THE MOLECULAR SIZE OF THE ANTITHROMBIN-BINDING SEQUENCE IN HEPARIN

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1. Introduction

The blood anticoagulant activity of heparin depends on its ability to bind, and thereby activate, antithrombin, a plasma protein that inhibits the proteinases of the so-called coagulation cascade [1]. Only a fraction (~1/3rd) of the molecules in heparin preparations binds with high affinity to antithrombin and this fraction accounts for most of the anticoagulant activity of the unfractionated material [2–4]. Attempts to define the structural basis for the interaction between heparin and antithrombin led to partially conflicting results. While it was claimed that the heparin structure required for binding to antithrombin is contained within a tetrasaccharide sequence [5], we proposed a more extended binding region [6]. Our conclusion was based on the isolation of oligosaccharides with high affinity for antithrombin, following partial depolymerization of heparin with bacterial heparinase [7] or with nitrous acid [6]. The oligosaccharides were tentatively identified as dodeca- or tetradecasaccharides. However, no attempt was made to define the extent of the actual binding sequence by selecting for the smallest possible oligosaccharide yet capable of binding with high affinity to antithrombin. Here, such a component has been isolated and identified as an octasaccharide. The location in the octasaccharide molecule of a tetrasaccharide structure (IdUA→GlcNac→GlcUA→$GlcNSO_3^-$), implicated in the antithrombin-binding sequence [5,6], has been determined.

Abbreviations: IdUA, L-iduronic acid; GlcUA, D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxyglucose; $GlcNSO_3^-$, 2-sulfamido-2-deoxyglucose; the location of *O*-sulfate groups is indicated in parentheses; glycosidic linkages are indicated by arrows (→)

2. Materials and methods

The heparin preparation used was as in [3]. Di-*O*-sulfated iduronosyl-2,5-anhydro-[1-^{3}H]mannitol was prepared from deamination products of heparin [8].

Methods for the determination of hexuronic acid and radioactivity have been reported [9]. Affinity chromatography on antithrombin–Sepharose was done as in [10], with the exception that human antithrombin (Kabi AB, Stockholm) was used; the procedure was adapted to a total gel volume of either 3 ml or 500 ml.

Gel chromatography of oligosaccharides was performed on a column (3 × 250 cm) of Sephadex G-50, eluted with 1 M NaCl at 16 ml/h. Periodate oxidation (pH 7, 37°C) followed by alkali treatment was done by the procedure in [11].

Degradation of heparin with nitrous acid was done as in [12], with the exception that the temperature was kept at −10°C. For additional experimental details, see the legends to figures.

3. Results and discussion

Heparin (1 g) was treated with nitrous acid at −10°C for 40 min and the products were fractionated by affinity chromatography on antithrombin–Sepharose (see fig.1). Material retained by the affinity matrix at low ionic strength (0.05 M NaCl–0.05 M Tris) was recovered by salt-gradient elution [10] and a portion of the product was reduced with sodium boro[^{3}H]hydride [8]. The resulting labeled oligosaccharides (with terminal 2,5-anhydro-[1-^{3}H]mannitol residues) were separated in a second affinity-chromatography step into 3 fractions with different affini-

ties for antithrombin (fig.1). The high-affinity fraction eluted in the same salt concentration range as a heparin fraction with an anticoagulant activity of ~250 units/mg (unpublished).

The high-affinity fraction (HA in fig.1) was mixed with unlabeled reference oligosaccharides, obtained by partial deamination of heparin, and was then subjected to gel chromatography on Sephadex G-50 (fig.2A). It separated into 3 peaks of labeled material, corresponding to octa-, deca- and dodecasaccharides, respectively. No labeled components smaller than octasaccharide were detected. The medium-affinity fraction (MA in fig.1), and the low-affinity fraction (LA in fig.1) both yielded essentially the same three peaks (^{3}H-labeled material in fig.2B and 2C, respectively), but in addition, a shoulder in the hexasaccharide region. In contrast, the material that lacked

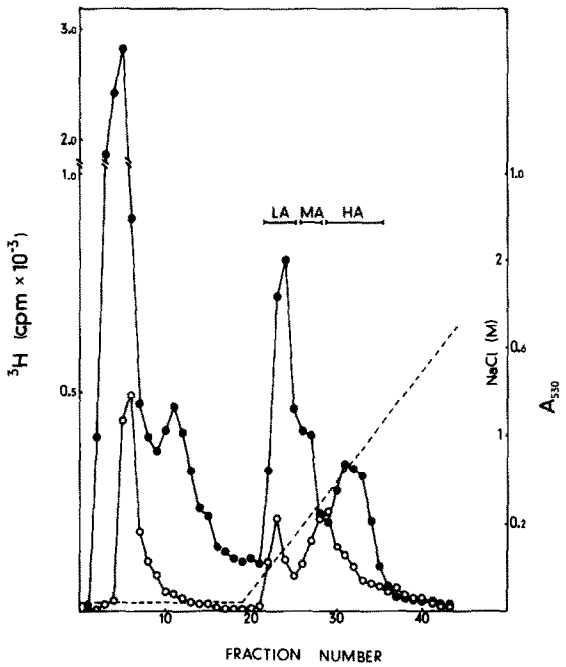

Fig.1. Affinity chromatography of heparin oligosaccharides on antithrombin–Sepharose. Heparin (1 g) was deaminated at −10°C in 500 ml total vol. [12]. After 40 min the reaction was interrupted by adjusting to pH 8.0 with 2 M Tris, and the mixture was concentrated and desalted by passage through a column of Sephadex G-15, eluted with 10% ethanol. The products were separated by affinity chromatography [10] on a column of antithrombin–Sepharose (500 ml bed vol.). Material eluted at 0.5–3.0 M NaCl (~6% of the starting material) was recovered, and a portion (100 μg uronic acid) was reduced with sodium boro[^{3}H]hydride, as in [8]. The ^{3}H-labeled oligosaccharides were mixed with 2 mg heparin and applied to a small column (3 ml) of antithrombin–Sepharose. Following gradient elution, effluent fractions were analyzed for uronic acid by the carbazole reaction (○) or for ^{3}H radioactivity (●). Fractions of low (LA, eluted at 0.05–0.45 M NaCl), medium (MA, 0.45–0.65 M NaCl) and high (HA, 0.65–1.2 M NaCl) affinity for antithrombin, respectively, were pooled and analyzed further by gel chromatography.

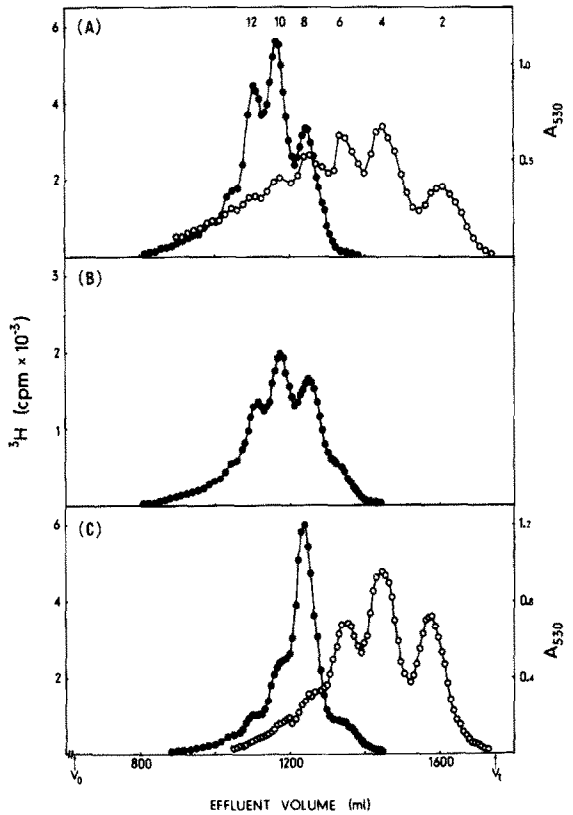

Fig.2. Gel chromatography on Sephadex G-50 of ^{3}H-labeled oligosaccharides with (A) high, (B) medium, and (C) low affinity for antithrombin. The isolation of the oligosaccharides is described in the legend to fig.1. The sample (100 × 10^3 cpm ^{3}H) in panel (A) was mixed with 100 mg reference oligosaccharides prior to chromatography; these oligosaccharides were prepared by partial deaminative cleavage of heparin (23 min reaction time), essentially as in fig.1. Effluent fractions were analyzed for ^{3}H radioactivity (●) or for uronic acid (○). The carbazole-positive material in panel (C) is a sample of the low-affinity oligosaccharides recovered with the break-through fraction of the initial, large-scale affinity chromatography in fig.1. The number of monosaccharide units in the various oligosaccharides is indicated above the appropriate peaks of reference oligosaccharides. These numbers were assigned on the assumption that each two consecutive species differ by a disaccharide unit; the identity of the most retarded component, a disaccharide, was ascertained by co-chromatography with di-*O*-sulfated iduronosyl-anhydro-[^{3}H]mannitol.

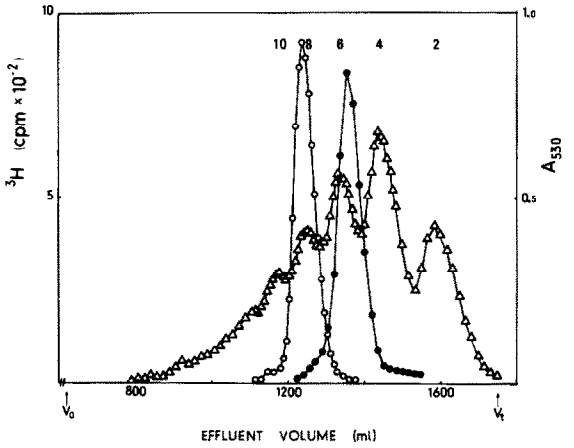

Fig.3. Gel chromatography on Sephadex G-50 of ^{3}H-labeled antithrombin-binding octasaccharide, before (○) and after (●) treatment with periodate-alkali. For preparation of octasaccharide heparin was treated with nitrous acid at −10°C for 35 min and the products precipitable with 10 vol. ethanol were fractionated by affinity chromatography on antithrombin–Sepharose. Material eluting at ≥0.9 M NaCl was recovered and fractionated by gel chromatography on Sephadex G-50. Effluent fractions corresponding to octasaccharides were pooled and reduced with sodium boro[^{3}H]hydride; the resulting labeled octasaccharide was purified by repeated affinity chromatography on antithrombin–Sepharose followed by gel chromatography on Sephadex G-50. Before analysis by gel chromatography the final sample of periodate-alkali-treated octasaccharide was mixed with 100 mg reference oligosaccharides (see the legend to fig,2); the elution pattern of these oligosaccharides was recorded by the carbazole reaction (△). For explanation of the numbering of the oligosaccharide peaks, see fig.2.

affinity for antithrombin at 0.05 M NaCl (i.e., the bulk of deamination products, ~90% of the starting material, removed in the initial, large-scale, affinity-chromatography step; carbazole-positive material in fig,2C) consisted largely of di-, tetra- and hexasaccharides. The absence of such components in the high-affinity fraction clearly demonstrates that the smallest heparin fragment with high affinity for antithrombin, that can be obtained by degradation with nitrous acid, is an octasaccharide.

Studies on the antithrombin-binding region in pig intestinal mucosa heparin showed the occurrence of a →IdUA→GlcNAc→GlcUA→GlcNSO$_3^-$ tetrasaccharide sequence, in which both uronic acid moieties were non-sulfated [5,6]. The location of this sequence in the antithrombin-binding, ^{3}H-labeled octasaccharide was established by periodate oxidation of the non-sulfated uronic acid units followed by alkaline cleavage of the products; the labeled fragment(s) formed should correspond to the interjacent sugar units between the glucuronic acid residue and the anhydro-[^{3}H]mannitol end group (see analogous experiment in [5]). The octasaccharide was quantitatively cleaved, yielding a distinct peak of labeled material that was somewhat retarded in relation to a reference hexasaccharide (fig.3) and would thus be identical with the pentasaccharide, [GlcNSO$_3^-$→IdUA(2-OSO$_3^-$)]$_2$→ anhydro-[^{3}H]mannitol (no 6-*O*-sulfate groups are indicated). The release of a labeled pentasaccharide can only be explained by the octasaccharide structure shown in fig.4, with the periodate-susceptible glucuronic acid unit located in position 3, and the non-sulfated iduronic acid component of the IdUA→ GlcNAc→GlcUA→GlcNSO$_3^-$→ sequence constituting the non-reducing terminal unit of the intact octasaccharide.

Our knowledge of the interaction between heparin and antithrombin is still fragmentary; little is thus known regarding which of the components of the octasaccharide structure are acutally involved in the binding to the protein. Since the nitrous acid reagent

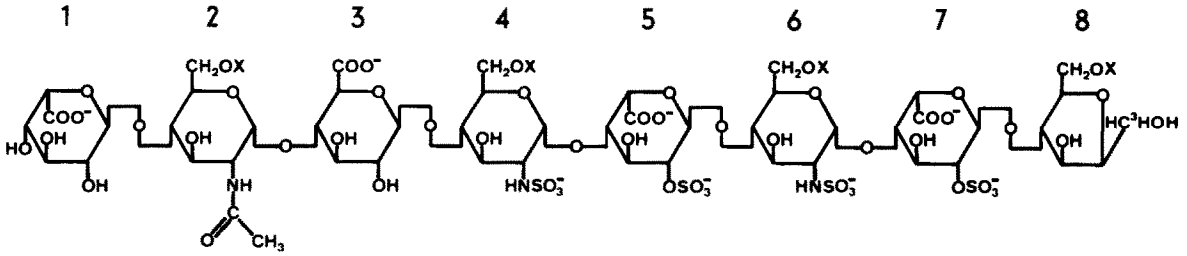

Fig.4. Tentative structure for the antithrombin-binding octasaccharide derived from pig mucosal heparin. X = H or SO$_3^-$. The ^{3}H-labeled 2,5-anhydro-mannitol residue in position 8 corresponds to an *N*-sulfated glucosamine unit in the intact polysaccharide. The structure shown represents the majority of antithrombin-binding sequences in pig mucosal heparin. However, structural variants compatible with affinity for antithrombin cannot be excluded (see [6] regarding the *N*-acetylglucosamine and glucuronic acid units in positions 2 and 3, respectively).

used to depolymerize heparin will attack at most every other glycosidic bond (the $GlcNSO_3^-\rightarrow$ linkages), it is obvious that the actual binding sequence will not necessarily span the entire octasaccharide structure; for instance, it seems likely that the anhydromannitol end group (position 8) may correspond to a glucosamine residue located outside the antithrombin-binding sequence in the intact heparin chain. Nevertheless, this region clearly extends beyond the tetrasaccharide segment (positions 1–4 in fig.4) implicated in [5]. Recent results demonstrate, in accord with this conclusion, that selective removal of either one of the two *N*-sulfate groups at positions 4 and 6, respectively, results in a distinct and appreciable loss in affinity of the octasaccharide for antithrombin (J. R., L. T., M. Höök, U. L., unpublished).

Acknowledgements

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