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Evidence for a 3-*O*-sulfated D-glucosamine residue in the antithrombin-binding sequence of heparin

(anticoagulant activity/nitrous acid deamination/heparin octasaccharide/periodate oxidation/disaccharide units in heparin)

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ABSTRACT **An octasaccharide with high affinity for antithrombin was isolated after partial deaminative cleavage of heparin with nitrous acid. After conversion of the 2,5-anhydro-D-mannose end group to anhydro[1-^{3}H]mannitol, labeled pentasaccharide was released from the octasaccharide by periodate–alkali treatment. Incubation of the pentasaccharide with a recently discovered 3-*O*-sulfatase from human urine resulted in desulfation, suggesting the occurrence of a 3-sulfate group on the terminal glucosamine residue. The same glucosamine residue was recovered as a 2,5-anhydro[1-^{3}H]mannitol derivative by a procedure involving deamination of the octasaccharide with nitrous acid, reduction of the products with sodium boro[^{3}H]hydride, isolation of ^{3}H-labeled tetrasaccharide by gel chromatography, and release of the labeled end-group by periodate–alkali treatment. Paper electrophoresis indicated disulfated anhydro[^{3}H]mannitol, presumably sulfated at C3 and C6, as a major component, along with smaller amounts of monosulfated (presumably 3-sulfated) anhydro[^{3}H]mannitol. Similar treatment of an analogous tetrasaccharide derived from heparin with low affinity for antithrombin failed to produce any disulfated anhydromannitol. These results suggest that 3-sulfated glucosamine is a unique component of high-affinity heparin, located at a specific position in the antithrombin-binding sequence of the molecule.**

The blood anticoagulant activity of heparin depends on its ability to bind with high affinity to antithrombin, a plasma protein that inhibits the proteinases involved in the coagulation mechanism (1). In commercially available heparin preparations more than 50% of the molecules lack this ability and are essentially devoid of anticoagulant activity (2–4). The antithrombin-binding sequence of the active heparin molecules was investigated by the isolation of oligosaccharides with high affinity for antithrombin, after partial depolymerization of heparin with nitrous acid (5, 6). A tentative structure of the smallest oligosaccharide obtained, an octasaccharide, is shown in Fig. 1. While some regions of this molecule appear to vary in structure, others are constant (5–7); except for the *N*-sulfate groups in positions 4 and 6, which are both essential for high-affinity binding to antithrombin (8), there is so far little information to indicate the role of the individual components. The distribution of 6-sulfate groups on the glucosamine units (X in Fig. 1) has not been elucidated.

The structure previously assigned to the antithrombin-binding sequence (Y = H in Fig. 1) was based on the assumption that *O*-sulfate groups may be present only on C2 of the iduronic acid and C6 of the glucosamine residues (cf. ref. 9). The recent discovery, in human urine, of an enzyme that removes the sulfate at C3 in 3-*O*,*N*-disulfated methyl glucosaminide strongly suggested that analogous 3-sulfated glucosamine residues occur in heparin and might play a unique role in the interaction of heparin with antithrombin (10). In the present study, evidence has been obtained that a 3-sulfated glucosamine residue (Y = SO_3^- in Fig. 1) is a unique component of the antithrombin-binding sequence in the heparin molecule.

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MATERIALS AND METHODS

Materials. Heparin oligosaccharides with high affinity for antithrombin were isolated by affinity chromatography on antithrombin-Sepharose, after partial deaminative cleavage of pig mucosal heparin with nitrous acid. Material eluted at ≥0.9 M NaCl was recovered. An octasaccharide fraction was isolated by gel chromatography on Sephadex G-50, as described (6). A portion of this material was reduced with sodium boro[^{3}H]hydride (11), yielding a product with terminal 2,5-anhydro[1-^{3}H]mannitol units (specific activity, about 1.0×10^5 cpm per μg of uronic acid). Labeled pentasaccharide corresponding to sugar units 4–8 (segment B in Fig. 1) was prepared by periodate–alkali treatment of the ^{3}H-labeled octasaccharide; the product was isolated by gel chromatography as described (6) and was then desalted. The pentasaccharide was homogeneous with regard to molecular size (see figure 3 in ref. 6). A tetrasaccharide derivative of segment A (positions 1–4 in Fig. 1), with a terminal 2,5-anhydro[1-^{3}H]mannitol residue corresponding to the glucosamine unit in position 4 (5, 6), was obtained by deaminative cleavage of unlabeled octasaccharide followed by reduction of the products with sodium boro[^{3}H]-hydride and gel chromatography on Sephadex G-25. Tetrasaccharides derived from pig mucosal heparin fractions with high affinity and with low affinity for antithrombin, respectively, were as described (5). Additional details on the preparation of labeled fragments from the heparin octasaccharide are given in the figure legends.

Glycuronosyl→2,5-anhydro[1-^{3}H]mannitol disaccharides with *O*-sulfate groups in various positions were prepared as described (11). 2,5-Anhydro[1-^{3}H]mannitol 6-sulfate was obtained by digesting glucuronosyl→2,5-anhydro[1-^{3}H]mannitol 6-sulfate with β-glucuronidase (11). A sample of 2,5-anhydro-D-[1-^{3}H]mannitol (1.44 μCi per μmol; 1 Ci = 3.7×10^{10} becquerels) was kindly donated by B. Weissmann, University of Illinois, Chicago, IL.

3-*O*-Sulfatase was partially purified from human urine (10); this enzyme acts on methyl 2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate, but not on the 4- or 6-sulfated analogs. The enzyme preparation contained some iduronate 2-sulfatase activity, as shown by incubation with iduronosyl 2-sulfate

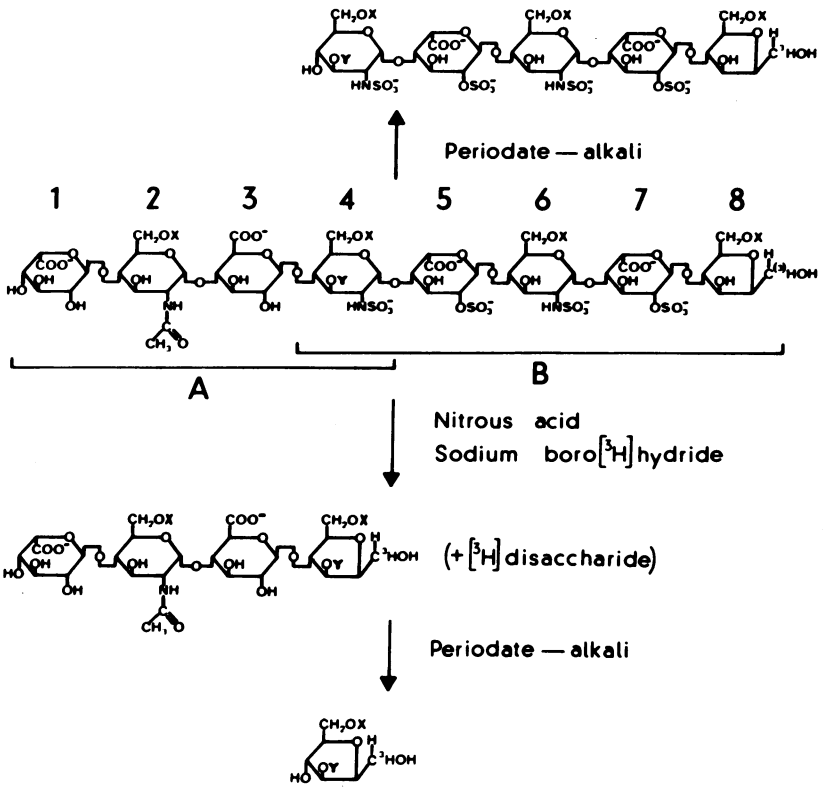

FIG. 1. Tentative structure for the antithrombin-binding octasaccharide derived from pig mucosal heparin. The structure shown represents the majority of antithrombin-binding sequences in pig mucosal heparin (5, 6). X is either a hydrogen atom or a sulfate group. Y on the glucosamine residue in position 4 has been tacitly assumed to be a hydrogen atom but is in the present investigation identified as a sulfate group. The figure outlines the degradative procedures employed to obtain ^3H-labeled fragments; only the labeled products of each reaction are shown. The cleavage site on periodate–alkali treatment is the nonsulfated uronic acid residue in position 3. For the isolation of pentasaccharide B, ^3H-labeled octasaccharide was used as starting material. The 2,5-anhydro[1-^3H]mannitol derivative of tetrasaccharide A was prepared from unlabeled octasaccharide, obtained by reducing the initial anhydromannose end group (position 8) with unlabeled sodium borohydride. The labeled disaccharide indicated within parenthesis was removed by gel chromatography.

→anhydro[^3H]mannitol 6-sulfate (9). A sample of purified iduronate 2-sulfatase, isolated from human serum, was a generous gift from Å. Wasteson, University of Uppsala, Sweden.

Methods. Radioactivity was determined as described (12). Paper electrophoresis was conducted on Whatman 3 MM paper in 1.6 M formic acid, pH 1.7 (40 V/cm). Paper chromatography was carried out in ethyl acetate/acetic acid/water, 3:1:1 (by volume).

Periodate oxidation (pH 7, 37°C, 24 hr) followed by alkali treatment was performed according to Fransson (13). *O*-Desulfation was achieved by treatment with dimethyl sulfoxide containing 10% (vol/vol) methanol at 100°C, as described by Nagasawa *et al.* (14).

RESULTS

Labeled pentasaccharide corresponding to residues 4–8 of the antithrombin-binding sequence (segment B in Fig. 1, with a terminal anhydro[^3H]mannitol unit) was fractionated by ion-exchange chromatography, as described in the legend to Fig. 2. Three distinct peaks of radioactivity were observed (Fig. 2*A*), a minor, less retarded (I), and two major, more retarded, fractions (II and III). Each of these components should contain two *N*-sulfate groups (corresponding to positions 4 and 6 in Fig. 1) and two sulfated iduronic acid units (positions 5 and 7) (6, 8). The different elution positions should therefore reflect variations in 6-sulfation (X in positions 4, 6, and 8), such that fractions I, II, and III contain either zero, one, and two or one, two, and three 6-sulfate groups, respectively, per molecule. Each of the isolated fractions was separately incubated with a sulfatase that specifically releases the 3-sulfate group from 3-*O*,*N*-disulfated methyl glucosaminide (10). This treatment removed a sulfate group from 60% of the molecules in fraction I, as shown by the appearance of a new, less anionic component on the ion-exchange chromatogram (Fig. 2*B*). Fraction II was similarly desulfated (Fig. 2*C*), although to a lesser extent (50%), whereas the most highly sulfated fraction (III) was unaffected by the enzyme (Fig. 2*D*). Prolonged incubation of fraction II with repeated addition of fresh enzyme did not result in further desulfation. These results suggest that a major proportion of the molecules in fractions I and II contain terminal glucosamine units with 3-*O*-sulfate groups that are susceptible to 3-sulfatase digestion. Removal of a sulfate group from the adjacent iduronic acid residue (position 5 in Fig. 1) by contaminating iduronate sulfatase was excluded, because purified iduronate sulfatase failed to induce any change in elution position on incubation (9) with pentasaccharide fraction II (not shown).

The partial resistance of fractions I and II, and the complete resistance of fraction III, toward enzymatic desulfation might indicate that only some of the terminal glucosamine residues in fractions I and II, and none of the corresponding units in fraction III, carry a 3-sulfate group. Alternatively, however, this resistance could reflect the occurrence of inhibitory 6-sulfate groups. Pending a more detailed study on the substrate specificity of the 3-sulfatase, it may be hypothesized that the enzyme will release a 3-sulfate group from a terminal glucosamine unit only in the absence of a sulfate group at C6.‡ Assuming that the glucosamine unit in position 4 of the antithrombin-binding sequence is indeed always 3-sulfated (Y = SO_3^- in Fig. 1), the 3-sulfate group in pentasaccharide fraction III would be resistant to the 3-sulfatase due to the invariable presence of a 6-sulfate group on the same glucosamine unit. In fractions I and II a proportion of the terminal glucosamine residues would not be sulfated at C6 and thus accessible to the 3-sulfatase. According to this alternative, fractions I, II, and III should contain one, two, and three 6-sulfate groups, respectively, per molecule, and the majority of the glucosamine residues in position 4 of the antithrombin-binding sequence

‡ Accordingly, the desulfation of a trisulfated glucosamine residue during enzymatic degradation of heparin would involve the consecutive action of a 6-*O*-sulfatase, a 3-*O*-sulfatase, and a sulfamidase, in the order mentioned.

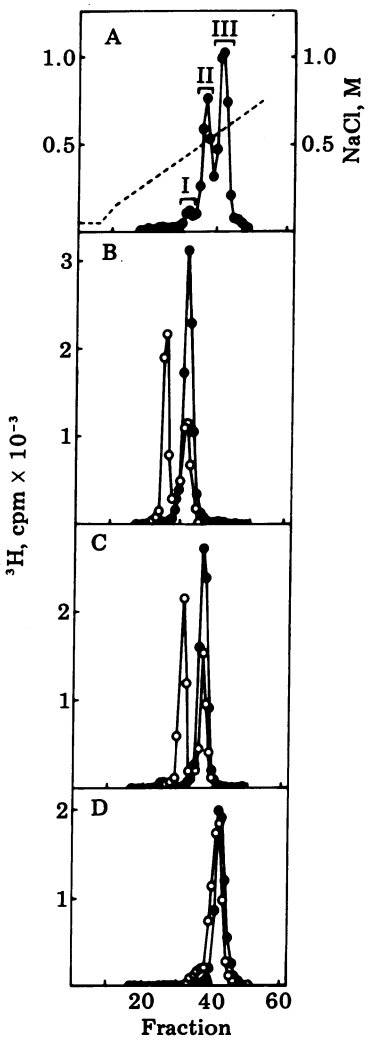

FIG. 2. Effect of 3-*O*-sulfatase on ^{3}H-labeled pentasaccharides corresponding to positions 4–8 of the antithrombin-binding sequence (segment B in Fig. 1). Pentasaccharide, prepared by periodate–alkali treatment of labeled octasaccharide, was fractionated on a 3-ml column of DEAE-cellulose (Whatman DE-52), eluted with a salt gradient prepared from 100 ml of 0.05 M LiCl and 100 ml of 1.5 M LiCl in 0.05 M acetate buffer, pH 4.0 (broken line in *A*). Fractions I–III were recovered as indicated and desalted by passage through a column of Sephadex G-15, eluted with 10% aqueous ethanol. About 20,000 cpm of each fraction was dissolved in 75 μl of 0.05 M Tris/maleate buffer, pH 6.3, and incubated at 37°C with 50 μg of 3-*O*-sulfatase; after 10 hr another 50 μg of enzyme was added and incubation was continued for a total period of 24 hr. Ion-exchange chromatograms of fractions I–III, before (●) and after (○) incubation with the 3-*O*-sulfatase, are shown in *B–D*, respectively.

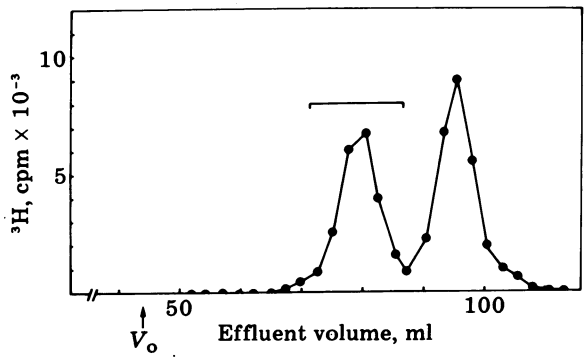

FIG. 3. Isolation by gel chromatography of ^{3}H-labeled tetrasaccharide corresponding to positions 1–4 of the antithrombin-binding sequence (segment A in Fig. 1). Unlabeled reduced octasaccharide (2,5-anhydromannitol in position 8), corresponding to about 20 μg of hexuronic acid, in 20 μl of water was mixed with 0.2 ml of deamination reagent (pH 1.5 procedure described in ref. 15) and left at room temperature for 10 min. The deamination products were reduced by adding 0.5 ml of 1 M Tris, followed by 0.5 μmol of sodium boro[^{3}H]-hydride (10 mCi per μmol). After 15 hr at room temperature the pH was adjusted to 4 with glacial acetic acid and then to 7 with 4 M NaOH. Labeled oligosaccharides were isolated by gel chromatography on a column of Sephadex G-15, equilibrated with 0.2 M NH_4HCO_3, and separated by gel chromatography on a column (1 × 190 cm) of Sephadex G-25, also in 0.2 M NH_4HCO_3. The fractions indicated by the horizontal bar were combined and lyophilized. The more retarded peak represents disaccharides essentially derived from sugar units 5 and 6 in Fig. 1.

should carry three sulfate groups, an *N*-sulfate group at C2 and *O*-sulfate groups at C3 and C6.

This interpretation was corroborated by the demonstration of di-*O*-sulfated glucosamine units, identified as the 2,5-anhydro[1-^{3}H]mannitol derivative (conversion outlined in Fig 1). Antithrombin-binding octasaccharide, reduced with unlabeled sodium borohydride, was degraded with nitrous acid and the products were labeled by reduction with sodium boro[^{3}H]-hydride, as described in the legend to Fig. 3. The tetrasaccharide corresponding to monosaccharide units 1–4 (segment A in Fig. 1) was isolated by gel chromatography (Fig. 3). The anhydro[^{3}H]mannitol end group, derived from the glucosamine unit in position 4 of the intact octasaccharide, was released, with its substituents, as a monosaccharide by periodate–alkali cleavage of the uronic acid residue in position 3; gel chromatography of the products on Sephadex G-15 indicated about 75% labeled monosaccharide. Paper electrophoresis showed a major component that migrated distinctly ahead of a di-*O*-sulfated disaccharide (iduronosyl→anhydromannitol) reference standard and, in addition, two smaller peaks of slower-migrating material (Fig. 4*A*). The fast-migrating compound was identified as di-*O*-sulfated anhydromannitol by partial desulfation, which yielded uncharged material and a component that migrated like anhydromannitol monosulfate (Fig. 5). Paper chromatography of the uncharged material, isolated by preparative paper electrophoresis, showed a single component with a R_F value similar to that of an anhydro[^{3}H]mannitol reference standard. Assuming that heparin is exclusively a 1,4-linked polymer, the two sulfate groups must be located at C3 and C6, because C4 of the parent glucosamine residue would be blocked by the glycosidic linkage to the adjacent uronic acid residue (see Fig. 1). In the intact polymer this glucosamine unit thus carries three sulfate groups, at C2, C3, and C6. The disulfated (at C2 and C3) glucosamine residue susceptible to 3-sulfatase digestion should be represented by (monosulfated) anhydromannitol 3-sulfate, presumably one of the two slower-migrating components appearing close to the anhydromannitol 6-sulfate reference standard (Fig. 4*A*). The excess of disulfated over monosulfated anhydromannitol in Fig. 4*A* is in agreement with the observation that only 20–25% of the total pentasaccharide B molecules (as calculated from the data given in Fig. 2) was susceptible to desulfation by the 3-sulfatase.

Disulfated anhydro[^{3}H]mannitol also occurred in tetrasaccharide isolated from undegraded high-affinity heparin (i.e., intact polysaccharide molecules containing the antithrombin-binding sequence) (Fig. 4*B*), although in reduced quantity compared to the tetrasaccharide obtained from the actual binding sequence (Fig. 4*A*). In contrast, no such component could be demonstrated in tetrasaccharides derived from low-affinity heparin (Fig. 4*C*). These findings strongly suggest that the 3-sulfated glucosamine residue is a unique component of the antithrombin-binding sequence in the heparin molecule.

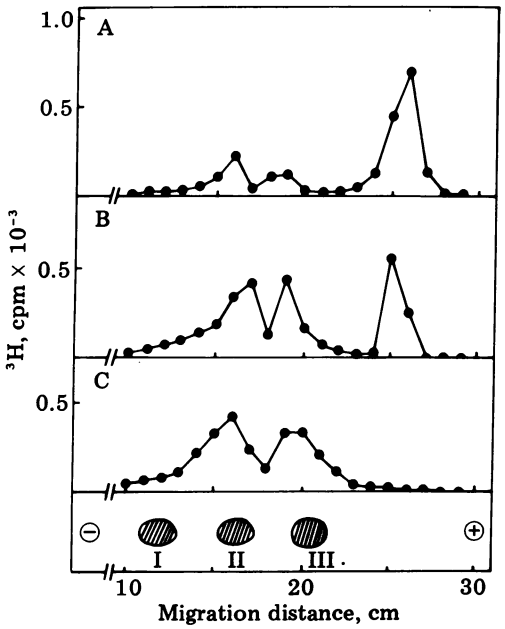

FIG. 4. Paper electrophoresis at pH 1.7 of products obtained on periodate–alkali treatment of ^{3}H-labeled tetrasaccharides isolated from: heparin octasaccharide with high affinity for antithrombin (*A*); heparin polysaccharide with high affinity for antithrombin (*B*); and heparin polysaccharide with low affinity for antithrombin (*C*). The tetrasaccharides were prepared as described in the legend to Fig. 3; the label derives from the 2,5-anhydro[1-^{3}H]mannitol end group. Tetrasaccharides (10–20 × 10^3 cpm) were dissolved in 50 μl of a solution containing 0.02 M $NaIO_4$ and 0.2 M $NaClO_4$ in 0.05 M sodium phosphate buffer, pH 7.0. After 24 hr at 37°C in the dark the samples were mixed with 20 μmol of D-mannitol and left at 37°C for another 30 min. The pH was adjusted to 11.5 by adding 40 μl of 0.1 M NaOH and the reaction mixtures were kept at room temperature for 30 min. Finally, the samples were neutralized with 0.1 M HCl and applied to the electrophoresis paper. The standards indicated below the tracings are: I, glucuronosyl→anhydro[^{3}H]mannitol 6-sulfate; II, anhydro[^{3}H]mannitol 6-sulfate; III, iduronosyl 2-sulfate→anhydro-[^{3}H]mannitol 6-sulfate.

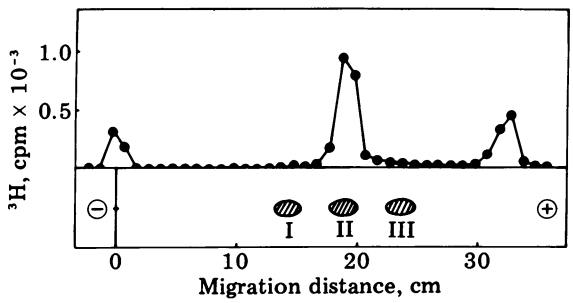

FIG. 5. Paper electrophoresis at pH 1.7 of the fast-migrating component shown in Fig. 4*A*, after partial desulfation. The component was isolated by preparative paper electrophoresis. A sample (30,000 cpm) was converted to the pyridinium salt and treated with 60 μl of dimethyl sulfoxide containing 10% water, at 100°C for 10 min. A 20-μl sample was applied to the electrophoresis paper. The standards shown below the tracing are the same as in Fig. 4. With increasing time of desulfation the uncharged component at the origin increased while the two migrating components decreased in amount; after 45 min the uncharged component only was present in significant amounts. This component was analyzed further by paper chromatography, as described in the text.

DISCUSSION

Some of the structural features required for binding of heparin to antithrombin have been defined previously (5–8). No qualitative differences in composition were detected between the binding sequence and other portions of the heparin molecule; the most conspicuous feature of the binding sequence was the invariable occurrence of nonsulfated iduronic acid, a minor constituent of the heparin molecule. Moreover, this component was located in a specific position, corresponding to the nonreducing terminal unit of an isolated octasaccharide with high affinity for antithrombin (6). Unexpectedly, the heptasaccharide obtained on digesting the octasaccharide with α-L-iduronidase retained high affinity for antithrombin (unpublished observation), suggesting that the nonsulfated iduronic acid is itself not essential to the interaction. This finding again raised the question as to the occurrence in the antithrombin-binding sequence of a unique component, previously not recognized in heparin.

A clue to the existence and nature of such a component emerged through the recent demonstration of an enzyme in human urine that is capable of desulfating a 3-sulfated glucosaminide (10). 3-Desulfation occurred only provided that the glucosaminide was also *N*-sulfated, thus strongly suggesting that the natural substrate for the enzyme is a heparin-like polysaccharide. The occurrence of 3-sulfated glucosamine residues in heparin was in fact suggested by Danishefsky *et al.* in 1969 on the basis of methylation analysis (16). The results of the present study indicate that the 3-sulfated glucosamine residue is indeed a specific component of the antithrombin-binding sequence in heparin. This residue may be either 6-sulfated (X = SO_3^- in position 4; Fig. 1), identified as the disulfated anhydro[^{3}H]-mannitol derivative in reducing-terminal position of tetrasaccharide A, or 6-unsubstituted (X = H), identified as a glucosamine residue susceptible to 3-sulfatase digestion, in nonreducing-terminal position of pentasaccharide B. The lack of disulfated anhydromannitol in tetrasaccharides derived from heparin with low affinity for antithrombin suggests, but does not prove, that the 3-sulfate group is actually confined to the antithrombin-binding sequence and does not occur elsewhere in the heparin molecule.

The evidence for a 3-sulfate group derives from two unrelated experiments, digestion of oligosaccharides with a 3-sulfatase and isolation of di-*O*-sulfated anhydromannitol. The interpretation of the enzymatic desulfation relies on the substrate specificity of the 3-sulfatase as defined with model glucosaminides (10); the occurrence in the enzyme preparation used of an unrecognized sulfatase, active with appropriate oligosaccharide substrates only, cannot be excluded. Moreover, the identification of the di-*O*-sulfated anhydromannitol as the 3,6-disulfated species is based on the assumption that C4 of the corresponding glucosamine residue is involved in glycosidic linkage. Again, the occurrence in heparin of minute proportions of glycosidic linkages in positions other than 1,4, although unlikely, has not been disproved. Although the evidence for the postulated 3-sulfate group is thus not quite conclusive, additional support for its existence was recently provided by ^{13}C NMR spectroscopy; a decasaccharide with high affinity for antithrombin yielded signals attributable to a glucosamine residue with a sulfate group at C3 (unpublished observation).

Previous experiments (6, 8) have shown that high-affinity binding of heparin to antithrombin requires a polymer sequence (Fig. 1) that extends beyond the tetrasaccharide structure (segment A) implicated by Rosenberg and Lam (7) as the site of interaction. However, the glucosamine unit in position 4, which is part of segment A, is of critical importance, because removal of its *N*-sulfate substituent abolished the interaction (8). The results of the present investigation suggest that the same glucosamine unit may have two additional sulfate substituents, at C3 and C6. Although the 3-sulfate group is probably essential to the interaction between heparin and an-

tithrombin, such a dependence has not yet been demonstrated.§ The 6-sulfate group at position 4 is sometimes missing and should therefore not be of functional importance.

Finally, the functional evaluation of 6-sulfate groups may be extended to positions 6 and 8 of the binding sequence. The partial resistance of fraction I toward 3-sulfatase digestion (Fig. 2*B*) suggests that its single 6-sulfate group may be located at position 4 as well as elsewhere (at position 6 or 8) in the molecule (the position numbers referring to the intact sequence in Fig. 1). If fraction I were indeed representative of the intact antithrombin-binding sequence, neither one of the 6-sulfate groups at positions 4, 6, or 8 would be required for high-affinity binding of heparin to antithrombin. In view of the low yield of fraction I (Fig. 2*A*), and the possibility of limited desulfation during the periodate–alkali treatment (used in the preparation of the pentasaccharide), this conclusion may not be valid. However, applying the same argument to fraction II, it seems safe to conclude that at most one of the three 6-sulfate groups can be essential to the anticoagulant action of heparin.

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§ Incubation of the antithrombin-binding heparin octasaccharide with the 3-sulfatase did not affect its affinity for antithrombin, as shown by affinity chromatography; however, this experiment is inconclusive because the enzyme would not be expected to attack an internal glucosamine residue. Treatment of the octasaccharide with periodate–alkali, which yields the pentasaccharide substrate for the 3-sulfatase, unfortunately also abolishes the affinity for antithrombin (unpublished).

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