing ANS \text{,6S} \rightarrow\) H1-H6 (5.8) 6.0 (6.4) 20.8 (8.6) 41.9

\[
\text{Table 5}
\]

Inter-residue and intra-residue experimental NOEs (in parentheses) and transferred NOEs of dodecasaccharide and dodecasaccharide-AT complexes, respectively

Spectra were measured at 600 MHz, 33 °C, in phosphate buffer (0.5 M NaCl) at five mixing times (150, 200, 300, and 400 ms). ND means not determined.

**Proton pair**

**NOE**

| Inter-residue | H1-H4 | 150 ms | 200 ms | 300 ms | 400 ms |
|---------------|-------|--------|--------|--------|--------|
| Inter-residue | H1-H5 | 1.3 (nd) | 15.3 | 41.3 | 57.1 |
| Inter-residue | H1-H6 | 1.3 (nd) | 15.3 | 41.3 | 57.1 |

**Intra-residue**

| Intra-residue | H1-H2 | 1.2 (nd) | 12.6 (4.0) | 18.0 |
| Intra-residue | H1-H3 | 1.2 (nd) | 12.6 (4.0) | 18.0 |

**Table 6**

Oligosaccharide glycosidic torsional angle pairs \(\psi/\omega\) (modeling description) of dodecasaccharide (dodeca) interacting with AT through the nonreducing \(\text{AGA*IA} \rightarrow\) and reducing \(\text{AGA*IA} \rightarrow\) (1st and 3rd column), octasaccharide (octa)-AT complexes obtained from octasaccharide 2 and 3 (2nd and 4th column), and \(\text{AT-D}_{2}\)-dodecasaccharide model (5th column)

| Glycosidic bond | AT/\(\text{AGA*IA} \rightarrow\)-dodeca | AT/octa-2 (15) | AT/\(\text{AGA*IA} \rightarrow\)-dodeca | AT/octa-3 (15) | \(\text{AT-D}_{2}\)-dodeca |
|----------------|--------------------------------|----------------|----------------|----------------|----------------|
| \(\Delta U\) | 57/9 | 55/1 | 48/0 | 70/3 |
| \(\text{ANAc,6S} \rightarrow\) \(\text{G}^{1}\) | -51/30 | -37/45 | -53/29 | -41/31 | -40/1 |
| \(\text{G}^{1} \rightarrow\) \(\text{ANAc,6S} \rightarrow\) \(\text{G}^{1}\) | -56/38 | -46/38 | -61/53 | -59/47 | 49/0 |
| \(\text{I}_{4} \rightarrow\) \(\text{ANAc,6S} \rightarrow\) \(\text{G}^{1}\) | 46/1 | 48/4 | 42/15 | -53/44 | -40/32 |
| \(\text{ANAc,6S} \rightarrow\) \(\text{I}_{4}\) | -52/38 | -38/32 | 33/10 | 49/2 | 47/32 |
| \(\text{I}_{4} \rightarrow\) \(\text{ANAc,6S} \rightarrow\) \(\text{G}^{2}\) | -35/14 | 46/3 | -45/18 | -45/29 | -45/18 |
| \(\text{G}^{2} \rightarrow\) \(\text{ANAc,6S} \rightarrow\) \(\text{G}^{2}\) | 50/2 | -52/37 | -58/52 | -52/33 | 49/4 |
| \(\text{ANAc,6S} \rightarrow\) \(\text{I}_{4}\) | -37/26 | 48/6 | 51/23 | 49/4 |
Figure 6. 800 MHz $^1$H NMR spectrum (a) and STD spectrum (b) of dodecasaccharide-AT complex.

Figure 7. Structures of 1:1 dodecasaccharide-AT assemblies. Dodecasaccharide interacting with nonreducing AGA*IA$^1$ (a) and with the reducing AGA*IA$^2$ (b).
responding to a retention time (RT) of nearly 101 min of LC separation provides the protein molecular weight ($M_r$ 57,877), in agreement with the theoretical AT mass value (Fig. 8A). The LC SEC/MS analysis of a mixture of AT and AGA*IA pentasaccharides at a 1:1 molar ratio produces the same chromatographic separation (consistent with the low SEC resolution), but the higher mass resolution and mass accuracy provided by the employed ESI-TOF instrument allow two different signal distributions to be recorded. The molecular weight calculated by deconvolution of the most intense signals corresponds to the protein-pentasaccharide complex (Fig. 8B): $M_r = 59,383 = (57,877 + 1506)$, where $M_r$ (AT) = 57,877 and $M_r$ (AGA*IA) = 1506.

A further confirmation of these results was established by running the same AT/AGA*IA 1:1 solution using denaturing experimental conditions, able to destabilize noncovalent interactions. More specifically, the ESI interface polarity was switched to negative mode, and a formic acid solution was introduced from a syringe pump (see “Experimental Procedures”) after the SEC separation and before the ionization source, allowing the weakly bound oligosaccharide to be dissociated from the interacting protein before detection. The mass spectrum shows the pentasaccharide molecular weight ($M_r$ 1506) and the loss of sulfate groups as usually observed for this type of compound in acidic conditions (Fig. 8C). The optimized LC-SEC/ESI-TOF method was then applied to the complex

![Figure 8](http://www.jbc.org/)

**FIGURE 8.** Evaluation of AT-AGA*IA interaction (1:1 molar ratio solution). A, LC SEC/UV profile at 280 nm (magenta line) and LC SEC/ESI-TOF MS profile (brown line). B, ESI-TOF spectrum (positive mode and normal conditions) corresponding to the AT-AGA*IA complex (RT range: 99.0–100.4 min; calculated $M_r = 59,383$). C, ESI-TOF spectrum (negative mode and denaturing conditions) of AGA*IA (RT range: 97.7–101.1 min; calculated $M_r = 1507$). mAU, absorbance unit from UV-visible spectroscopy.
between AT and the dodecasaccharide. Results for the AT-dodecasaccharide complex, run both in normal and denaturing conditions, are reported in Fig. 9. The lower peak at \( R_t = 82 \) min, attributable to a protein, does not produce any mass information as observed for the AT-pentasaccharide complex. The evidence of a 1:1 noncovalent complex between the AT protein and the dodecasaccharide was proven showing the corresponding peak with \( M_r = 61,118 \). The mass difference between the complex and the free AT corresponds to the molecular weight of the interacting oligosaccharide (\( M_r = 3300 \)). The same mass was also measured by negative mass spectrum in denaturing conditions (Fig. 9C).

**DISCUSSION**

Whereas natural variants of the AT-binding pentasaccharide (i.e. N-sulfation instead of N-acetylation of the first amino sugar residue and 6-O-desulfation of the 3-O-sulfated residue) have been described (25), the location of the pentasaccharide along the heparin chain is still uncertain. Although an early study suggested that this domain was located prevalently toward the nonreducing end of the molecule (26), other studies suggested a more random distribution (27). In recent studies by our groups, the presence of the pentasaccharide in both sulfated and unsulfated sequences was shown, suggesting a more random distribution of the pentasaccharide along the heparin chain (14). The different order of elution on the AT affinity column of an octasaccharide mixture suggested the active role of the flanking residue in the binding of the pentasaccharide sequence with AT (15). Moreover, structural modifications within the AGA*IA sequence, originating with the process used to depolymerize the polysaccharide, such as the presence of the 1,6-anhydrohexasamine residue at the reducing end or naturally present in the parent heparin, such as a pentasaccharide with two A* residues, have a strong influence in the AT binding properties of these oligosaccharides (16, 17). The possibility of heparin chains with high AT-binding site density was suggested for high molecular weight fractions of porcine mucosal heparins (10), clam heparins (27), “macromolecular” rat skin heparins (28), and high affinity low molecular weight heparins (11). However, two active pentasaccharides in a row were never reported. It should be noted that the difficulties of isolating and purifying long heparin oligosaccharides and the fact that both enzymatic and chemical depolymerization procedures were not sufficiently selective to produce short chains containing this partic-

![FIGURE 9. Evaluation of AT-dodecasaccharide interaction (1:1 molar ratio). A, LC SEC/UV profile at 280 nm (blue line) and LC SEC/ESI-TOF MS profile (red line). B, ESI-TOF spectrum (positive mode and normal conditions) corresponding to the AT-dodecasaccharide complex (RT range: 91.3–95.0 min; calculated \( M_r = 61,118 \)). C, ESI-TOF spectrum (negative mode and denaturing conditions) of dodecasaccharide (RT range: 91.6–94.8 min; calculated \( M_r = 3300 \)). mAU, absorbance unit from UV-visible spectroscopy.](http://www.jbc.org/Downloaded_fromhp://www.jbc.org/gi1)
ular dodecasaccharide containing two active sites could explain this. For example, the enzymatic depolymerization procedure with heparinase I depolymerizes the pentasaccharide sequence at the level of A* and generates truncated and inactive sequences (29), which is likely the reason why such sequences were not isolated by this methodology (21). The possible presence of more than one pentasaccharide sequence in a given heparin chain was supported by data obtained by fluorescence spectroscopy on the stoichiometry of the interaction of AT with active high molecular weight heparin fractions (10). This finding was confirmed by quantification, by NMR spectroscopy, of the glucuronic acid residue linked to A* (G-A)3. This disaccharide, contained only in the pentasaccharide sequence and considered the real marker of the AT-binding sequence, was found to be present in high affinity fractions of LMWHs (i.e. dalteparin and enoxaparin), with contents slightly higher than those corresponding to one disaccharide per chain (11).

The isolation of a dodecasaccharide containing two contiguous AT-binding sequences was only possible due to the particular alkaline β-eliminative cleavage method used to prepare the ULMWH semuloparin (see under “Experimental Procedures”). The combined use of phosphazene base and aprotic solvent in the depolymerization process protected the AT binding region from cleavage, allowing isolation of longer AT-binding oligosaccharides (19). Moreover, as the two pentasaccharide sequences within the dodecasaccharide are resistant to phosphazene depolymerization, they accumulate in the dodecasaccharide fraction of semuloparin. As can be seen in the chromatogram (Fig. 2), dodecasaccharide is one of the most abundant compounds in this fraction, although it was not detected in other LMWHs obtained by using different β-elimination procedures. The higher affinity of the dodecasaccharide for AT compared with fondaparinux is combined with a higher anti-FXa activity, both in agreement with the presence of two AT-binding sequences. The possibility of the dodecasaccharide binding AT in two different molecular assemblies enhances the probability of the binding and consequently the affinity to the protein. STD experiments confirmed that both pentasaccharide sequences interact with AT, even though the binding with the nonreducing pentasaccharide (AGA*IA)1 is slightly preferred to that in which the reducing AGA*IA2 binds to the protein. Notably, these data are in agreement with the slightly higher affinity found for the octasaccharide with a structure identical to the nonreducing side of dodecasaccharide (Fig. 1c), with respect to that of the octasaccharide corresponding to the reducing part (Fig. 1a) (15). In addition to these results, the unexpected conformational equilibrium in the bound state between Ic4 and 2S0 forms of both I25 and I2S residues also suggested the simultaneous presence of two dodecasaccharide/AT assemblies.

However, a “sandwich” assembly in which the ligand is located between two protein molecules, similarly to what already observed in the complex between heparin and FGF1 (30), cannot be excluded. Whereas LC-MS data did not detect the 2:1 complex, STD results are compatible with a possible contribution of AT1-dodecasaccharide assembly. All these data support the idea that a second AT-binding sequence contributes to reinforcement of the binding with AT, through both the 1:1 interactions and the possible 2:1 tertiary complex, both of them being present at the equilibrium.

The discovery of this dodecasaccharide confirms the extreme heterogeneity and structural complexity of heparin, extending the action of enzymes involved in the last step of the biosynthetic pathway (8) to 3-O-sulfation of two contiguous AGAIA sequences. There are different hypotheses on the abundance and distribution of the AT-binding site along the heparin chains. Whereas some authors envisage a limited access of polysaccharide to membrane-bound enzymes (i.e. 3-O-sulfotransferase) in the Golgi apparatus, others suggest that the distribution of recognition sites for the 3-O-sulfotransferase may control the synthesis of the AT-binding site (28, 31). Differences in structure and specificity of different 3-O-sulfotransferases, each of them exhibiting unique substrate recognition properties and distinct functional roles, may also influence the distribution of the AT-binding site in both physiological and pathophysiological conditions (9, 32). At the present state of knowledge on the actual physiological function of heparin (26), the significance of biosynthesis of two contiguous active sites for AT remains elusive.

The occurrence of more than one AT-binding sequence in a specific oligosaccharide was described for the first time in this work, and this finding was made possible by the peculiar chemo-selective depolymerization procedure and original separation techniques used. Other depolymerization methods to prepare LMWHs and ULMWHs can affect the structure and the distribution of the active sequences along the originated fragments, without preserving the complex multiple AT-binding sequences. Knowledge of the detailed structure of heparin may help in designing new LMWHs and ULMWHs as potential drugs in different therapeutic fields, and the dodecasaccharide described in this study is a particularly attractive target for chemical and/or chemo-enzymatic synthesis of a novel antithrombotic drug.

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