The Antithrombin-binding Sequence in Heparin
IDENTIFICATION OF AN ESSENTIAL 6-O-SULFATE GROUP*

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An octasaccharide with high affinity for antithrombin, isolated after partial deaminative cleavage of heparin, was previously found to have an L-iduronosyl-N-acetylglucosaminyl-6-O-sulfate nonreducing terminal disaccharide unit. After digestion of this octasaccharide with α-L-iduronidase and N-acetylglucosamine-6-sulfate sulfatase, two fractions, with high and low affinity for antithrombin, respectively, were isolated by affinity chromatography on antithrombin-Sepharose. Structural analysis showed that the high affinity fraction contained intact octasaccharide, whereas the low affinity fraction consisted of the expected heptasaccharide, lacking a 6-sulfate group on the terminal N-acetylglucosamine residue. Digestion of the octasaccharide with α-L-iduronidase only yielded heptasaccharide which was identical with the low affinity species except for the presence of this 6-sulfate group. This less degraded heptasaccharide retained high affinity for antithrombin. It is concluded that the 6-sulfate group on the N-acetylglucosamine residue is of critical importance to the interaction between heparin and antithrombin.

Commercially available heparin preparations are heterogeneous in that only a fraction, generally less than half, of the molecules are capable of binding with high affinity to the protease inhibitor antithrombin (1–3). These high affinity components, which account for most of the blood anticoagulant activity of the unfractiated material, contain a specific oligosaccharide sequence which mediates the binding to the inhibitor. A tetrasaccharide structure, \( \text{IdUA-GlcNAc(6-OSO}_3)\text{-GlcUA-GlcNSO}_3(6\text{-OSO}_3) \), found to be overrepresented in heparin molecules with high affinity for antithrombin (as compared to the corresponding low affinity species) (4) could be recovered in heparin oligosaccharides isolated by affinity chromatography on antithrombin-Sepharose (5). The smallest such antithrombin-binding oligosaccharide, formed by deaminative cleavage of heparin with nitrous acid, was an octasaccharide, the nonreducing half of which (units 1–4 in Fig. 1) consisted of the tetrasaccharide sequence described (Ref. 6 and references therein). Units 7 and 8 of this octasaccharide displayed extensive structural variability and were therefore considered to fall outside the actual antithrombin-binding sequence (6). The extension of the binding region toward the nonreducing terminus was established by affinity chromatography following chemical and/or enzymatic modification of the octasaccharide (6). N-Decacylation of the N-acetylglucosamine unit 2 had no significant effect on the affinity of the octasaccharide for antithrombin, whereas removal of the entire nonreducing terminal disaccharide unit (1–2) was accompanied by a drastic loss of binding affinity. On the other hand, release of only the terminal iduronosyl unit 1 by digestion of the octasaccharide with α-L-iduronidase had no effect on the binding properties. It was therefore concluded that the actual binding region is comprised of the pentasaccharide sequence 2–6 (within brackets in Fig. 1).

Further characterization of the antithrombin-binding octasaccharide revealed a novel structural feature, a 3-O-sulfate group located at the glucosamine unit 4 (7). Since this substituent appeared to be unique to the antithrombin-binding region of the heparin molecule (8, 9), it is presumably essential to the interaction with the inhibitor. The functional roles of various other sulfate groups have been amenable to experimental evaluation. Selective N-desulfation of the octasaccharide thus indicated that the N-sulfate group of residues 4 and 6 are both required for binding to antithrombin (10). Furthermore, the loss of bioaffinity caused by removal of the nonreducing terminal disaccharide (units 1 and 2), as contrasted to the retention of affinity after release of unit 1 only, tentatively implicated the 6-sulfate group on unit 2 (6). In the present study, this conclusion was verified by selective removal of the 6-sulfate group, using a specific N-acetylglucosamine-6-sulfate sulfatase.

EXPERIMENTAL PROCEDURES

Materials—Heparin from pig intestinal mucosa (Stage 14 material) was obtained and purified as described (6).

A heparin octasaccharide with high affinity for antithrombin was isolated, reduced (unit 8) with unlabeled NaBH₄, and N-desacetylated (unit 2) by hydrazinolysis (6). The N-desacetylated octasaccharide was radiolabeled by treatment with [14C]acetic anhydride (6), yielding 2-[14C](1–8) (Fig. 1) with a specific activity of 65 × 10³ cpm of 14C/μg of uronic acid.

UA-[3H]aMan disaccharides were prepared from heparin as described and separated into mono-O- and di-O-sulfated species by
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RESULTS AND DISCUSSION

A sample (1.5 × 10^6 cpm) of the antithrombin-binding N-[14C]acetyl-labeled heparin octasaccharide [2,3,4,5,6,7,8-[14C]) was digested with α-L-iduronidase and acetylglucosaminidase as described under "Experimental Procedures." After desalting by passage through a column (1 × 70 cm) of Sephadex G-15 equilibrated with 10% aqueous ethanol, a portion (1 × 10^6 cpm) of the digested material was incubated further in the presence of N-acetylgalactosamine-6-sulfate sulfatase. The final product, expected to contain the heptasaccharide [2,3,4,5,6,7-[14C)] lacking a 6-sulfate group on unit 2 (Fig. 2), was subjected to analytical affinity chromatography on antithrombin-Sepharose. Two distinct fractions were observed (Fig. 3B), one with high affinity for antithrombin, similar to the intact octasaccharide (not shown), and one with low affinity which appeared to be unretarded by the immobilized protein. In contrast, digestion with α-L-iduronidase only (expected to release the nonreducing terminal iduronic acid unit 1) did not significantly affect the affinity properties of the antithrombin-binding octasaccharide (Fig. 3A; see also Ref. 6). The formation of a low affinity component on consecutive digestions of the octasaccharide (Fig. 3A) was introduced as indicated. For additional information, see the text.

Fig. 1. Structure of the antithrombin-binding octasaccharide isolated after partial deaminative cleavage of heparin with nitrous acid. The 2,5-anhydro-α-d-mannitol unit 8 corresponds to an N-sulfated glucosamine residue in the intact polysaccharide. The pentasaccharide extending from position 2 to position 6 represents the actual binding sequence (within brackets). Structural variants are indicated by alternate groupings of the main structure (6). The sulfate groups marked by an asterisk are unique to the antithrombin-binding region and have not been found elsewhere in the heparin molecule (7-9). The sulfate groups marked with an (e) were previously shown to be essential for high affinity binding of the octasaccharide to antithrombin (10); the present report deals with the role of the 6-O-sulfate group in position 2. A radioactive [14C] label (circled) was introduced as indicated. For additional information, see the text.

Preparative paper electrophoresis (11). Tetrasaccharides with the general structure U-α-GlcNAc-GlcUA-[3,(6-di-)OS03] were obtained by a similar procedure, involving HNO2-NaBH4 treatment of heparin, followed by isolation of labeled oligosaccharides by gel chromatography (6). Unlabeled reference oligosaccharides were prepared following partial random depolymerization of heparin with nitrous acid (6). α-L-Iduronidase from human kidney (12) was a gift from Dr. Leonard H. Rome, University of California, Los Angeles, CA. α-N-Acetylgalactosaminidase (13) and N-acetylglucosamine-6-sulfate sulfatase (14), both isolated from human urine, were gifts from Dr. Kurt von Figura, University of Munster, Munster, West Germany.

Analytical Methods—Hexuronic acid was determined by the carbazole reaction with p-glucuronic acid as standard (15). The methods used to determine radioactivity are described in Ref. 16. Gel chromatography was performed with columns of Sephadex G-15 (1 × 75 cm) or Sephadex G-50 (1 × 70 cm), both eluted with 0.15 M NaCl at ~5.5 ml/h. High voltage paper electrophoresis (50 V/cm) was conducted on Whatman No. 3MM paper in 1.6 M formic acid (pH 1.7). Affinity chromatography on antithrombin-Sepharose was performed as described (6).

Chemical and Enzymatic Modification Procedures—Treatment of saccharides with nitrous acid (pH 1.5) followed by reduction of the deamination products was performed as described (6), except that unlabeled NaBH4 (10 mM) was substituted for NaB3H4 in the reduction step. The reduced oligosaccharides were isolated by gel chromatography on Sephadex G-15 in 0.2 M H4NCO3 and were then desalted by lyophilization.

For enzymatic degradation, oligosaccharides (30 µg or less of hexuronic acid) were digested with either 1 unit of a-L-iduronidase in 250 µl of 0.05 M acetate buffer (pH 4.3) containing 0.1 M NaCl, 3 mM NaN3, and 0.05% Triton X-100; 20 microunits of N-acetylglucosamine-6-sulfate sulfatase (17), both isolated from human urine, were gifts from Dr. Kurt von Figura, University of Munster, Munster, West Germany.

Fig. 2. Enzymatic modification procedures involving units 1 and 2 of the antithrombin-binding heparin octasaccharide. The sequence shown comprises units 1-4-5-6-7. The tetrasaccharide sequence extending from unit 5 to unit 8; X, H or SO3; (see Refs. 6 and 7). In addition, the points of cleavage on treatment with nitrous acid, followed by reduction (HNO2-NaBH4), are indicated. The glucosaminidic linkage between units 4 and 5 will be split, and the internal N-sulfated glucosamine unit 4 converted into a terminal 2,5-anhydro-β-mannitol residue; the structure of the tetrasaccharide fragment 1-4-5 will thus be GlcN(14C)Ac([6-O(3,6-di-)SO3])-GlcUA-aMan-(3,6-di-)so3).
trophoresis (pH 1.7) of the same components. The tetrasaccharide with α-L-iduronidase and N-acetylgalcosamine-6-sulfate sulfatase should therefore presumably be due to release of the 6-sulfate group from the N-acetylgalcosamine 6-O-sulfate unit 2, exposed by the action of a-L-iduronidase. Conversely, the high affinity fraction would consist either of the 6-sulfate group of unit 4 (indicated by the arrow in Fig. 1) or of the hepta-sulfate unit 2, exposed by the action of a-L-iduronidase. In order to confirm and differentiate these alternative interpretations, the products of enzymatic modification were subjected to isolation of the labeled fragments obtained on deaminative digestion of the unlabeled octasaccharide (Fig. 4A). The two peaks, a and b, were observed also with the α-L-iduronidase-digested material (Fig. 5A), in accord with the conclusion that a fraction of the octasaccharide molecules had escaped degradation by the exoglycosidase. However, in addition, two novel major components, a1 and b1, were detected, which had migrated somewhat faster than compounds a and b, respectively (Fig. 5A). The migration positions of these components were as expected, assuming that compounds a, b, a1, and b1 were trisaccharides, differing from the corresponding tetrasaccharides.

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Fig. 3. Affinity chromatography on antithrombin-Sepharose of products (10 x 10^6 cpm) obtained on digesting the ^14C-labeled antithrombin-binding octasaccharide [2-^14C] (1-8) with α-L-iduronidase (A) (●) or α-L-iduronidase and N-acetylgalcosamine-6-sulfate sulfatase (B) (○). Samples (2 mg) of commercial heparin were included as internal standard (○, hexuronic acid determined by the carbazole reaction). The separation into fractions of high (HA) and low (LA) affinity for antithrombin is indicated by the horizontal bars in A. The arrow indicates the start of the NaCl gradient. ---, NaCl concentration (M), shown only in B.
iduronidase was generally followed by release of the exposed corresponding to the structure GlcNAc(6-OSO3)-GlcUA-
peaks, indicating that removal of the iduronic acid unit 6-sulfate group in the subsequent sulfatase digestion. Deamination 
yielded a labeled tetrasaccharide (Fig. 4A). These molecules would be expected to resist also the action of 
portion of the octasaccharide molecules had remained intact, intact octasaccharide (Fig. 4B). Again, two peaks were observed, but now with migration position corresponding to those expected for mono- and disulfated trisaccharides, respectively. The structures assigned to these compounds would thus be GlcNAc-GlcUA-aMan(3-OSO3) for the slower migrating and GlcNAc-GlcUA-aMan(3,6-di-OSO3) for the faster-migrating species.

The experiments described lead to the tentative conclusion that the low affinity fraction consisted of heptasaccharide which lacked not only the iduronic acid unit 1 but also the 6-
sulfate group on the N-acetylglucosamine unit 2. Since the heptasaccharide still carrying a 6-sulfate group on unit 2 (obtained by digesting the octasaccharide with a-L-iduronidase only) retained high affinity for antithrombin, the loss of bioaffinity must be explicitly attributed to the loss of this particular sulfate group. In order to conclusively establish this relationship, it was essential to confirm the absence of a 6-
sulfate group on the terminal N-acetylglucosamine residue in the low affinity heptasaccharide. To this end, the low affinity

FIG. 5. Paper electrophoresis at pH 1.7 of 14C-labeled oligosaccharides obtained by HNO3-NaBH4 treatment of anti-thrombin-binding heparin octasaccharide [2-14C](1-8) before (O) and after (D) digestion with a-L-iduronidase (A) or obtained by HNO3-NaBH4 treatment of fractions with high (O) and with low (D) affinity for antithrombin recovered after digesting the octasaccharide with a-L-iduronidase and N-acetylglucosamine-6-sulfate sulfatase (B). The standards shown below the tracings are monosulfated (I) and disulfated (II) UA-[3H] aMan disaccharides. The designations of the various peaks in A are used in the text.

disaccharide molecules had been attacked by the a-L-iduronidase.

In order to characterize the components with high and low affinity for antithrombin detected after digestion of the octasaccharide with both a-L-iduronidase and N-acetylglucosamine-6-sulfate sulfatase, respectively, the affinity chromatography illustrated in Fig. 3B was reproduced on a preparative scale (~800 \times 10^3 cpm of 14C). The resulting fractions, 40% high affinity and 60% low affinity material, were desalted by passage through Sephadex G-15. Since characterization of the material digested only with a-L-iduronidase indicated that a portion of the octasaccharide molecules had remained intact, these molecules would be expected to resist also the action of the sulfatase and would therefore be recovered in the final high affinity fraction. Accordingly, deamination of this fraction yielded a labeled tetrasaccharide (Fig. 4C), which separated on paper electrophoresis (Fig. 5B) into the same two peaks, a and b, that were observed after deamination of the intact octasaccharide (Fig. 5A). It is noted that peak b, corresponding to the structure GlcNAc(6-OSO3)-GlcUA-aMan(3,6-di-OSO3), is essentially absent in Fig. 5B, suggesting that removal of the iduronic acid unit 1 by the a-L-iduronidase was generally followed by release of the exposed 6-sulfate group in the subsequent sulfatase digestion. Deamination of the low affinity fraction produced a labeled product which behaved like a trisaccharide on gel chromatography (Fig. 4D). This trisaccharide peak was slightly but significantly shifted in relation to that derived from the octasaccharide after digestion with a-L-iduronidase only (Fig. 4B); the difference in size would be due to the 6-sulfate group (on unit 2) released by the sulfatase (Fig. 2). The electrophoretic properties of the trisaccharide obtained from the low affinity fraction conformed to the postulated distribution of sulfate groups (Fig. 5B). Again, two peaks were observed, but now with migration position corresponding to those expected for mono- and disulfated trisaccharides, respectively. The structures assigned to these compounds would thus be GlcNAc-GlcUA-aMan(3-OSO3) for the slower migrating and GlcNAc-GlcUA-aMan(3,6-di-OSO3) for the faster-migrating species.

FIG. 6. Gel chromatography on Sephadex G-50 of products obtained on digesting the antithrombin-binding octasaccharide [2-14C](1-8) with a-L-iduronidase and N-acetylglucosamine-6-sulfate sulfatase: fraction with high affinity for antithrombin (A and B), fraction with low affinity for antithrombin (C and D), before further incubation with a-N-acetylglucosaminidase (A and C), and after incubation with a-N-acetylglucosaminidase (B and D). Before chromatography, each sample (~15 \times 10^3 cpm of 14C) was mixed with unlabeled reference oligosaccharides (7 mg of hexuronic acid) prepared by partial deamination of heparin. Eluate fractions were analyzed for radioactivity (O) and for hexuronic acid (---). The number of monosaccharide residues in the various reference oligosaccharides is indicated above the appropriate peaks.
fraction was digested with an \( \alpha \)-\( N \)-acetylglucosaminidase known to act only on nonsulfated terminal \( N \)-acetylglucosamine units (17). Since the single \( N \)-acetylglucosamine residue in the potential substrate was \( N \)-\( ^{14} \)C-acetyl-labeled, an effect of the enzyme would be readily manifested on subsequent gel chromatography by the appearance of a labeled monosaccharide (Fig. 2). Before digestion with the \( \alpha \)-\( N \)-acetylglucosaminidase, the low affinity fraction appeared on gel chromatography (Sephadex G-50) as a distinct peak of \( ^{14} \)C activity, corresponding to the elution position of a heptasaccharide (Fig. 6C). After incubation with the enzyme, most of the radioactive activity appeared in the elution position of a monosaccharide (Fig. 6D). This result demonstrated that the terminal \( N \)-\( ^{14} \)C acetylglucosamine unit in the low affinity heptasaccharide was nonsulfated. The occurrence of significant \( N \)-acetylglucosamine-6-sulfate sulfatase activity in the \( \alpha \)-\( N \)-acetylglucosaminidase preparation was excluded, since the enzyme failed to release any labeled monosaccharide from the material recovered after digesting the high affinity octasaccharide with \( \alpha \)-L-iduronidase only (not shown). The high affinity fraction recovered after iduronidase and sulfatase digestion of the octasaccharide apparently consisted of intact molecules that had escaped the initial degradation by the iduronidase. As expected, these components were unaffected by treatment with \( \alpha \)-\( N \)-acetylglucosaminidase, but emerged as labeled octasaccharide (Fig. 6B) as well as before (Fig. 6A) incubation with the enzyme. Due to lack of enzyme, the reason for the partial resistance of the octasaccharide preparation against iduronidase digestion was not further investigated.

Taken together, the results obtained in the present study indicate that the 6-sulfate group on the \( N \)-acetylglucosamine unit 2 of the antithrombin-binding octasaccharide, i.e., the nonreducing terminal unit of the actual binding sequence (6), is of crucial importance to the polysaccharide-protein interaction. The difference in elution position on antithrombin-Sepharose caused by removal of this sulfate group is of the same magnitude as that observed for native heparin species with high or low affinity for antithrombin. Determination of the binding constants for such fractions by spectrophotometric techniques indicated affinities differing by about a 1000-fold (18, 19).

**Acknowledgments**—We thank Dr. L. H. Rome for providing the \( \alpha \)-L-iduronidase and Dr. K. von Figura for the gifts of \( N \)-acetylglucosamine-6-sulfate sulfatase and \( \alpha \)-\( N \)-acetylglucosaminidase.

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