Glycoconj J (2014) 31:409–416
DOI 10.1007/s10719-014-9543-9

Antithrombin-binding oligosaccharides: structural diversities in a unique function?

Marco Guerrini · Pierre A. J. Mourier ·
Giangiacomo Torri · Christian Viskov

Abstract Heparin-antithrombin interaction is one of the most documented examples of heparin/protein complexes. The specific heparin sequence responsible for the binding corresponds to a pentasaccharide sequence with an internal 3-*O*-sulfated glucosamine residue. Moreover, the position of the pentasaccharide along the chain as well as the structure of the neighbor units affects the affinity to antithrombin. The development of separation and purification techniques, in conjunction with physico-chemical approaches (mostly NMR), allowed to characterize several structural variants of antithrombin-binding oligosaccharides, both in the free state and in complex with antithrombin. The article provides an overview of the studies that lead to the elucidation of the mechanism of interaction as well as acquiring new knowledge in heparin biosynthesis.

Keywords Antithrombin · Heparin · Oligosaccharides · NMR · Protein-ligand interaction

Introduction

Heparin (UFH) and low-molecular-weight heparins (LMWHs) are the most common anticoagulant and antithrombotic drugs used in medicine [1]. Heparin and its low molecular weight versions are composed by linear polysaccharidic chains constituted by alternating disaccharide sequences of either L-iduronic acid (IdoA) or D-glucuronic acid (GlcA), and glucosamine (GlcN). The GlcN residues can be *N*-sulfated (GlcNS) or *N*-acetylated (GlcNAc) at position 2, *O*-sulfated at position 6 (GlcN6S), and more rarely, also at position 3 (GlcNS,3S, designated A*). The uronic acid residues, IdoA and more rarely GlcA, can be *O*-sulfated at position 2 (IdoA2S/GlcA2S). LMWHs can present further structural heterogeneity due to the presence of different reducing and nonreducing residues at the site of cleavage, characteristic of each depolymerization procedure [2]. Although the dependence of the chain sulfation, degree, and distribution related to the animal and organ source is known, the exact sequence of each heparin polymer chains is still not easy to fully disclose [3, 4]. The activity of heparin is principally based on the binding and activation of antithrombin (AT), the major heparin cofactor in the inhibition of several serine proteases of the coagulation system, particularly factors Xa, IXa, and IIa (thrombin) [5]. On the other hand, LMWHs exert their antithrombotic and anticoagulant activities principally via factor-Xa enzyme, whereas their inhibition of factor-IIa is considerably reduced, since it needs the formation of an AT-thrombin-heparin ternary complex involving oligosaccharide chains of at least 18 residues containing AGA*IA (defined later) toward the nonreducing end [6]. Consequently, the anti-factor-Xa/anti-factor-IIa ratio, defined as 1 for UFH, increases up to 4 for LMWH, with a decreasing risk of bleeding and heparin-induced thrombocytopenia [7].

The mechanism of interaction of heparin and LMWHs with AT is one of the best studied examples of heparin/protein complexes [8, 9], representing currently the unique evidence of a highly selective recognition between a glycosaminoglycan and a protein. The specific heparin sequence responsible for the binding corresponds to the pentasaccharide $A_{NAc,6S}$-G-$A_{NS,3,6S}$-I_{2S}-$A_{NS,6S}$ [AGA*IA] that, inducing a conformational change in AT, enhances by several hundred fold the rate at which serine proteinases can be inhibited. The interaction of the pentasaccharide is mainly governed by specific electrostatic interactions between its sulfate groups and positively charged amino acids of the heparin AT-binding [ATB] region. The molecular basis of the AT-pentasaccharide interaction was

M. Guerrini (✉) · G. Torri
Istituto di Ricerche Chimiche e Biochimiche "G. Ronzoni", via G. Colombo 81, 20133 Milan, Italy
e-mail: guerrini@ronzoni.it

P. A. J. Mourier · C. Viskov
Sanofi, 13 Quai Jules Guesde, 94403 Vitry sur Seine, France

first revealed by crystal structure studies [10, 11] using a synthetic high-affinity variant of the natural pentasaccharide, known as idraparinux (compound 1, Table 1), differing in the pattern of sulfation and full *O*-methylation (Table 1) [12]. The availability of the synthetic pentasaccharide Fondaparinux (compound 2, Table 1, corresponding to the α-methyl-glycoside of *N*-deacetylated-*N*-sulfated AGA*IA) closer to the natural ATB sequence, contributed in defining the interaction at molecular level between AT and one of the main naturally occurring pentasaccharides in porcine mucosal heparin [13, 14]. All studies indicated that protein-specific interactions involved mainly Arg46, Arg47, Lys114, Lys125, and Arg129. Such residues interact with four sulfates and two carboxylate groups of the pentasaccharide (Fig. 1), among which the 3-*O*-sulfate group of the internal glucosamine residue is the most important for the antithrombin activation. Actually, its interaction with Lys114 favors the initial recognition of AT by the $A_{NAc,6S}$-G-$A_{NS,3,6S}$trisaccharide and the consecutive binding of the reducing -IdoA2S-GlcNS,6S disaccharide [9, 15, 16].

Structural ATB site variants have been isolated from porcine and bovine heparin [17]. Their characterization showed that the glucosamine at the nonreducing end can be *N*-sulfated instead of *N*-acetylated and that the internal 3-*O*-sulfated glucosamine can be 6-*O*-desulfated. A consistent number of heparin fragments containing the ATB sequence AGA*IA, endowed with high affinity for AT, have been identified [18, 19]. Advances in analytical and separation methods have also 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 [20–22]. Chemo enzymatic synthesis, allowing to generate different AT-oligosaccharides, recently opened new perspectives in the design of "tailored" homogeneous very-low-molecular-weight heparins with an accurate prediction of their anti-Xa and anti-IIa activities [23, 24]. The detailed study of the interaction between AT and AGA*IA-containing oligosaccharides became a very important challenge in the future development of this class of drugs, particularly in understanding the role of the position of the AGA*IA sequence along the chain as well as the function of its extensions toward the reducing and nonreducing terminals.

Table 1 Heparin sequences studied in complexes with AT (1–11) and the octadecasaccharide containing three ATB sequences (12)

1. (fully *O*-methylated)Glc2,6S-GlcA-Glc2,3,6S-IdoA2S-Glc2,3,6S[10]
2. GlcNS,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S-OMe[14]
3. GlcNS,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S-IdoA2S-GlcNS,6S[49]
4. ΔU-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S-IdoA2S-GlcNS,6S[31]
5. ΔU-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S-IdoA-GlcNAc,6S[31]
6. ΔU2S-GlcNS,6S-IdoA-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S[31]
7. ΔU2S-GlcNS,6S-GlcA-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S[31]
8. ΔU2S-GlcNS,6S-IdoA-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-1,6anGlcNS[44]
9. ΔU2S-GlcNS,6S-IdoA-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-1,6anManNS[44]
10. ΔU-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,3,6S-IdoA2S-GlcNS,6S[45]
11. ΔU-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S-IdoA-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S[46]
12. ΔU-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S-IdoA-GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S-IdoA- GlcNAc,6S-GlcA-GlcNS,3,6S-IdoA2S-GlcNS,6S[55]

The present review illustrates the main mechanisms governing the interaction of heparin fragments with AT, focusing on the role of both the reducing and nonreducing end extensions in affecting the protein binding. Together with X-ray analysis [10, 11, 14], isothermal titration calorimetry [25], and surface plasmon resonance [26], the NMR-based approach in the study of the AT binding affinity of different AGA*IA-containing oligosaccharides provided new insights for understanding the mechanism of these interactions.

AT binding sequences

Structural variability of heparin AT binding site was considered several years ago, after the isolation and characterization of oligosaccharides obtained by partial enzymatic depolymerization of heparins from different species and tissue sources [17, 27, 28]. For example, substitution of the acetyl with a sulfate group at the *N*-position of the nonreducing glucosamine of the pentasaccharide increased the AT affinity by two folds, whereas lack of 6-*O*-sulfation in the 3-*O* sulfated glucosamine did not significantly affect the binding affinity [29]. However, the simplistic idea that the AT binding depends exclusively on the pentasaccharide, independently of the structure of the whole oligosaccharide, was recently overcome by several studies on HS and heparin oligosaccharides, which demonstrated the active role in the binding to and activation of antithrombin of residues extending the active sequence toward both its reducing and nonreducing sides [30, 31].

This aspect is of primary relevance to the wide clinical use of LMWHs, for which different depolymerization processes generate various structural variants, including cleavage within the pentasaccharide sequence that strongly affects the AT binding properties of the LMWHs. Particularly, this aspect has to be taken into the account in the preparation of generic drugs to guarantee the biosimilarity between the branded and generic products [32]. However, the lack of sufficient amounts of homogeneous oligosaccharides containing an AT binding site has impaired the detailed investigation of AT-heparin

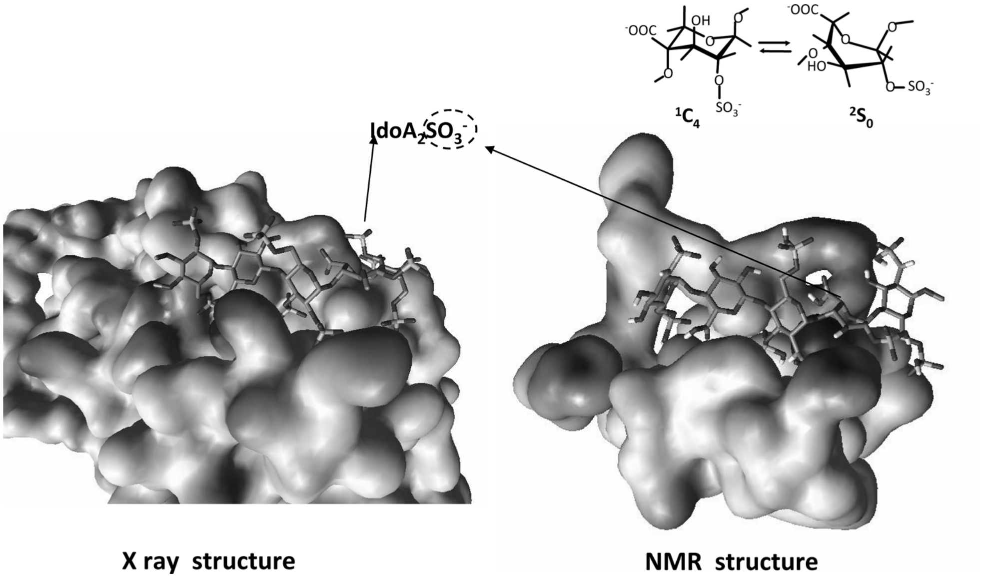

Fig. 1 Crystal structure and NMR structure of the AT-pentasaccharide complexes [10, 13]. The IdoA2S, having the sulfate group oriented outside the AT-binding site, is in $^2S_0$ conformation in both structures

interactions for several years, allowing only studies with synthetic models. The combination of orthogonal separation techniques, including gel permeation chromatography, HPLC, and AT affinity chromatography on semi-preparative scale, made possible to isolate from LMWHs and ULMWHs pure AT-binding oligosaccharides having a size from hexa- up to octadeca-saccharides, and detailed studies of the influence of structural variability of chain on the AT binding and the related anti-Xa activity [20]. The sequences of the AT-binding oligosaccharide variants so far isolated and studied in complex with AT are shown in Table 1.

Iduronic acid conformational properties

The allosteric conformational changes in AT induced by binding the pentasaccharide promote the extension of the helix D of AT, allowing the expulsion of the N-terminal portion of the reactive center loop from β-sheet A and so render the P1 arginine more accessible to target proteinase [8, 33]. Previous studies indicated that the nonreducing end trisaccharide$A_{NAc,6S}$-G-$A_{NS,3,6S}$- is crucial for the initial binding, while the disaccharide -I_{2S}-$A_{NS,6S}$ at the reducing end is responsible for the stabilization of the high affinity, activated conformation [34]. This two-step interaction mechanism is assisted by the conformational "plasticity" of L-iduronic acid residue, a characteristic that, besides facilitating the docking of the oligosaccharide to AT, favors the interaction of iduronic acid containing glycosaminoglycans with other heparin-binding proteins [35].

The iduronic acid residue in aqueous solution exists in equilibrium mostly between the chair $^1C_4$ and the skew $^2S_0$ conformation [36]. The relative population of these forms in solution depends on the presence of 2-*O*-sulfated group and the structure of the neighboring units. In particular, in the trisulfated disaccharide domains of heparin, the ratio $^1C_4/^2S_0$ of IdoA2S is approximately 60:40, whereas in the pentasaccharide sequence, the corresponding ratio is approximately reversed (i.e., 40:60, $^1C_4/^2S_0$) (Fig. 1) [37].

Whereas the overall geometry of the helical chains of heparin remains similar for the two IdoA conformers, the spacing between sulfate groups on adjacent residues is significantly different for the two local conformations, clustering the sulfate groups in different orientation along the chain. Such extra degree of freedom, unique of IdoA-containing oligosaccharides, together with rotational freedom at the glycosidic linkages, can contribute to a more favorable fitting of the ligand to the binding sites of a protein receptor [38]. Analysis of a large number of heparin oligosaccharide-protein complexes has led to the general concept that the linear propagation of GAG chains is interrupted at the binding site level by "kinks" associated with a conformation of an iduronic acid different from that prevailing in the portion of chain not involved in the binding [39]. It is worth noting that in case of interaction with AT, although the sulfate group of IdoA has no direct electrostatic interactions with any residue of the AT binding site, it facilitates the shifting toward the appropriate conformation of the IdoA residue ($^2S_0$), enhancing the contacts between the AGA*IA and basic amino acid residues in the ATB region (Fig. 1). This behavior was observed for all AGA*IA-containing oligosaccharides bound to AT so far described (Table 1).

Experimental approaches

The first data recapitulating the binding of a pentasaccharide to AT were obtained from analysis of X-ray diffraction patterns in the solid state [10]. However, in almost all studies, the geometry of the pentasaccharide sequence was not sufficiently detailed and information regarding the bound conformation of sugar moieties can be retrieved only from published crystal co-ordinates. Despite some structural differences of the AGA*IA sequences contained in the synthetic oligosaccharides co-crystalized with AT, both glycosidic geometries and proton-proton inter-residue distances appeared very similar among the different complexes (Table 2). However, since the electron density map of the oligosaccharide moiety is not often sufficient to precisely establish the coordinates of the sugar atoms, the structure is usually built based on the published heparin coordinates [40]. For instance, the structure of the complex between a heparin decasaccharide and FGF1 showed only the central hexasaccharide moiety, as the low resolution limited the detection only to those residues directly involved in the binding [41].

In the last two decades, the use of NMR spectroscopy in the investigation of transiently forming complexes under near-physiological conditions resulted in an increasing number of papers. Despite describing various carbohydrate/protein interactions, only very few studies on sulfated glycosaminoglycans and proteins were published [31, 42–47]. To our knowledge, the early one was the study of the complex between the synthetic pentasaccharide Fondaparinux and AT [13], in which authors confirmed that AT stabilizes the $^2S_0$ conformation of IdoA2S, observing a variation of glycosidic linkage geometries with respect to the free ligand in solution (Fig. 1). Noteworthy, this study was also the first example of application of transferred NOE experiments to glycosaminoglycans and the subsequent interpretation of the results with full relaxation and conformational exchange matrix analysis [48].

Further studies were focused on the structural role and molecular conformation of different disaccharide extensions on both sides of the AGA*IA sequence in modulating the AT affinity (Table 3) [31, 49]. When bound to AT, transferred NOE experiments indicated that the conformation of IdoA2S residue inside the pentasaccharide displayed always the $^2S_0$ conformation. However, IdoA and IdoA2S residues located immediately before or after the AGA*IA sequence assumed either the $^1C_4$ or the $^2S_0$ conformation independently of the prevalent form they have in solution in absence of the protein. For instance, the IdoA2S and IdoA located after the pentasaccharide, respectively, in the octasaccharides 4 and 5, both displayed the $^1C_4$ conformation when bound to AT, while in the free state, the population of $^2S_0$ conformer for IdoA2S is significantly higher than that of IdoA. This finding suggests that the $^1C_4$ conformation of the IdoA residues located after the pentasaccharide enhances the AT affinity of the AGA*IA-containing oligosaccharides, by optimizing contacts between AGA*IA and AT. Thus, being $^1C_4$ the prevalent conformation assumed in solution by the IdoA residue nearer to the final binding conformation, it is possible to explain the higher affinity of the octasaccharide 5 with respect to the octasaccharide 4 [31]. In confirmation of this hypothesis, the energetically less favorable $^2S_0$ form assumed by IdoA located at the nonreducing side in the octasaccharide 4 in the bound state results in a further minor affinity to the protein. Notably, the substitution of IdoA with a GlcA residue in the octasaccharide 7 induces one order of magnitude increase of affinity to AT. As demonstrated by fluorescence titration experiments and interpreting the structures obtained by fitting experimental transferred NOEs with those calculated on the

Table 2 Inter-glycosidic proton-proton distances and dihedral angles of pentasaccharide moieties drown from published structures

Inter-glycosidic linkage		AT/AGA*IA	AT/Thr/AGA*IA	AT/fXa/AGA*IA	AT/AGA*IA
		X-ray structure[10]	X-ray structure[8]	X-ray structure[14]	NMR structure[13]
	Distance (nm)				
A1-G4		0.243	0.226	0.216	0.312
G1-A4*		0.229	0.215	0.203	0.226
A1*-I4		0.297	0.290	0.275	0.253
A1*-I3		0.277	0.256	0.272	0.291
I1-A4$_{red}$		0.236	0.230	0.229	0.247
	Dihedral angles				
A-G	ϕ	−31	−43	−19	−56
	ψ	−22	−21	−40	−60
G-A*	ϕ	35	36	35	40
	ψ	8	11	−2	12
A*-I	ϕ	−66	−64	−60	−32
	ψ	−35	−42	−36	−43
I-A$_{red}$	ϕ	48	53	51	45
	ψ	5	12	14	16

Table 3 Equilibrium dissociation constant Kd (μm) for the interaction of human AT heparin oligosaccharides purified from LMWH and ULMWH with respect to that of the pentasaccharide fondaparinux determined at 25 °C in 0.05 M HEPES, pH 7.4 containing 0.1 M NaCl

Saccharides	Kd (0.1 M NaCl)
4	2.1×10^{-7}
5	0.5×10^{-7}
6	1.2×10^{-7}
7	1.5×10^{-9}
8	0.9×10^{-6}
9	1.2×10^{-6}
10	1.1×10^{-9}
11	1.0×10^{-8}
Fondaparinux	2.1×10^{-8}

basis of models obtained by docking and molecular modeling procedures, the $^4C_1$ conformation of GlcA contributes to establish further nonionic interactions between hydroxyl groups of GlcA and the D-helix of AT [31].

The most versatile NMR technique to characterize protein-ligand interactions and to determine the binding epitope of the ligands is the saturation transfer difference (STD) [50]. The experiment is based on the transfer of saturation via spin diffusion from the macromolecule to the bound ligand. A reference spectrum can be also acquired without saturating the proteins, and the final saturation difference spectrum, obtained by subtracting the saturated spectrum from the reference spectrum, shows only ligand protons having the "strongest" contacts to the protein. The application of this method to AT/oligosaccharide complexes allowed identifying which residues flanking the pentasaccharide are involved in the binding. Although, as expected, the most intense STD signals were those of residues belonging to the pentasaccharide sequence, signals of residues outside the pentasaccharide could be also observed. STD signals of the uronate residue preceding the AGA*IA sequence, independently if it was IdoA or 4,5-unsaturated uronic acid residue (ΔU), were observed, indicating that this residue could also be involved in the binding (Fig. 2). This experimental evidence was supported by the significantly higher AT affinity showed by the hexasaccharide containing an additional IdoA residue at the nonreducing end with respect to the pentasaccharide [51].

Antithrombin-binding oligosaccharide variants

As mentioned before, many of the AT-binding oligosaccharides described so far have been isolated from LMWHs and ULMWHs. The type of depolymerization process applied to UFH to prepare LMWHs generates several structural variants that can affect the affinity and the resulting activity, mainly if occurring within the pentasaccharide. In case of enoxaparin, 1,6-anhydro-hexosamine residues (both D-manno and D-glucopyranosidic configurations) are generated at the reducing end of the chains during alkaline depolymerization of heparin benzyl ester [52, 53]. These unique bicyclic units were found to be present also at the reducing end of the AGA*IA sequence (octasaccharides 8 and 9 of Table 1) [44]. These octasaccharides showed reduced AT affinity of about 10- to 50-fold with respect to the octasaccharide 6 bearing the intact GlcNS,6S residue at the reducing end. As this decrease was found more important than that observed just after 6-*O*-desulfation of the terminal reducing glucosamine of the pentasaccharide [15], it was proposed that the reduced affinity can be correlated with the lower population of the skew $^2S_0$ conformer of the neighboring IdoA2S residue, induced by the presence of 1,6-anydro residue [44].

More recently, the development of new depolymerization procedures more suitable to preserve the AT-binding sequences allowed to isolate from ULMWHs new ATB sequences not previously evidenced by enzymatic procedures [54]. The existence of heparin/HS structures with an additional 3-*O*-sulfated residue appears highly plausible in view of the substrate specificities defined for various members of the 3-*O*-sulfotranferase family [22]. Moreover, compositional analysis of high-affinity fractions of LMWH suggested that some of the chains can contain more than one GlcNS,3,6S residue [21]. Recently, three oligosaccharides containing an additional GlcNS,3,6S residue have been isolated and characterized. The first is an octasaccharide containing a second GlcNS,3,6S residue at the reducing end (compound 10, Table 1), whereas the second and the third ones are a dodecasaccharide and a octadecasaccharide containing two and three pentasaccharide sequences, respectively (compounds 11 and 12, Table 1) [46, 55].

The presence of a second GlcNS,3,6S residue at the reducing end of the pentasaccharide in compound 10 increases the affinity of about 200-fold with respect to the analog octasaccharide 4 lacking in the 3-*O*-sulfated group at the reducing residue (Table 3). This higher affinity was explained by the increasing interaction of both *N*- and 3-*O*-sulfated group of the second A* with Arg^{46} and Arg^{47} as well as by the higher population of the $^2S_0$ form of the IdoA2S inside the pentasaccharide [45]. A similar increase of $^2S_0$ population of IdoA2S was previously described for a synthetic pentasaccharide bearing an extra 3-*O*-sulfo group at the reducing end residue [56].

The identification of oligosaccharides containing more than one pentasaccharide sequence, including a new one with three consecutive AT-binding pentasaccharides, confirms the extreme heterogeneity and structural complexity of heparin [46, 55]. Although the possibility of interaction with more than one AT molecule was not proved, STD experiments showed that the dodecasaccharide 11 (Table 1) can bind AT with both reducing and nonreducing end pentasaccharides, the latter being slightly preferred. This suggests the possibility

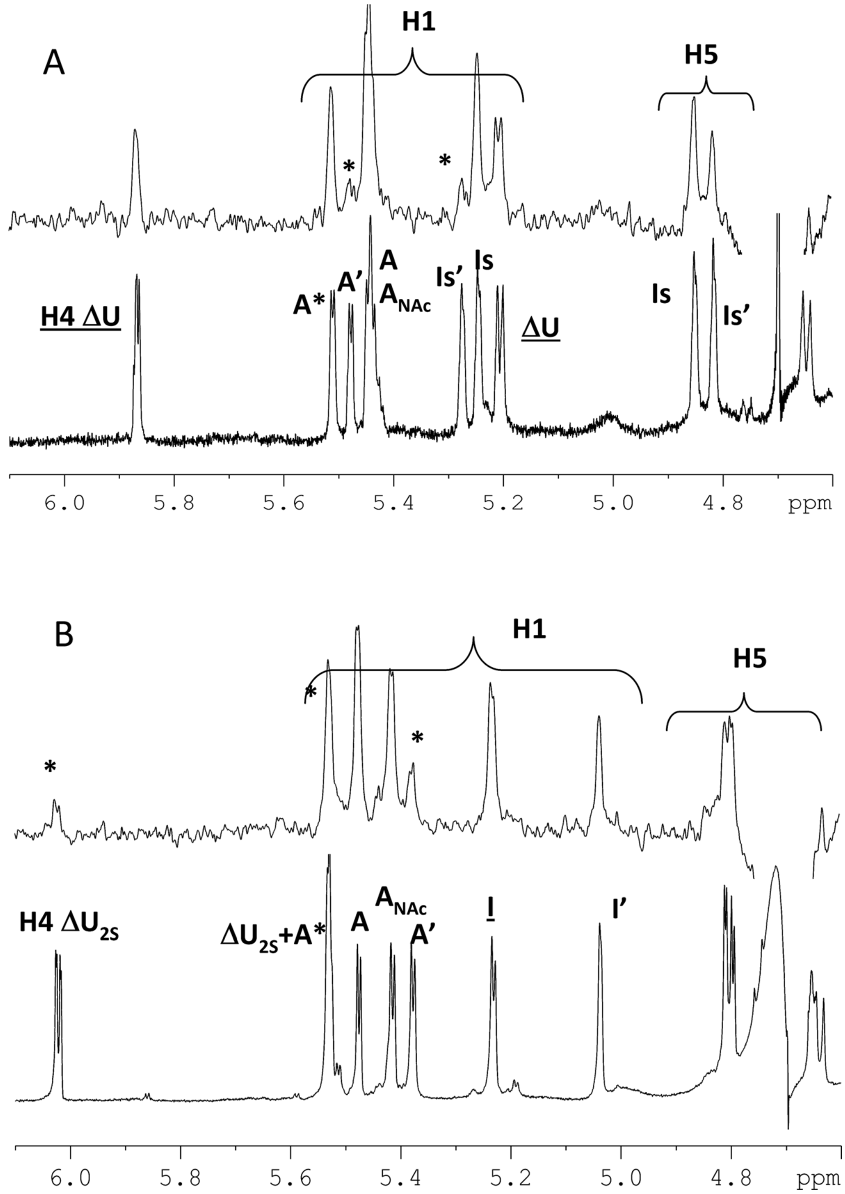

Fig. 2 Saturation transfer difference spectra of compound 4 (**a**) and compound 6 (**b**) in comparison with the corresponding reference proton spectra. Signal belonging residues not involved in the binding and those belonging to the uronate residues preceding the pentasaccharide sequences are *highlighted with asterisk* and *underlined*, respectively

that these oligosaccharides bind AT in different molecular assemblies, thus enhancing the probability of the binding and consequently the affinity to the protein. Noteworthy, the relatively low anti-FIIa activity of the octadecasaccharide, compared with that of other AGA*IA bearing heparin fractions of the same size, suggests that the multiple and contiguous assembling of AT-binding oligosaccharides decreases the possibility to form the ternary complex with thrombin.

Although the abundance and position of ATB sequences in oligosaccharides can vary according to the different glycosaminoglycans (i.e., heparan sulfate or heparin) and type of animal tissue, the significance and real physiological function of biosynthesis of contiguous active sites for AT remains elusive. The existence of seven 3-*O*-sulfotransferase enzymes, each of them exhibiting unique substrate recognition, may influence also the distribution of the GAG AT binding site in both physiological and pathological conditions [22, 57]. A major breakthrough in the study of structural motifs responsible for AT interactions should relate to more efficient separation and purification methods and new synthetic approach to obtain short chains containing biologically relevant sequences. The new chemoenzymatic method recently proposed, overcoming pure synthetic approaches, could generate a series of oligosaccharides of defined new structures at multimilligram scale, to be used both as models to further investigate the role of AT-binding oligosaccharides in other biological functions [23, 24] and as tools for identifying specific interactions with relevant proteins and enzymes. Such approach could enable the opening of new therapeutic horizons for heparin and other glycosaminoglycans.

Acknowledgments We thank Drs. Benito Casu and Giuseppe Cassinelli for their critical reading of the manuscript and helpful comments.

References

1. Lever, R., Mulloy, B., Page, C.P.: Heparin: a century of progress. Springer, Berlin (2012)
2. Guerrini, M., Guglieri, S., Naggi, A., Sasisekharan, R., Torri, G.: Low molecular weight heparins: structural differentiation by bidimensional nuclear magnetic resonance spectroscopy. Semin Thromb Hemost **33**, 478–487 (2007)
3. Casu, B., Guerrini, M., Naggi, A., Torri, G., De-Ambrosi, L., Boveri, G., Gonella, S.: Differentiation of beef and pig mucosal heparins by NMR spectroscopy. Thromb Haemost **74**, 1205–1209 (1995)
4. Mulloy, B., Gray, E., Barrowcliffe, T.W.: Characterization of unfractionated heparin: comparison of materials from the last 50 years. Thromb Haemost **84**, 1052–1056 (2000)
5. Gray, E., Hogwood, J., Mulloy, B.: The anticoagulant and antithrombotic mechanisms of heparin. In: Lever, R., Mulloy, B., Page, C.P. (eds.) Heparin: a century of progress, pp. 281–306. Springer, Berlin (2012)
6. Lane, D.A., Denton, J., Flynn, A.M., Thunberg, L., Lindahl, U.: Anticoagulant activities of heparin oligosaccharides and their neutralization by platelet factor 4. Biochem J **218**, 725–732 (1984)
7. Gray, E., Mulloy, B., Barrowcliffe, T.W.: Heparin and low-molecular-weight heparin. Thromb Haemost **99**, 807–818 (2008)
8. Huntington, J.A.: Serpin structure, function and dysfunction. J Thromb Haemost **9**(Suppl. 1), 26–34 (2011)
9. Petitou, M., Barzu, T., Herault, J.P., Herbert, J.M.: A unique trisaccharide sequence in heparin mediates the early step of antithrombin III activation. Glycobiology **7**, 323–327 (1997)
10. Jin, L., Abrahams, J.P., Skinner, R., Petitou, M., Pike, R.N., Carrell, R.W.: The anticoagulant activation of antithrombin by heparin. Proc Natl Acad Sci U S A **94**, 14683–14688 (1997)
11. McCoy, A.J., Pei, X.Y., Skinner, R., Abrahams, J.P., Carrell, R.W.: Structure of beta-antithrombin and the effect of glycosylation on antithrombin's heparin affinity and activity. J Mol Biol **326**, 823–833 (2003)
12. Petitou, M., van Boeckel, C.A.: A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? Angew Chem Int Ed Engl **43**, 3118–3133 (2004)
13. Hricovini, M., Guerrini, M., Bisio, A., Torri, G., Petitou, M., Casu, B.: Conformation of heparin pentasaccharide bound to antithrombin III. Biochem J **359**, 265–272 (2001)
14. Johnson, D.J., Li, W., Adams, T.E., Huntington, J.A.: Antithrombin-S195A factor Xa-heparin structure reveals the allosteric mechanism of antithrombin activation. EMBO J **25**, 2029–2037 (2006)
15. van Boeckel, C.A.A., Petitou, M.: The unique antithrombin III binding domain of heparin: a lead to new synthetic antithrombotics. Angew Chem Int Ed Engl **32**, 1671–1818 (1993)
16. Desai, U.R.: New antithrombin-based anticoagulants. Med Res Rev **24**, 151–181 (2004)
17. Loganathan, D.H., Wang, M., Mallis, L.M., Linhardt, R.J.: Structural variation in the antithrombin III binding site region and its occurrence in heparin from different sources. Biochemistry **29**, 4362–4368 (1990)
18. Linhardt, R.J., Gunay, N.: Production and chemical processing of low molecular weight heparins. Semin Thromb Hemost **25**, 5–16 (1999)
19. Casu, B., Torri, G.: Structural characterization of low-molecular weight heparins Semin. Thromb Hemost **25**, 17–26 (1999)
20. Mourier, P.A., Viskov, C.: Chromatographic analysis and sequencing approach of heparin oligosaccharides using cetyltrimethylammonium dynamically coated stationary phases. Anal Biochem **332**, 299–313 (2004)
21. Bisio, A., Vecchietti, D., Citterio, L., Guerrini, M., Raman, R., Bertini, S., Eisele, G., Naggi, A., Sasisekharan, R., Torri, G.: Structural features of low molecular weight heparins affecting their affinity to antithrombin. Thromb Haemost **102**, 865–873 (2009)
22. Thacker, B.E., Xu, D., Lawrence, R., Esko, J.D.: Heparan sulfate 3-O-sulfation: a rare modification in search of a function. Matrix Biol **35**, 60–72 (2013)
23. Xu, Y., Masuko, S., Takieddin, M., Xu, H., Liu, R., Jing, J., Mousa, S.A., Linhardt, R.J., Liu, J.: Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins. Science **334**, 498–501 (2011)
24. Xu, Y., Pempe, E.H., Liu, J.: Chemoenzymatic synthesis of heparin oligosaccharides with both anti-factor Xa and anti-factor IIa activities. J Biol Chem **287**, 29054–29061 (2012)
25. Fu, L., Zhang, F., Li, G., Onishi, A., Bhaskar, U., Sun, P., Linhardt, R.J.: Structure and activity of a new low-molecular-weight heparin produced by enzymatic ultrafiltration. J Pharm Sci **103**, 1375–1383 (2014)
26. Tyler-Cross, R., Sobel, M., McAdory, L.E., Harris, R.B.: Structure–function relations of antithrombin III-heparin interactions as assessed by biophysical and biological assays and molecular modeling of peptide–pentasaccharide-docked complexes. Arch Biochem Biophys **15**, 206–213 (1996)
27. Lindahl, U., Thunberg, L., Bäckstrőm, G., Riesenfeld, J., Nordling, K., Bjork, I.: Extension and structural variability of the antithrombin-binding sequence in heparin. J Biol Chem **259**, 12368–12376 (1984)
28. Toida, T., Hileman, R.E., Smith, A.E., Vlahova, P.I., Linhardt, R.J.J.: Enzymatic preparation of heparin oligosaccharides containing antithrombin III binding site. J Biol Chem **271**, 32040–32047 (1996)
29. Choay, J., Petitou, M., Lormeau, J.C., Sinaÿ, P., Casu, B., Gatti, G.: Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. Biochem Biophys Res Commun **116**, 492–499 (1983)
30. Chen, J., Jones, C.L., Liu, J.: Using an enzymatic combinatorial approach to identify anticoagulant heparan sulfate structures. Chem Biol **14**, 986–993 (2007)
31. Guerrini, M., Guglieri, S., Casu, B., Torri, G., Mourier, P., Boudier, C., Viskov, C.: 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. J Biol Chem **283**, 26662–26675 (2008)
32. Harenberg, J., Walenga, J., Torri, G., Dahl, O.E., Drouet, L., Fareed, J.J.: Update in the recommendations on biosimilar low-molecular weight heparin from the Scientific Subcommitee on Control of Anticoagulation of the International Society of Thrombosis and Haemostasis. Thromb Haemost **334**, 1421–1425 (2013)
33. Whisstock, J.C., Pike, R.N., Jin, L., Skinner, R., Pei, X.Y., Carrell, R.W., Lesk, A.M.: Conformational changes in serpins: II. The mechanism of activation of antithrombin by heparindagger. J Mol Biol **301**, 1287–1305 (2000)
34. Desai, U.R., Petitou, M., Bjőrk, I., Olson, S.T.: Mechanism of heparin activation of antithrombin. Role of individual residues of the pentasaccharide activating sequence in the recognition of native and activated states of antithrombin. J Biol Chem **273**, 7478–7487 (1998)
35. Hricovíni, M., Guerrini, M., Bisio, A., Torri, G., Naggi, A., Casu, B.: Active conformations of glycosaminoglycans.NMR determination of the conformation of heparin sequences complexed with antithrombin and fibroblast growth factors in solution. Semin Thromb Hemost **28**, 325–333 (2002)
36. Ferro, D.R., Provasoli, A., Ragazzi, M., Torri, G., Casu, B., Gatti, G., Jacquinet, J.C., Sinay, P., Petitou, M., Choay, J.: Evidence for conformational equilibrium of the sulfated l-iduronate residue in heparin

and in synthetic heparin mono- and oligosaccharides: NMR and force-field studies. J Am Chem Soc **108**, 6773–6778 (1986)

37. Ragazzi, M., Ferro, D.R., Perly, B., Sinay, P., Petitou, M., Choay, J.: Conformation of the pentasaccharide corresponding to the binding site of heparin for antithrombin III. Carbohydr Res **165**, c1–c5 (1987)
38. Casu, B., Petitou, M., Provasoli, M., Sinaÿ, P.: Conformational flexibility: a new concept for explaining binding and biological properties of iduronic acid-containing glycosaminoglycans. Trends Biochem Sci **13**, 221–225 (1988)
39. Raman, R., Sasisekharan, V., Sasisekharan, R.: Structural insights into biological roles of protein-glycosaminoglycan interactions. Chem Biol **12**, 267–277 (2005)
40. Mulloy, B., Forster, M.J., Jones, C., Davies, D.B.: N.M.R. and molecular-modelling studies of the solution conformation of heparin. Biochem J **293**, 849–858 (1993)
41. DiGabriele, A.D., Lax, I., Chen, D.I., Svahn, C.M., Jaje, M., Schlessinger, J., Hendrickson, W.A.: Structure of a heparin-linked biologically-active dimer of fibroblast growth factor. Nature **393**, 812–817 (1998)
42. Guerrini, M., Hricovini, M., Torri, G.: Interaction of heparins with fibroblast growth factors: conformational aspects. Curr Pharm Des **13**, 2045–2056 (2007)
43. Nieto, L., Canales, A., Fernández, I.S., Santillana, E., González-Corrochano, R., Redondo-Horcajo, M., Javier Cañada, F., Nieto, P., Martín-Lomas, M., Giménez-Gallego, G., Jiménez-Barbero, J.: Heparin modulates the mitogenic activity of fibroblast growth factor by inducing dimerization of its receptor. a 3D view by using NMR. Chem Biol Chem **14**, 1732–1744 (2013)
44. Guerrini, M., Elli, S., Gaudesi, D., Torri, G., Casu, B., Mourier, P., Herman, F., Boudier, C., Lorenz, M., Viskov, C.: Effects on molecular conformation and anticoagulant activities of 1,6-anhydrosugars at the reducing terminal of antithrombin-binding octasaccharides isolated from low-molecular-weight heparin enoxaparin. J Med Chem **53**, 8030–8040 (2010)
45. Guerrini, M., Elli, S., Mourier, P., Rudd, T.R., Gaudesi, D., Casu, B., Boudier, C., Torri, G., Viskov, C.: An unusual antithrombin-binding heparin octasaccharide with an additional 3-O-sulfated glucosamine in the active pentasaccharide sequence. Biochem J **449**, 343–351 (2013)
46. Viskov, C., Elli, S., Urso, E., Gaudesi, D., Mourier, P., Herman, F., Boudier, C., Casu, B., Torri, G., Guerrini, M.: Heparin dodecasaccharide containing two antithrombin-binding pentasaccharides: structural features and biological properties. J Biol Chem **288**, 25895–25907 (2013)
47. Yu, F., Roy, S., Arevalo, E., Schaeck, J., Wang, J., Holte, K., Duffner, J., Gunay, N.S., Capila, I., Kaundinya, G.V.: Characterization of heparin–protein interaction by saturation transfer difference (STD) NMR. Anal Bioanal Chem **406**, 3079–3089 (2014)
48. Moseley, H.N.B., Curto, E.V., Krishna, N.R.: Complete relaxation and conformational exchange matrix (CORCEMA) analysis of NOESY spectra of interacting systems; two-dimensional transferred NOESY. J Magn Reson **108**, 243–261 (1995)
49. Belzar, K.J., Dafforn, T.R., Petitou, M., Carrell, R.W., Huntington, J.A.: The effect of a reducing-end extension on pentasaccharidebinding by antithrombin. J Biol Chem **275**, 8733–8741 (2000)
50. Mayer, M., Meyer, B.: Characterization of ligand binding by saturation transfer difference NMR spectroscopy. Angew Chem Int Ed **38**, 1784–1788 (1999)
51. Choay, J., Lormeau, J.C., Petitou, M., Sinaÿ, P., Fareed, J.: Structural studies on a biologically active hexasaccharide obtained from heparin. Ann N Y Acad Sci **370**, 644–649 (1981)
52. Mourier, P., Viskov, C.: US Pat. 2005/0119477 A1, 2005. Chem Abstr **142**, 89363 (2005)
53. Mascellani, G., Guerrini, M., Torri, G., Liverani, L., Spelta, F., Bianchini, P.: Characterization of di- and monosulfated, unsaturated heparin disaccharides with terminal N-sulfated 1,6-anhydro- β-D-glucosamine or N-sulfated 1,6-anhydro β-D-mannosamine residues. Carbohydr Res **342**, 835–842 (2007)
54. Mourier, P., and Viskov, C.: Polysaccharides comprising two antithrombin III-binding sites, Preparation thereof and use thereof as antithrombotic medicaments. WO 2012/140580 A1 (2012)
55. Mourier, P.A.J., Guichard, O.J., Herman, F., Viskov, C.: Isolation of a pure octadecasaccharide with anti-thrombin activity from a ultralow-molecular weight heparin. Anal Biochem **453**, 7–15 (2014)
56. van Boeckel, C.A.A., van Beetz, T., Aelst, S.F.: Synthesis of a potent antithrombin activating pentasaccharide: a new heparin-like fragment containing two 3-O-sulphated glucosamines. Tetrahedron Lett **29**, 803–806 (1988)
57. Moon, A.F., Xu, Y., Woody, S.M., Krahn, J.M., Linhardt, R.J., Liu, J., Pedersen, L.C.: Dissecting the substrate recognition of 3-O-sulfotransferase for the biosynthesis of anticoagulant heparin. Proc Natl Acad Sci U S A **109**, 5265–5270 (2012)