Antithrombotic Properties of a Dermatan Sulfate Hexadecasaccharide Fractionated by Affinity for Heparin Cofactor II

By P. Sie, D. Dupouy, C. Caranobe, M. Petitou, and B. Boneu

The relationship between the antithrombotic activity of dermatan sulfate (DS) in vivo and its catalytic effect on the inhibition of thrombin by heparin cofactor II (HC II) in vitro was investigated. DS was depolymerized by Smith degradation and the fragments obtained were separated by gel filtration. The fragment of minimal size with full catalytic activity was a hexadecasaccharide, which was further fractionated by affinity for immobilized HC II. Only a small proportion by weight (6.7%) was recovered in the high-affinity fraction, which had about 10 times more catalytic activity than the unfractionated oligosaccharide; the change in activity was primarily caused by the removal of inert materials, recovered in the low-affinity fraction. 1H-NMR spectra indicated strengthening of the signal given by ido A (2SO4) in the high-affinity fraction compared with that of the low-affinity fraction. The anticoagulant activity of the high-affinity fraction was exclusively HC II-dependent. The antithrombotic potency was evaluated in rabbits using the Wessler-thromboplastin model. Half-maximal prevention of thrombosis was obtained after injection of 250 µg/kg DS, of 500 µg/kg hexadecasaccharide, or of 60 µg/kg of its high-affinity fraction. The low-affinity fraction was ineffective at the highest dose tested (1,200 µg/kg) and did not potentiate the effect of the high-affinity fraction. These results show that the antithrombotic effect of DS is essentially dependent on HC II binding and activation and that HC II is therefore a suitable target for antithrombotic drugs.

FOR MANY YEARS, heparin and its derivatives have been the only glycosaminoglycans used as antithrombotic agents. Recently, nonheparin glycosaminoglycans such as dermatan sulfate (DS) have become available for experimental and clinical studies. DS-containing proteoglycans are present on the surface of cells and in the extracellular matrix. DS is a linear polymer made up of alternating units of N-acetyl-D-galactosamine and uronic (mainly iduronic) acid. The chain length may vary from 25 to 90 disaccharide units. Chemical heterogeneity results from varying degrees of O-sulfation and from the type of uronic acid: D-glucuronic or L-iduronic.1

DS enhances the action of heparin cofactor II (HC II), a serine protease inhibitor present in the plasma at micromolar concentrations that solely inhibits thrombin among the various proteases of the clotting system.2 The level of thrombin inhibition is increased by about 1,300-fold at optimal concentrations of DS.4 As a result of thrombin inhibition, DS has an anticoagulant activity about 50 times less potent than heparin on a weight basis.5 In vitro, the anticoagulant activity is entirely mediated through HC II.6

It has long been recognized that in a commercial preparation of DS, only a small proportion of the DS chains bind a column of immobilized HC II.7 The catalytic activity is likely to be achieved through a template mechanism that implies that both HC II and thrombin bind to a single glycosaminoglycan chain, as shown for heparin.8 In agreement with this, the smallest DS oligosaccharide to retain a catalytic activity is of 12 to 14 sugar residues whereas shorter oligosaccharides still bind to HC II.9 The structure of a DS hexa-saccharide that binds HC II with high affinity has been elucidated by Maimon and Tollefsen.10

The antithrombotic activity of DS was shown for the first time by Buchanan et al11 using a Wessler type thrombosis model in rabbits and has been confirmed in various other models and animal species.12-15 DS is effective in humans for the prophylaxis of deep venous thrombosis after hip fracture16 and for maintaining anticoagulation during hemodialysis.17

The mechanism of the antithrombotic activity of DS has not been firmly established. Considering the relatively high doses of DS needed to achieve a full antithrombotic effect, a mechanism independent of HC II (eg, a direct effect on vascular endothelium) cannot be ruled out. Fernandez et al12 showed a good correlation between the antithrombotic potency of different glycosaminoglycans and their respective abilities to catalyze thrombin inhibition by HC II or antithrombin (AT).11. But in our hands, the enhancement of the catalytic activity of DS by chemical sulfation failed to increase its antithrombotic potency.18

Whether or not HC II activation is a key mechanism for the antithrombotic effect of DS is an important question as it implies that HC II may be a suitable target for antithrombotic drugs. To address this question, we prepared well-defined DS oligosaccharide fractions with high and low affinity for HC II and we examined the effects of the hexadecasaccharide fractions in an experimental model of thrombosis.

MATERIALS AND METHODS

Depolymerisation of DS. To a solution of DS of porcine origin (2 g) dissolved in a sodium acetate buffer (0.05 mol/L; pH 5; 180 mL) previously cooled to 4°C, sodium periodate (0.1 mol/L in the same buffer) was added (20 mL). After 20 hours at 4°C, sodium borohydride (600 mg dissolved in 10 mL of water) was then added, and after 1 hour at room temperature, was followed by sulfuric acid (1 mol/L, 20 mL). The resulting solution was heated for 2 hours at 60°C, then neutralized by addition of sodium hydroxide and concentrated to a final volume of 60 mL. Salts were removed by passage through a column of Sephadex G-25 (Kabi-Pharmacia, Uppsala, Sweden) eluted with water and DS fragments were obtained (1.65 g) after lyophilization.

The mixture of different sized oligosaccharides thus obtained was then fractionated on a Sephadex G-50SF (Kabi-Pharmacia) column from the Laboratoire d'Hémostase, Centre de Transfusion Sanguine CHU Purpan, Toulouse; and Sanofi-Chaoy, Gentilly, France.

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(2.15 cm × 2 m) equilibrated in 0.2 mol/L sodium chloride. To obtain a suitable resolution, no more than 120 mg of the oligosaccharide mixture was applied per run. Under such conditions it was possible to use the humps on the chromatogram (see Fig 1) to pool the different fractions. The homogeneity of the individual fractions was checked by high performance liquid chromatography (HPLC) analysis under conditions already reported.19

Oligosaccharide concentration was determined by the carbazole assay.20

Fractionation by HC II affinity chromatography. Thirty milligrams of HC II, purified from human plasma,21 was noncovalently coupled to 10 mg of Concanavalin-A Sepharose (Kabi-Pharmacia) as described by Griffith and Marbet.7 The column was washed with 2 mol/L NaCl, 20 mmol/L Tris HCl pH 7.4 and equilibrated with 50 mmol/L NaCl, 20 mmol/L Tris HCl pH 7.4 before use. The capacity of the column varied with the oligosaccharide size, from 0.9 to 0.3 mg for the hexadeca to the decasaccharidic preparation, respectively. As we noted that on re-use the capacity tended to decrease, we prepared a second column similar to the first one and we continued the fractionation with the new column. The materials obtained using the two successive columns were pooled.

Twenty milligrams of each oligosaccharide diluted in the equilibration buffer was applied to the HC II Concanavalin-A Sepharose (Kabi-Pharmacia) column and washed with the same buffer at a flow rate of 50 mL/h. Bound oligosaccharide was eluted with 0.5 mol/L NaCl 20 mmol/L Tris HCl pH 7.4 buffer because preliminary experiments had shown that no significant amount of material was eluted below 0.25 mol/L or above 0.5 mol/L NaCl. The flow-through effluent was applied to the regenerated column and the process was repeated twice more. Although small amounts of material may bind to the affinity gel during the fourth run, we did not attempt to fractionate the material further. Bound material of the four runs was pooled (high-affinity fractions) and the flow-through effluent of the last run represented the low-affinity fraction. These steps were repeated until about 60 mg of hexadecasaccharide, tetradecasaccharide, decasaccharide, and decasaccharide had been chromatographed. Finally, the materials were desalted and concentrated by lyophilisation.

1H-NMR analysis. 1H-NMR experiments were performed, using a 500 MHz instrument, according to methods already reported.22

Kinetic analysis of thrombin inhibition by HC II in the presence of DS oligosaccharides. The ability of DS oligosaccharides to increase the rate of thrombin inhibition by HC II or antithrombin III was determined as previously described.19 The oligosaccharide concentrations required to inhibit half the thrombin activity under the assay conditions were determined (IC 50). The IC 50 of undegraded DS in the same series of experiments was also determined (usually 3 to 4 µg/mL). The activity of each fraction was expressed relative to the activity of DS by calculating the ratio of the respective IC 50. By definition, 1 unit of catalytic activity was equivalent to the activity of 1 µg of undegraded DS.

A more detailed kinetic analysis was performed with the hexadecasaccharide fractions because it was these fractions that were selected for the antithrombotic experiments (see below).

All reactions were performed at 37°C in polypropylene tubes in 150 mmol/L NaCl, 20 mmol/L Tris HCl pH 7.4 buffer containing 0.1% polyethylene glycol 6000. The reaction was initiated by adding thrombin to a solution containing HC II and the oligosaccharide to be tested, under a final volume of 100 µL. At suitable time intervals (from 2 seconds in the presence of high-affinity oligosaccharide to 4 minutes in the absence of any oligosaccharide), 10µL samples were removed and placed into disposable plastic cuvettes containing 490 µL of 0.2 mmol/L H-D-Phe-PipArg pNA (S 2,238; Kabi-Pharmacia) and 0.5 mg/ml Polybrene (Aldrich, Milwaukee, WI) in the above buffer. After 4 minutes, the amidolytic reaction was stopped by addition of acetic acid. The precise moments of sampling and stopping were recorded on a personal computer by using push button equipped pipettes. Absorbance at 405 nm was measured on a spectrophotometer connected to the same computer and the amidolytic activity was automatically calculated (instrument and program designed by C. Hemker, Rijkuniversiteit, Limburg). Assays were performed in triplicate.

Over the time chosen for each experiment, which varied from about 30 seconds in the presence of the high-affinity fraction to 15 minutes in the absence of oligosaccharide, the HC II-thrombin reaction followed apparent pseudo-first order kinetics. In a first series of experiments, thrombin and HC II concentrations were fixed (50 and 500 mmol/L, respectively) and oligosaccharide concentrations were varied. In a second series the HC II-thrombin reaction velocity

Fig 1. Gel filtration of DS oligosaccharides on Sephadex G 50-SF. Periodate oxidation theoretically only cleaves unsulfated iduronic and glucuronic acid residues that are converted into dialdehydes. The chemical structures generated by Smith degradation (periodate treatment followed by reduction and mild acid hydrolysis) of DS end up with a hydroxylated alpha-, beta-, or gamma-trihydroxylated carboxylic acid. In the text we have considered this residue as a monosaccharide and therefore we are dealing with even numbers of monosaccharide residues in oligosaccharides. Chain length was determined by HPLC and oligosaccharide concentration by the carbazole assay. The catalytic activity in the presence of purified HC II and thrombin was determined relative to undegraded DS and the specific activity (left ordinate axis) was expressed on a molar basis, taking into account the molecular weights of each fraction determined by HPLC.
was measured versus HC II concentration (0.25 to 3 μmol/L) in the presence of low oligosaccharide concentrations (12.5 nmmol/L for the high-affinity, and 300 nmmol/L for the unfractonated and low-affinity preparations).

Assessment of anticoagulant activities. Plasmas depleted of AT III, HC II, or both were prepared by immunoadsorption as previously described. Residual AT III and HC II were undetectable by rocket immunoelectrophoresis. A control plasma was prepared by passage through uncoupled Sepharose 4B (Kabi-Pharmacia) in the same experimental conditions. Low- and high-affinity hexadecasaccharidic preparations). The APTT was measured using the Actin FS reagent (Dade, Aguado, Puerto Rico). The TCT was measured using a solution of human thrombin (Fbrinex; Ortho-Diagnostics, Raritan, NJ) so that the baseline TCT was 18 to 20 seconds. Clotting times were recorded in duplicate on a KC 10 apparatus (Amelung, Lienne, Germany).

Assessment of antithrombotic properties. Preliminary studies had shown that the antithrombotic activity of low molecular weight DS administered by bolus injection was reduced compared to unfractonated DS. More precisely, below tetradecasaccharides, this activity decreased sharply (unpublished data, Sie, Petitou, and Boneu, May 1990). Under the hypothesis that antithrombotic and catalytic activities varied proportionally, it was calculated that a minimal amount of 3 mg of the high-affinity fraction of the hexadecasaccharide would be required for a proper assessment of the antithrombotic activity in our model. This quantity was available. In contrast, it was expected that the antithrombotic activity of the high-affinity fraction of shorter oligosaccharides could not be investigated because of the simultaneous decrease of catalytic activity and recovery (Table 1). So, we restricted our study to the hexadecasaccharidic fractions (and to natural DS for comparison).

Antithrombotic activities were investigated in rabbits according to the Wesler model using human brain thromboplastin as thrombogenic stimulus. Briefly, New Zealand male rabbits (2 to 3 kg) were anesthetized by intramuscular administration of Ketamine hydrochloride (Imalgene 1000; Rhône-Merieux, Lyon, France) and 5 mg·kg⁻¹ of xylazine (Rompun; Bayer, Leverkusen, Germany). Both jugular veins were isolated between two loose sutures. The left carotid artery was cannulated to allow the injection of 125I-labeled rabbit fibrinogen (5 μCi) followed 10 minutes later by the oligosaccharides and 3 minutes later by human thromboplastin at 1 mg·kg⁻¹ (Thromborel; Behringwerke, Marburg, Germany). Thirty seconds after the end of the thrombotic challenge, the jugular vein segments were occluded by the distal and proximal sutures and was maintained for 15 minutes. Then, the occluded segments were opened and the size of the formed thrombus was measured by radioactive counting. In saline-treated animals (n = 13), thrombus size was 123 ± 18 μL (mean ± SEM). The percentage of volume reduction in the treated group was used to quantitate the antithrombotic activity. In these experimental conditions, we repeatedly found that standard heparin gave a 50% reduction of thrombus size at the dose of 30 μg/kg.

Selected doses of each compound were injected to groups of four to six animals. Citrated blood samples were taken 3 minutes after treatment, just before thromboplastin injection, to determine the APTT and the TCT. In addition, plasma oligosaccharide concentrations were determined as previously described using the respective eluates (high affinity) were pooled separately, desalted, and concentrated. Concentrations were measured by the carbazole assay. Catalytic activities with purified HC II and thrombin were determined and expressed in units/μg relative to undegraded DS that was arbitrarily assigned an activity of 1 unit/μg.

The results of HC II affinity chromatography are summarized in Table 1. The amount of bound oligosaccharide decreased with each pass through the column. The catalytic activity of the last flow-through effluent was low. The relatively active materials, or so-called low-affinity fractions, represented the majority of the oligosaccharide mass. Only a small proportion by weight was recovered in the high-affinity fraction and it decreased with the oligosaccharide size. The activity of the high-affinity fraction also decreased with size. In the presence of purified AT III no catalytic activity was detectable indicating absence of contamination by traces of heparin-derived oligosaccharides (not shown).

H-NMR analysis. H-NMR analysis of the four fractions obtained after affinity chromatography showed the presence in the high-affinity material of signals at 5.1 to 5.2 ppm. Because of the small amount of material available, the high-affinity parts of the four different size fractions were pooled for further investigation using 2-D NMR techniques. It was clearly shown, using "inadequate" proton-proton experiments, that these signals corresponded to H-2 of 2-sulfated iduronic residues in dermatan sulfate. It is notable that these signals and the corresponding sulfated uronic acid residues were missing in the low-affinity material (Fig 2). Quantitative analysis of the proportion of sulfated and unsulfated iduronic acid residues indicated that 2-sulfated residues accounted for two thirds of total uronic acid in the pool of high-affinity material.

Kinetic properties of fractionated DS hexadecasaccharide. Figure 3 shows the changes in the second order rate constants of the HC II-thrombin reaction according to the concentration of each fraction. On a weight basis, activities

<table>
<thead>
<tr>
<th>Chain Length (monosacchar.)</th>
<th>MW (daltons)</th>
<th>Unfrac.</th>
<th>High Aff.</th>
<th>Low Aff.</th>
<th>% Weight in the High Aff. Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadeca</td>
<td>4,505</td>
<td>0.60</td>
<td>7.63</td>
<td>0.05</td>
<td>6.7</td>
</tr>
<tr>
<td>Tetradeca</td>
<td>4,114</td>
<td>0.36</td>
<td>4.14</td>
<td>0.07</td>
<td>4.5</td>
</tr>
<tr>
<td>Dodeca</td>
<td>3,515</td>
<td>0.13</td>
<td>1.81</td>
<td>0.03</td>
<td>2.2</td>
</tr>
<tr>
<td>Deca</td>
<td>2,865</td>
<td>0.03</td>
<td>0.29</td>
<td>&lt;0.01</td>
<td>1.2</td>
</tr>
</tbody>
</table>

DS was partially depolymerized by Smith degradation and the resulting fragments were isolated by gel filtration on Sephadex G50-5F. Apparent MWs were calculated from HPLC experiments using either synthetic or semisynthetic glycosaminoglycan fragments as standards. Indexes of dispersity found by HPLC varied between fractions from 1.04 to 1.07. DS oligosaccharides of various lengths were then applied to a column of HC II noncovalently linked to ConcanaVail A Sepharose. After successive recycling, the flow-through fraction (low affinity) and the NaCl eluates (high affinity) were pooled separately, desalted, and concentrated.

<table>
<thead>
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<th>RESULTS</th>
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Fractionation of DS oligosaccharides. Figure 1 shows the results of size fractionation of DS oligosaccharides after Smith degradation of DS. The catalytic activity sharply decreased in the decasaccharide fraction. Oligosaccharides containing 16 or more sugar residues had a fairly constant catalytic activity, above 60% of that of undegraded DS. So we decided to investigate the hexadecasaccharide, tetradecasaccharide, dodecasaccharide, and decasaccharide preparations. About 100 mg of each fraction was available.

The results of HC II affinity chromatography are summarized in Table 1. The amount of bound oligosaccharide decreased with each pass through the column. The catalytic activity of the last flow-through effluent was low. The relatively active materials, or so-called low-affinity fractions, represented the majority of the oligosaccharide mass. Only a small proportion by weight was recovered in the high-affinity fractions and it decreased with the oligosaccharide size. The activity of the high-affinity fraction also decreased with size. In the presence of purified AT III no catalytic activity was detectable indicating absence of contamination by traces of heparin-derived oligosaccharides (not shown).

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Fig 2. NMR spectroscopy. 500 mHz 1H-NMR spectra of low-affinity (top) or high-affinity (bottom) fractions. The signal at 5.2 ppm in the high-affinity fraction (arrow) is characteristic of 2-sulfated iduronic acid residues.

considerably varied between the three preparations as indicated by the differences in kd values. The unfractionated preparation was about five times less active than the high-affinity fraction and over 50 times more active than the low-affinity fraction. The maximum rate constants at saturating concentrations of each fraction (k° max) were very close. So, the difference in activity was probably caused by the presence of inactive materials in the unfractionated and low-affinity preparations. In support of this hypothesis, saturation kinetics with respect to HC II at low oligosaccharide concentrations (Fig 4) indicated that the affinity constant (Km) found for the three fractions were not substantially different. Apparent maximum velocities cannot be compared because they were obtained at different oligosaccharide concentrations.

Anticoagulant activities of fractionated DS hexadecasaccharides. To ensure that the effect of the materials used for antithrombotic experiments on the coagulation system was exclusively dependent on HC II, we prepared plasmas depleted of AT III and/or HC II and we performed APTT and TCT tests in the presence of the high- and low-affinity fractions of the DS hexadecasaccharide.

As shown in Table 2, the concentrations required for doubling the baseline APTT of the control plasma were 40 and 500 μg/mL for the high- and low-affinity fractions, respectively. At these concentrations, the same effect on the APTT was found in the AT III-depleted plasma, whereas in HC II-depleted plasmas the high-affinity fraction was not anticoagulant and the effect of the low-affinity fraction was largely reduced. The prolongation of the TCT by the two preparations was exclusively dependent on HC II.

Antithrombotic potencies of fractionated DS hexadecasaccharides. The results of the antithrombotic experiments are summarized in Table 3. Fifty percent thrombus prevention was obtained after the administration of an intravenous bolus dose of 500 μg/kg of the hexadecasaccharide. This was not significantly different from the antithrombotic effect of natural DS at the same dose. However, at 250 μg/kg the hexadecasaccharide had no antithrombotic activity unlike non-depolymerized DS. The low-affinity hexadecasaccharide had no activity at the maximum dose tested. The high-affinity hexadecasaccharide, in contrast, displayed a strong antithrombotic effect at relatively low doses. The same antithrombotic score (about 50% thrombus prevention) was obtained after the administration of 60 and 500 μg/kg of the high-affinity and unfractionated preparations, respectively. At 30 μg/kg the high-affinity fraction had no significant antithrombotic effect. The simultaneous administration of high- and low-affinity fractions at 30 and 1,200 μg/kg, respectively, remained ineffective.

No anticoagulant effect was detected 3 minutes after the intravenous bolus injection of any product at any dose using the APTT test. A small prolongation of the TCT was observed after the administration of 120 μg/kg of the high-affinity fraction (plus 5.6 ± 0.5 seconds) or of 500 μg/kg of non-depolymerized DS (plus 6.9 ± 1.9 seconds).

DISCUSSION

The present experiments were designed to investigate the relationships between the catalytic activity of DS on HC II-thrombin reaction and its antithrombotic properties in vivo. To this end, we depolymerized DS, selected a fragment of minimum size with a catalytic activity close to that of natural DS, and prepared fractions with low and high catalytic activities by affinity chromatography for HC II.

DS depolymerization was performed by Smith degradation. Using a similar method, Tollefsen et al9 found that the catalytic activity was lost in the decasaccharide fraction. In our hands, decasaccharides had a detectable activity. As the index of dispersity of this fraction was 1.05, contamination by adjacent dodecasaccharides is unlikely to account for this low activity. The molar specific activity above the tetradecasaccharide was fairly constant. On a weight basis, it was about

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Fig 3. Oligosaccharide-catalyzed inactivation of thrombin at varying concentrations of unfractonated, high-affinity, and low-affinity preparations of a DS hexadecasaccharide. Rates of thrombin inhibition by HC II were measured under pseudo first order conditions at fixed concentrations of thrombin (50 nmol/L) and HC II (500 nmol/L) in the presence of varying concentrations of oligosaccharide. Residual thrombin activity was discontinuously monitored with a chromogenic substrate as described in Materials and Methods. The apparent first order rate constant of the reaction (k') was obtained from the slope of a semilog plot of thrombin activity against time. The second order rate constant (k") was calculated by dividing k' by the concentration of HC II in the reaction. In the absence of oligosaccharide, k'' averaged \( \Delta k'' = 5.82 \times 10^{-5} \text{ mol/min} \). This value was subtracted from the rate constant measured in the presence of the oligosaccharide. The difference (AK'') represented the rate constant caused by catalysis by the oligosaccharide. Double reciprocal plotting of the data allowed the determination of the maximum rate constant (AK" max) and of the dissociation constant (kd) indicated for this particular experiment.

60% of that of natural DS. Taking into account the higher molecular mass of natural DS (range 12,000 to 45,000 daltons, peak 25,000 daltons: a molar specific activity cannot be calculated for such a heterogeneous compound), one can estimate that the catalytic activity per molecule does not increase above a chain length of 16 sugar residues. Therefore, although shorter DS oligosaccharides may have some catalytic activity\(^9^{10}\) (and the present study) the minimum structure required for full catalysis of the HC II-thrombin reaction appeared to be a hexadecasaccharide.

Fractionation of DS oligosaccharide by affinity chromatography indicated that only a small proportion (7% or less) of chains bound HC II with high affinity. This contrasts with the values of 25% to 45% reported by Tollefsen et al.\(^9\) As we deliberately overloaded the HC II-Sepharose column, we may have selected the molecules having the highest affinity for HC II. In support of this, the ratio between the specific activities of high-affinity fractions and those of the unfractonated preparation was 10 to 12 (Table 2). These ratios were only 2 to 4 in Tollefsen's study, indicating that in the con-

Fig 4. Oligosaccharide-catalyzed inactivation of thrombin by HC II: saturation kinetics with respect to HC II. The inhibition of thrombin (50 nmol/L) by HC II (0.25 to 3 \( \mu \text{mol/L} \)) in the presence of unfractonated (300 nmol/L), high-affinity (12.5 nmol/L) and low-affinity (300 nmol/L) preparations of a DS hexadecasaccharide was determined as described in Materials and Methods. The initial velocity of the reaction \( (V_i = k \cdot [\text{thrombin}] ) \) was plotted against the HC II concentration according to the following equation: \( \frac{[\text{HC II}]}{V_i} = \frac{[\text{HC II}]}{V_m} + \frac{K_m}{V_m} \). where \( V_m \) is the apparent maximum reaction velocity at saturation with HC II and \( K_m \) the oligosaccharide HC II dissociation constant. The equation is derived from the equilibrium of a reaction mechanism analogous to random order, bireactant enzyme catalysis.\(^6\)
The residual activity of the low-affinity fraction was not distinguishable from that of the high-affinity fraction. Whether or not these were supported by molecules having the same structure remains to be determined.

The high-affinity HC II binding region has recently been elucidated as a hexasaccharide made of a repeat of a single disulfated disaccharide Ido A (2S04) Gal Nac (4S04).10 Hexasaccharides containing four or five sulfate groups per molecule bind HC II with less affinity. In agreement with these data, NMR spectra indicated strengthening of the signal given by Ido A (2S04) in the high-affinity fraction compared with the unfractionated preparation. We had also noticed in the past that the main difference in the NMR spectra of highly active and weakly active DS fractions obtained by ion exchange chromatography occurred in the intensity of the signals at 5.2 ppm (Petitou and Sié, unpublished data, May 1989).

The ratio of sulfated to unsulfated iduronic acid units in the active fractions is compatible with the occurrence of a hexasaccharide sequence in our products. The fact that unsulfated iduronic acid is missing in the low-affinity material could have gone undetected by NMR spectroscopy.

The proportion of material bound to HC II decreased from the hexadecasaccharide to the decasaccharide fractions, by about 85%. This was estimated by the carbazole reaction, which is probably not influenced by the sulfate group substituted on the uronic acid. This decrease was rather unexpected since, in the Smith degradation procedure, cleavage does not occur randomly and the HC II-binding region, which does not contain unsulfated iduronic acid residues, should be preserved. Assuming that the molecular weight decreased from 4,505 daltons for the hexadecasaccharide to 2,865 daltons for the decasaccharide, the mass associated to a constant number of HC II-binding sites would decrease in the same proportions (ie, by only 36%). A possible explanation could be that sulfated uronic acids are clustered during the biosynthesis of the DS polymer, extending beyond the minimal hexasaccharide HC II-binding site to form longer sequences, uncleavable by Smith’s procedure.

The unfractionated hexadecasaccharide was slightly less effective than natural DS in preventing thrombosis in Wessler’s thromboplastin model. This may be caused by its lower catalytic activity, which was only 60% of that of natural DS on a weight basis. Alternatively, the hexadecasaccharide may have rapidly diffused toward the extravascular compartment, a phenomenon that occurs with low molecular weight DS but not with natural DS.23 Indeed, 3 minutes after the intravenous injection, the plasma concentrations of hexadecasaccharide were significantly lower than those of DS.

Because of the shortage of material, a single dose of low-affinity hexadecasaccharide was investigated. It was ineffective, indicating that the antithrombotic activity of DS is essentially dependent on HC II-binding and activation. Although it cannot be concluded from a single-dose experiment...
that low-affinity fractions do not potentiate high-affinity fractions, our results contrast with findings reported for heparin oligosaccharides having different affinities for AT III where inactive species may offer some protection from neutralization or scavenging processes in vivo by competing with active species.

High-affinity hexadecasaccharide was about 10 times more antithrombotic than the unfractionated material. As the ratio of catalytic activities in vitro was also about 10, this supported the idea that the antithrombotic potency in vivo was directly proportional to the catalytic activity on thrombin-HC I1 reaction in vitro. We previously found that heavily sulfated DS was not superior to natural DS as an antithrombotic despite its higher catalytic activity in vitro. It must be recalled that in this case the average number of sulfate groups per disaccharide was 3.7, i.e., significantly higher than 2 in the "canonic" hexaxasaccharide. In addition, position six of the galactosamine moieties was quantitatively substituted, whereas in the present study Ido A (250) is the marker of active species. Finally, oversulfation of DS resulted in anticoagulant effects independent of HC II where similar to those of polyanionic polymers. This was not the case for the high-affinity DS hexadecasaccharide that required HC II for its anticoagulant effect. Thus, it is likely that selective sulfation at position two of iduronic acid is preferable to extensive and nonselective sulfation to improve the antithrombotic potency of natural DS. As shown in the model used in this study, efficient doses of high-affinity DS hexadecasaccharide approached those of standard heparin on a weight basis, and we conclude that selective activation of HC II is a promising approach in the search for new antithrombotic agents.

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