Structural and functional properties of heparin analogues obtained by chemical sulphation of *Escherichia coli* K5 capsular polysaccharide

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Capsular polysaccharide from *Escherichia coli* K5, with the basic structure $(GlcA\beta1\text{-}4GlcNAc\alpha1\text{-}4)_n$, was chemically modified through N-deacetylation, N-sulphation and O-sulphation [Casu, Grazioli, Razi, Guerrini, Naggi, Torri, Oreste, Tursi, Zoppetti and Lindahl (1994) Carbohydr. Res. **263**, 271–284]. Depending on the reaction conditions, the products showed different proportions of components with high affinity for antithrombin (AT). A high-affinity subfraction, M_r approx. 36000, was shown by near-UV CD, UV-absorption difference spectroscopy and fluorescence to cause conformational changes in the AT molecule very similar to those induced by high-affinity heparin. Fluorescence titrations demonstrated about two AT-binding sites per polysaccharide chain, each with a K_d of approx. 200 nM. The anti-(Factor Xa) activity was 170 units/mg, similar to that of the IIId international heparin standard and markedly higher than activities of previously described heparin analogues. Another preparation, M_r approx. 13000, of higher overall *O*-sulphate content, exhibited a single binding site per chain, with K_d approx. 1 μM, and an anti-(Factor Xa) activity of 70 units/mg. Compositional analysis of polysaccharide fractions revealed a correlation between the contents of -GlcA-GlcNSO$_3$(3,6-di-OSO$_3$)- disaccharide units and affinity for AT; the 3-O-sulphated GlcN unit has previously been identified as a marker component of the AT-binding pentasaccharide sequence in heparin. The abundance of the implicated disaccharide unit approximately equalled that of AT-binding sites in the 36000-M_r polysaccharide fraction, and approached one per high-affinity oligosaccharide (predominantly 10–12 monosaccharide units) isolated after partial depolymerization of AT-binding polysaccharide. These findings suggest that the modified bacterial polysaccharide interacts with AT and promotes its anticoagulant action in a manner similar to that of heparin.

INTRODUCTION

Heparin is a complex sulphated glycosaminoglycan produced by connective-tissue-type mast cells. The best studied of its many biological effects is its ability to prevent the coagulation of blood (for a recent review, see ref. [1]); as the result of this property, heparin is widely used in the clinic for prevention and treatment of thromboembolic disease. The anticoagulant activity of heparin is expressed through binding of the polysaccharide to antithrombin (AT), which leads to acceleration of the rate at which this proteinase inhibitor complexes with and thereby inactivates the enzymes involved in blood coagulation. Heparin and the related polysaccharide, heparan sulphate, which is produced by a variety of cell types, have heterogeneous and variable structures, such that even highly purified preparations display a multitude of different saccharide sequences. They are generated by partial enzymic modification of a basic $(GlcA\beta1\text{-}4GlcNAc\alpha1\text{-}4)_n$ polymer, involving N-deacetylation and N-sulphation of GlcNAc residues, C-5 epimerization of GlcA to IdoA units, and O-sulphation at various positions (for reviews of the biosynthetic process, see refs. [1–3]). The anticoagulant activity of heparin and heparan sulphate is due to the occurrence of a specific pentasaccharide sequence that mediates the interaction with AT [4–7]; the structure is shown in Figure 1. The distinguishing structural feature of the AT-binding region is the 3-O-sulphated glucosamine residue, which is present in about one-third of the heparin chains in commercial heparin. These components bind with high affinity ($K_d \leqslant 10^{-8}$ M) to AT (HA-heparin), whereas polysaccharide chains lacking 3-*O*-sulphate groups show much weaker interaction ($K_d \sim 10^{-5}$ M) (LA-heparin). The specific AT-binding pentasaccharide has been generated by chemical synthesis [8]. Moreover, attempts have

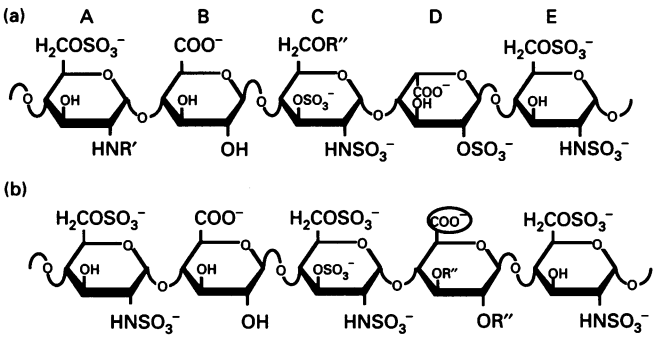

Figure 1 Structures of AT-binding regions in heparin (a) and compound Bb-2 (b)

R′ = -COCH$_3$ or -SO$_3$; R″ = -H or -SO$_3$. The circled carboxyl group indicates the main structural difference between the two sequences. For additional information see the text.

Abbreviations used: aMan$_R$, 2,5-anhydro-D-mannitol residue obtained by reduction of terminal 2,5-anhydro-D-mannose residue with $NaBH_4$; AT, antithrombin; GlcA, D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine); HA-heparin, heparin with high affinity for AT; IdoA, L-iduronic acid; LA-heparin, heparin with low affinity for AT. The positions of *O*-sulphate groups in a polysaccharide chain are indicated in parentheses.

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been made to produce polysaccharides that mimic the anticoagulant properties of heparin, by chemical modification of polysaccharides from various sources [9]. Most of these attempts have met with limited success, mainly because of the difficulties in simulating the specific AT-binding pentasaccharide sequence.

We recently described the generation of a series of heparin-like products through regioselective sulphation of the capsular polysaccharide from *Escherichia coli* K5 [10]. This polymer, which has the same $(GlcA\beta1\text{-}4GlcNAc\alpha1\text{-}4)_n$ structure as the biosynthetic precursor of heparin/heparan sulphate (sometimes referred to as *N*-acetylheparosan), provided a novel starting material for the semisynthetic approach to the formation of heparin-like compounds. The *E. coli* K5 polysaccharide was first converted into sulphaminoheparosan by N-deacetylation (through hydrazinolysis) followed by N-sulphation (treatment with trimethylamine sulphur trioxide complex). Controlled O-sulphation of the product yielded a series of novel compounds with varied O-sulphation patterns and anticoagulant activities. Chromatography of one of these compounds (Bb-2) on immobilized AT revealed a fraction with an apparent affinity for AT that was intermediate between those of HA- and LA-heparin. Preliminary structural analysis suggested the occurrence in this fraction of 3-O-sulphated glucosamine units.

The present study was undertaken to verify that the chemical modification of the *E. coli* K5 polysaccharide had indeed generated saccharide sequences capable of specific interaction with AT. The results indicate, similarly to previous studies of heparin biosynthesis [11], that the formation of components with high affinity for AT and high anticoagulant activity depends on 3-O-sulphation of glucosamine residues. Moreover, it is demonstrated that interaction with such a saccharide causes a conformational change in AT similar to that induced by HA-heparin. A subfraction obtained by affinity chromatography on immobilized AT showed an anti-(Factor Xa) activity comparable with that of heparin and appreciably higher than that of any previously described heparin analogue.

EXPERIMENTAL

Materials

NaB^3H_4 (5–15 Ci/mmol) was purchased from New England Nuclear (Dreeich, Germany). Sephadex G-50 (superfine grade), Sephadex G-15 and Sephacryl S-300 HR were obtained from Pharmacia Biotechnology. Bovine liver β-D-glucuronidase (type B-10) was purchased from Sigma, and the chromogenic substrate (S-2222) for determination of Factor Xa from Pharmacia. Human AT was isolated as described by Miller-Andersson et al. [12]. Bovine Factor Xa was a gift from J. Stenflo, Malmö Allmänna Sjukhus, Sweden.

E. coli K5 capsular polysaccharide was chemically modified (extensively N-deacetylated and N-sulphated, variously O-sulphated) as described previously [10]. Two preparations, Bb-2 and Bc-2, with anti-(Factor Xa) activities that were 7 and 28% respectively of that of a reference heparin preparation, were selected for further characterization. Compound Bb-2 was obtained by treating the tributylammonium salt of the N-sulphated intermediate with anhydrous dimethylformamide and pyridine sulphur trioxide complex (5 equiv. of SO_3 per equiv. of free OH groups) at 0 °C for 1 h. The product contained a total of 2.1 sulphate residues per disaccharide unit. To obtain compound Bc-2, the intermediate was treated twice in a similar fashion, yielding a product with 2.5 sulphate residues per disaccharide unit.

The two modified polysaccharides were subjected to affinity chromatography on AT–Sepharose as described [10]. In a preparative fractionation of compound Bb-2, subfractions with increasing affinity for AT were pooled as shown in Figure 2(a). The high-affinity fraction 5 of compound Bb-2 and not affinity-fractionated compound Bc-2 were separated further by gel chromatography on a column (3 cm × 100 cm) of Sephacryl S-300, eluted with 0.2 M NH_4HCO_3. Fractions around the peak region of the elution profiles, about 60% of the polysaccharides applied (results not shown), were pooled and desalted by lyophilization. For analytical gel chromatography the products were run on a column (1 cm × 100 cm) of Sephacryl S-300 in 1 M NaCl. The column was calibrated with samples of porcine mucosal heparin of known M_r (8400, 13200, 21500 and 34700), determined by sedimentation-equilibrium ultracentrifugation [13]. Polysaccharide concentrations were determined using the carbazole reaction for hexuronic acid [14].

Pig mucosal heparin, and its subfractions of low and high affinity for AT, were as described previously [15].

Preparation and analysis of oligosaccharides

N-Sulphated polysaccharides were degraded to GlcA-[1-^{3}H]aMan$_R$ disaccharides by treatment with HNO_2 (pH 1.5) followed by reduction of the products with NaB^3H_4, as described [16]. The products were analysed by anion-exchange HPLC on a Partisil-10 SAX column connected to a radioflow detector [17]. Alternatively, they were separated by high-voltage paper electrophoresis on Whatman no. 3 MM paper (40 V/cm) in 1.6 M formic acid (pH 1.7). ^{3}H-labelled mono- and di-saccharide standards were as described previously [18].

Partial O-desulphation of disaccharides was achieved by treatment with 0.2 M trifluoroacetic acid at 100 °C for 30 min in sealed glass tubes, followed by removal of the acid in a rotary evaporator. Digestion of disaccharides with β-D-glucuronidase was performed as described previously [19].

Oligosaccharides with high affinity for AT were obtained as follows. A sample (50 mg) of compound Bb-2 fraction 4 was partially degraded by limited deamination, involving treatment with 10 μl of HNO_2 reagent (pH 1.5; see ref. [20]) in a total volume of 2 ml of water, acidified to pH 1.5 with H_2SO_4. After 3 h at room temperature, the reaction mixture was neutralized with Na_2CO_3 and then reduced with 5 mCi of NaB^3H_4. Radiolabelled oligosaccharides were recovered by gel chromatography through a column (2 cm × 125 cm) of Sephadex G-15, equilibrated with 0.2 M NH_4HCO_3, and then lyophilized. After further separation by gel chromatography on a column (1 cm × 190 cm) of Sephadex G-50 in 1 M NaCl, fractions ranging from tetrasaccharides to dodecasaccharides were pooled, desalted and subjected to affinity chromatography on AT–Sepharose. The column (1.5 cm × 6 cm) was eluted with a salt gradient (50 mM to 3 M NaCl in 0.05 M Tris/HCl, pH 7.4). The most retarded components were subjected to compositional analysis based on exhaustive deamination followed by reduction of disaccharides with NaB^3H_4 [17].

Spectroscopic methods

Near-UV CD was performed at 22±2 °C in 20 mM sodium phosphate/0.1 M NaCl, pH 7.4, with a Jasco J41A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) and a 1 cm-pathlength cell [21]. The AT concentration was 25 μM and the molar ratio of polysaccharide to protein was 1.5:1.

UV-absorption difference spectra were measured at 22±2 °C with a Cary model 3 spectrophotometer (Varian Techtron, Victoria, Australia) essentially as described previously [21]. The

buffer and the protein and polysaccharide concentrations were as for the measurement of CD spectra.

Fluorescence was measured at 25±0.2 °C in an SLM 4800 S spectrofluorimeter (SLM-Aminco, Urbana, IL, U.S.A.) with an excitation wavelength of 280 nm. Cells of 1 cm pathlength were used, and samples were continuously stirred during measurements. The buffer consisted of 20 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 100 μM EDTA and 0.1% poly(ethylene glycol). Emission spectra were recorded with excitation and emission bandwidths of 4 nm, at AT and polysaccharide concentrations of 1 and 5 μM respectively. The spectra were corrected for the wavelength-dependent response of the photomultiplier tube. Titrations of AT with the modified K5 polysaccharides for determination of stoicheiometries and binding constants were monitored at the wavelength of the maximal fluorescence change (339–340 nm), with excitation and emission bandwidths of 4–8 and 16 nm respectively. The measurements were made after successive additions of 5 μl of either Bb-2 fraction 5 or Bc-2 (10–100 μM) to a cuvette with 2 ml of AT (0.1–2 μM). Stoicheiometries and binding constants were evaluated by non-linear regression analysis of the titration curves [22].

^{1}H-n.m.r. spectra were obtained at 500 MHz with a Bruker AMX 500 instrument equipped with a 5 mm ^{1}H/X inverse probe. Samples were dissolved in ^2H_2O (0.5 ml; 99.99% ^{2}H). Chemical shifts are given in p.p.m. downfield from internal sodium 3-(trimethylsilyl)propionate.

Anticoagulant activity

The ability of modified K5 polysaccharide to potentiate the inactivation of Factor Xa by AT was determined as described [23]. The rate of Factor Xa inhibition by AT in the presence of different concentrations (0.25–2 nM) of either the IIId international heparin standard or modified K5 polysaccharide was measured at 37 °C in 0.05 M Tris/HCl buffer, pH 7.4, containing 0.1 M NaCl and 1% poly(ethylene glycol) by a two-step procedure. First, 45 μl of 160 nM AT was preincubated for 5 min at 37 °C with 30 μl of 1–8 nM polysaccharide and then 45 μl of 68 nM Factor Xa was added. After an additional 5 min, the second step was performed by measuring residual Factor Xa activity. A 100 μl aliquot of the reaction mixture was added directly to a cuvette containing 500 μl of 50 mM Tris/HCl/0.1 M NaCl/0.2 mg/ml polybrene, pH 8.3, and 100 μl of 2 mM chromogenic substrate S-2222 in water. Changes in A_{405} were measured for 3–5 min in a Cary model 3 spectrophotometer. Factor Xa activity was calculated from the initial slopes of the resulting curves.

RESULTS

Fractionation of polysaccharides

Affinity chromatography of compound Bb-2 on AT–Sepharose yielded two distinct peaks, one of which, corresponding to about half of the total material, was essentially unretarded, indicating low affinity for the proteinase inhibitor (Figure 2a). The second fraction emerged at an elution position intermediate between those of LA- and HA-heparin. The separated polysaccharide was subdivided into five fractions as shown in Figure 2(a). Preparative gel chromatography of fraction 5 on Sephacryl S-300 (see the Experimental section) gave a broad elution pattern indicating polydispersity (results not shown). The pool selected for further studies had a relatively high M_r, with an estimated average of approx. 36000, as indicated by analytical rechromatography on a Sephacryl S-300 column calibrated with heparin fractions of known M_r (Figure 3).

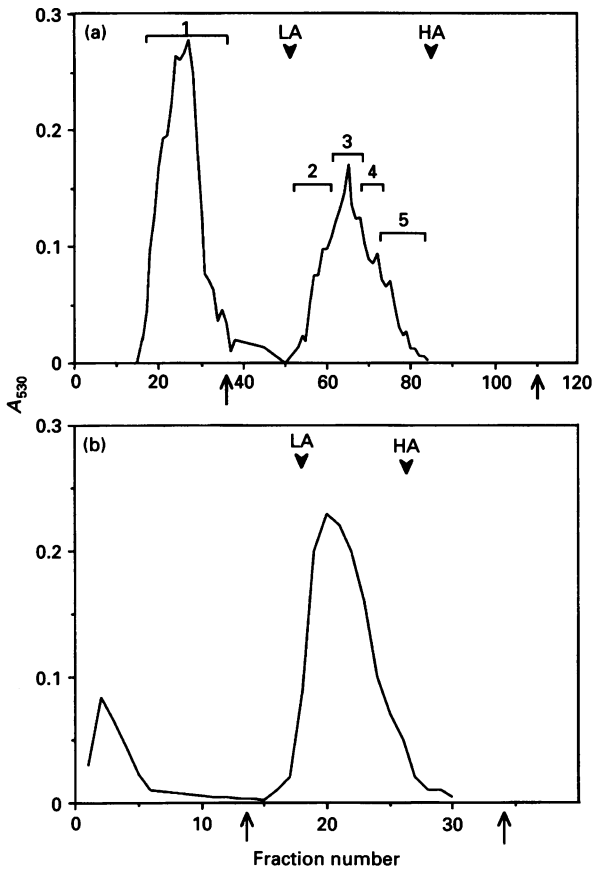

Figure 2 Affinity chromatography of chemically modified K5 polysaccharide on immobilized AT

(**a**) Preparative fractionation of compound Bb-2 on a 300 ml column (24 cm × 4 cm) of AT–Sepharose, eluted with a linear salt gradient (0.05 M NaCl to 3 M NaCl in 0.05 M Tris/HCl, pH 7.4, extending between the arrows; total gradient volume 3 litres). Effluent fractions were analysed for hexuronic acid by the carbazole reaction. Fractions were recovered as indicated and desalted by dialysis against water. For additional experimental details, see ref. [10]. (**b**) Analytical fractionation of preparation Bc-2 on a 3 ml column of AT–Sepharose. The same salt gradient was used as in (**a**), but the total volume was 80 ml. The peak elution positions of standard LA- and HA-heparin are indicated by arrowheads.

Chromatography on AT–Sepharose of compound Bc-2 showed a prominent fraction (about 85% of the total material) at a retarded elution position, similar to that of compound Bb-2 (Figure 2b). The same material (without subfractionation of AT–Sepharose) was subjected to preparative gel chromatography on Sephacryl S-300. The resultant elution pattern (not shown) was again rather broad but shifted toward a more retarded elution position than that of compound Bb-2 fraction 5. Analytical gel chromatography of a fraction obtained after removal of the extreme high- and low-M_r components indicated an average M_r of about 13000 (Figure 3).

Interaction with AT

Interaction between compound Bb-2 fraction 5 and AT was studied by near-UV CD, UV-absorption difference spectroscopy

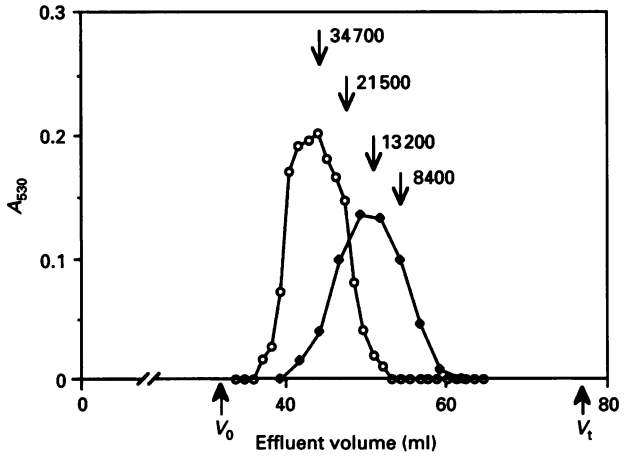

Figure 3 Analytical gel chromatography of size-fractionated chemically modified K5 polysaccharides

Samples were first fractionated on a preparative column of 300 ml of Sephacryl S-300 in 0.2 M NH_4HCO_3. Fractions around the peak regions of the elution profiles were pooled and lyophilized. Samples of the recovered material were then analysed on a column (1 cm × 100 cm) of Sephacryl S-300 eluted with 1 M NaCl; effluent fractions were analysed for hexuronic acid by the carbazole reaction. ○, Fraction 5 of AT-affinity-fractionated compound Bb-2; ◆, compound Bc-2 (not fractionated with regard to affinity for AT). The peak elution positions of heparin standards of known M_r are indicated by arrows. For additional information see the Experimental section.

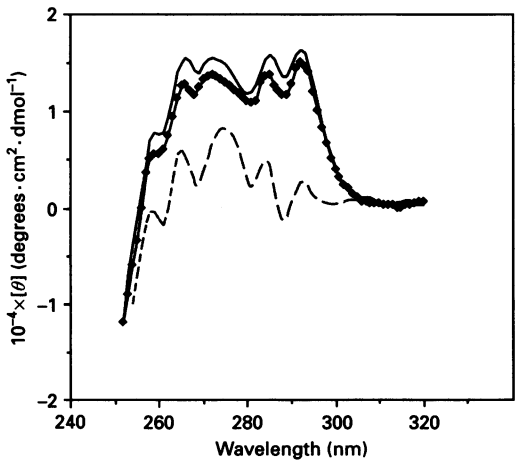

Figure 4 Effects of polysaccharides on the near-UV CD spectrum of human AT

Spectra were recorded for human AT alone (– – –) or for AT in the presence of either HA-heparin (——) or compound Bb-2 fraction (◆). The protein concentration was 25 μM and the molar ratio of polysaccharide/AT was 1.5 for both heparin and the modified K5 polysaccharide. The unit on the ordinate is molar ellipticity.

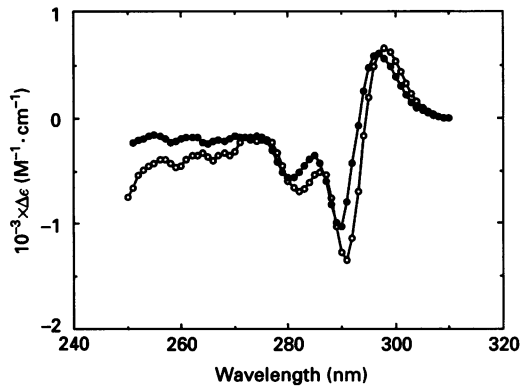

Figure 5 UV-absorption difference spectra of human AT induced by HA-heparin (○) or fraction 5 of compound Bb-2 (●)

The protein concentration and molar ratios of polysaccharide to protein were as given in the legend to Figure 4. The small shift in the wavelengths may be due to the fact that the experiments were performed on different occasions with different batches of AT.

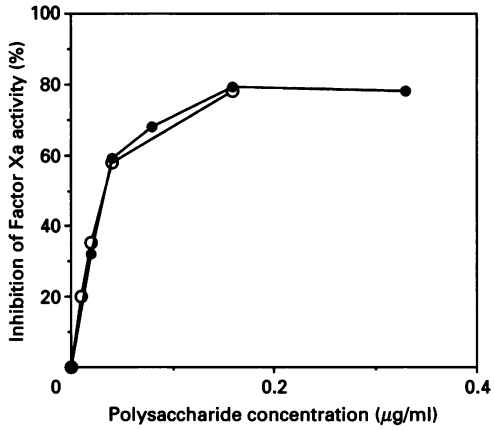

Figure 6 Inhibition of Factor Xa

Effects of fraction 5 of compound Bb-2 (○) and of standard heparin (●) on inhibition of Factor Xa activity by AT were determined as described in the Experimental section. The results are expressed as percentage inhibition of Factor Xa activity induced by different concentrations of the polysaccharides, above that induced by AT alone.

and fluorescence, under conditions giving 96% or more saturation of AT with the polysaccharide, as inferred from the binding stoicheiometry and affinity measurements described below. The modified K5 polysaccharide induced changes in both the CD and absorption difference spectra of AT that were very similar to those caused by HA-heparin (Figures 4 and 5). The polysaccharide also enhanced the fluorescence-emission intensity of AT about 25% without shifting the wavelength of the maximum emission, 340 nm (results not shown). This enhancement is intermediate between those caused by HA- and LA-heparin, i.e. 40% and 10% respectively [21].

An apparent polysaccharide/protein binding stoicheiometry of 0.52 ± 0.08 (mean ± S.E.M., $n = 4$) was obtained for compound Bb-2 fraction 5 by titrations of AT with the polysaccharide, monitored by the fluorescence increase at 340 nm, at protein concentrations of 1–2 μM. Similarly, a dissociation constant of 200 ± 36 nM (mean ± S.E.M., $n = 6$) was measured for the complex by titrations at protein concentrations of 0.1–2 μM. A binding stoicheiometry of about 1 and a K_d of approx. 1 μM was estimated for unfractionated compound Bc-2 in a single titration at 1 μM AT concentration.

Anticoagulant activity

The rate of Factor Xa inactivation by AT was determined in the presence of compound Bb-2 fraction 5, compound Bc-2 or the IIId international heparin standard. Figure 6 shows the extent of

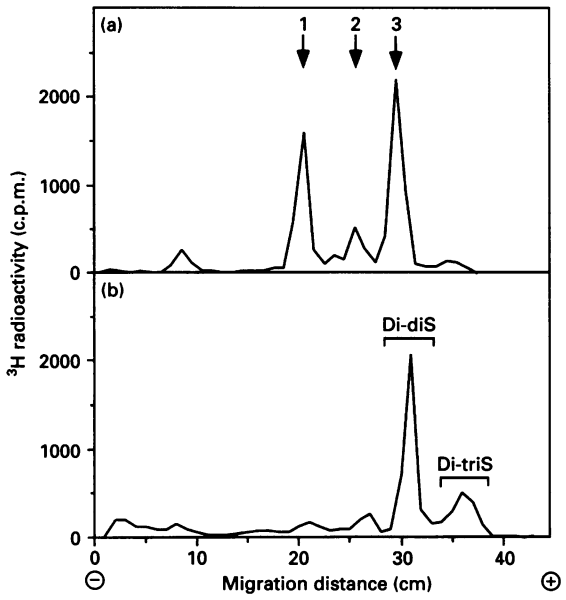

Figure 7 High-voltage paper electrophoresis of disaccharides

The ^{3}H-labelled disaccharide deamination products were derived from (**a**) compound Bb-2 fraction 5 or (**b**) compound Bc-2. Polysaccharide samples were subjected to HNO_2 (pH 1.5)/NaB^3H_4 treatment and the resultant disaccharides were recovered by gel chromatography and then further separated by paper electrophoresis at pH 1.7. The migration positions of the standards are indicated by the numbered arrows: 1, GlcA-aMan$_R$(6-OSO$_3$) (monosulphated disaccharide); 2, aMan$_R$(6-OSO$_3$) (monosulphated monosaccharide); 3, IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$) (disulphated disaccharide). The separation illustrated in (**a**) was analytical scale (about 10000 c.p.m.) whereas that in (**b**) was preparative (about 1000000 c.p.m.; narrow guide-strip counted). The di- and tri-sulphated disaccharides derived from compound Bc-2 were recovered as indicated by the horizontal bars, and were eluted from the paper strips for further analysis.

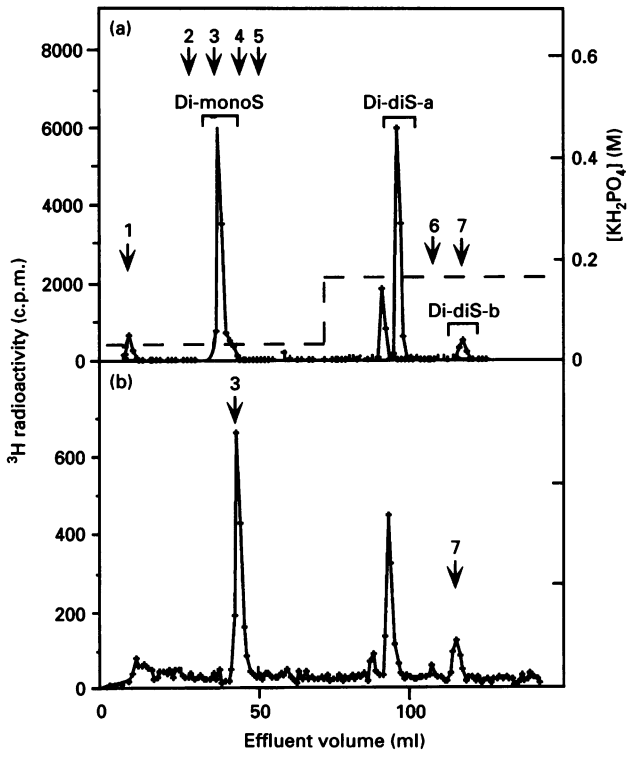

Figure 8 Anion-exchange HPLC of disaccharides

The ^{3}H-labelled disaccharide deamination products were derived from (**a**) compound Bb-2, fraction 5 or (**b**) oligosaccharides with high affinity for AT, isolated after partial deaminative cleavage of compound Bb-2 fraction 4. The separations were performed on a Partisil-10 SAX column (0.46 cm × 25 cm) (Whatman) eluted at a rate of 1 ml/min using step gradients with increasing concentrations of aq. KH_2PO_4. Monosulphated disaccharides were eluted with 0.026 M and disulphated disaccharides with 0.15 M KH_2PO_4, as indicated by the dashed line. The elution positions of internal standards, all containing [1-^{3}H]aMan$_R$ units, are indicated by numbered arrows: 1, aMan$_R$(6-OSO$_3$) and non-sulphated HexA-aMan$_R$; 2, GlcA(2-OSO$_3$)-aMan$_R$; 3, GlcA-aMan$_R$(6-OSO$_3$); 4, IdoA-aMan$_R$(6-OSO$_3$); 5, IdoA(2-OSO$_3$)-aMan$_R$; 6, IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$); 7, GlcA-aMan$_R$(3,6-di-OSO$_3$). Effluent fractions of volume 1 ml were collected and analysed for radioactivity. (**a**) Preparative run, where fractions corresponding to monosulphated and disulphated disaccharides were pooled as indicated by the horizontal bars. Disaccharides were desalted on 1 ml columns of DEAE-cellulose, that were washed with 0.05 M NH_4HCO_3 after application of the samples, and then eluted with 0.5 M NH_4HCO_3. The 0.5 M eluates contained the disaccharides and were lyophilized. For additional information, see the Experimental section.

Factor Xa inhibition, measured at different concentrations of compound Bb-2 fraction 5 and expressed as percentage inhibition of the activity initially present. The linear portion of the resultant curve virtually coincides with that of the heparin standard, indicating similar specific anti-(Factor Xa) activity (approx. 170 units/mg). The activity for compound Bc-2 (which had not been fractionated by affinity chromatography on AT–Sepharose) was lower (about 70 units/mg).

Structural analysis

The two polysaccharide preparations compound Bb-2 fraction 5 and compound Bc-2, were subjected to compositional analysis based on identification of GlcA-[1-^{3}H]aMan$_R$ disaccharides generated by HNO_2/NaB^3H_4 treatment of the polymers. Of particular interest was the occurrence of the 3-O-sulphated glucosamine residue previously implicated as a marker component of the specific AT-binding pentasaccharide region (see the Introduction).

Disaccharides accounted for about 90% of the labelled deamination products obtained from compound Bb-2 fraction 5, as expected for an extensively N-sulphated polysaccharide. High-voltage paper electrophoresis indicated major components of mono-O- and di-O-sulphated disaccharides, with much smaller amounts of non-sulphated and tri-O-sulphated species (Figure 7a). This pattern was verified by anion-exchange HPLC (Figure 8a). A major peak (designated Di-monoS) appeared at the elution position of the monosulphated disaccharide, GlcA-aMan$_R$(6-OSO$_3$). A second peak of approximately similar size (Di-diS-a), immediately preceded by a smaller peak (not characterized further), emerged in the approximate elution position of disulphated disaccharides but separate from any of the disaccharide standards available to us. In addition, a smaller peak (Di-diS-b) was seen at the position expected for GlcA-aMan$_R$(3,6-di-OSO$_3$). Component Di-monoS was completely eliminated by digestion of the disaccharide sample with β-D-glucuronidase before HPLC, and was replaced by a less retarded peak in the aMan$_R$-monosulphate region (results not shown), thus confirming the identification of GlcA-aMan$_R$(6-OSO$_3$). Components Di-diS-a and -b were recovered and desalted on small columns of DEAE-cellulose as described in the legend to Figure 8. Digestion of Di-diS-b with β-D-glucuronidase resulted in the formation of labelled disulphated aMan$_R$ monosaccharide, as demonstrated by paper electrophoresis (see Figure 10a) and by anion-exchange HPLC (results not shown), thus identifying the parent disaccharide as GlcA-aMan$_R$(3,6-di-OSO$_3$) (3–4% of the total ^{3}H-labelled disaccharide units). Di-diS-a resisted

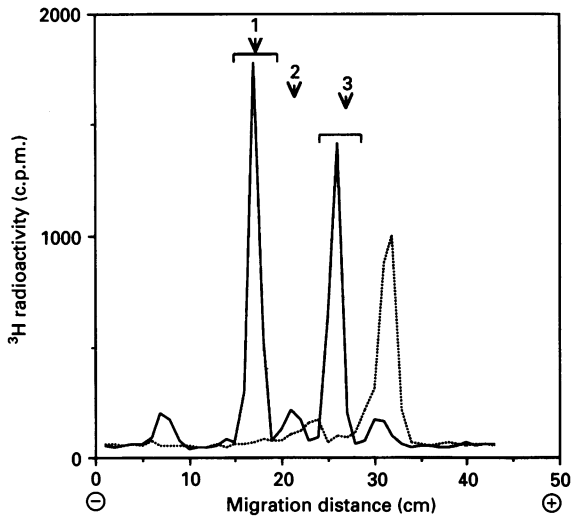

Figure 9 Partial desulphation of trisulphated disaccharide

High-voltage paper electrophoresis of ^{3}H-labelled trisulphated disaccharide deamination products from compound Bc-2, before (----) and after (——) partial desulphation with trifluoroacetic acid. The trisulphated disaccharide, isolated by preparative electrophoresis as shown in Figure 7b, was subjected to partial hydrolytic desulphation (see the Experimental section) and electrophoresis was repeated. The resulting mono- and di-sulphated disaccharides were recovered as indicated by the brackets. The standards indicated by numbered arrows are the same as in Figure 7.

digestion with β-D-glucuronidase as indicated by paper electrophoresis of the products (results not shown), suggesting that the corresponding disaccharide(s) contained a sulphated GlcA residue. Partial desulphation (see the Experimental section) converted about 30% of Di-diS-a into monosulphated disaccharide (the remaining 70% being unaffected judging from the paper electrophoresis pattern). The monosulphated product appeared at the elution position of GlcA-aMan$_R$(6-OSO$_3$) on anion-exchange chromatography and was susceptible to digestion with β-D-glucuronidase (results not shown). It is concluded that Di-diS-a contains mainly GlcA(2/3-OSO$_3$)-aMan$_R$(6-OSO$_3$), the sulphate group(s) on the GlcA residue being more susceptible to hydrolytic desulphation than the sulphate on the aMan$_R$ residue.

High-voltage paper electrophoresis of the ^{3}H-labelled disaccharides derived from polysaccharide Bc-2 yielded a major peak (Di-diS) which migrated similarly to a di-*O*-sulphated disaccharide standard (Figure 7b). An additional component (designated Di-triS; about 25% of the total radioactivity) migrated faster than Di-diS, and thus was assumed to represent a tri-O-sulphated disaccharide. Fraction Di-diS, isolated by preparative paper electrophoresis, gave a major peak on anion-exchange HPLC at an elution position similar to that of component Di-diS-a from sample Bb-2 (results not shown). This component also resisted digestion with β-D-glucuronidase, and thus was identified as a disulphated disaccharide with at least one sulphate group on the GlcA residue. Fraction Di-triS was also recovered by preparative paper electrophoresis from the deamination products of polysaccharide Bc-2. The predicted basic structure of this component, involving three sulphate substituents on a GlcA-aMan$_R$ disaccharide, was verified by paper electrophoresis after partial desulphation. The parent disaccharide was virtually eliminated by this treatment and was replaced by two novel components, generated in approximately equal proportions, which migrated like mono-O- and di-O-sulphated disaccharide standards (Figure 9). The desulphated

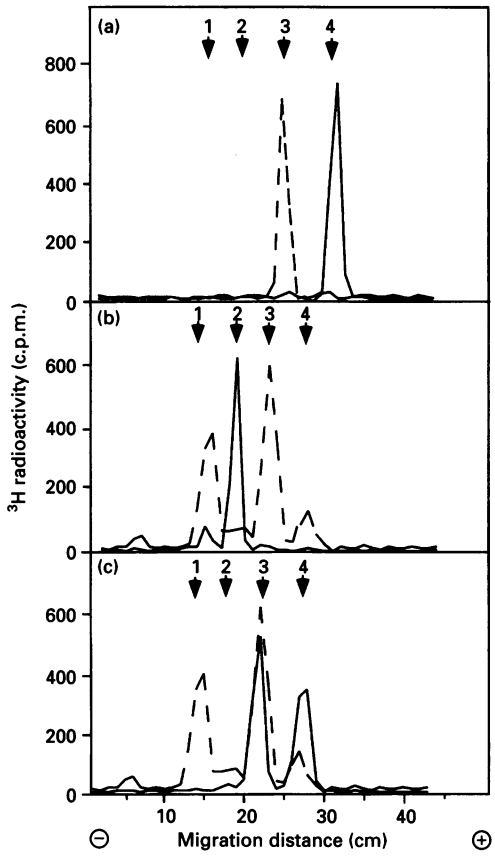

Figure 10 Digestion of disaccharides with β-D-glucuronidase

Digestion products were analysed using high-voltage paper electrophoresis. ——, Digested sample; ----, undigested sample. (**a**) Disulphated disaccharide Di-diS-b derived from compound Bb-2, fraction 5, and isolated by preparative anion-exchange HPLC (Figure 8a); (**b**) monosulphated disaccharide obtained by partial desulphation of trisulphated disaccharide from compound Bc-2 (see Figure 9); (**c**) disulphated disaccharide obtained by partial desulphation of trisulphated disaccharide from compound Bc-2 (Figure 9). The undigested reference pattern in (**b**) and (**c**) shows the mixture of the entire desulphation products, before separation by preparative electrophoresis. The migration positions of standard saccharides are indicated by numbered arrows: 1, GlcA-aMan$_R$(6-OSO$_3$); 2, aMan(6-OSO$_3$); 3, GlcA-aMan$_R$(3,6-di-OSO$_3$) [co-migrates with IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$)]; 4, aMan$_R$(3,6-di-OSO$_3$).

products were recovered by preparative paper electrophoresis and were separately subjected to digestion by β-D-glucuronidase. The monosulphated disaccharide was almost completely converted into labelled aMan$_R$ monosulphate (Figure 10b). About half of the disulphated disaccharide, corresponding to about 5% of the total initial radiolabelled disaccharide derived from compound Bc-2, yielded aMan$_R$(3,6-di-OSO$_3$) monosaccharide, whereas the remainder resisted enzymic cleavage (Figure 10c). These results indicate that fraction Di-triS contains the disaccharide, GlcA(2/3-OSO$_3$)-aMan$_R$(3,6-di-OSO$_3$), possibly along with components involving 2,3-di-O-sulphated GlcA residues (linked to mono-O-sulphated aMan$_R$).

Composition of AT-binding oligosaccharides

In order to gain more information about the structure of the actual AT-binding region(s), AT-binding oligosaccharides derived from chemically modified bacterial polysaccharide were isolated and subjected to compositional analysis. Fraction 4 of compound Bb-2 was partially depolymerized by deaminative cleavage, and the resulting oligosaccharides were radiolabelled

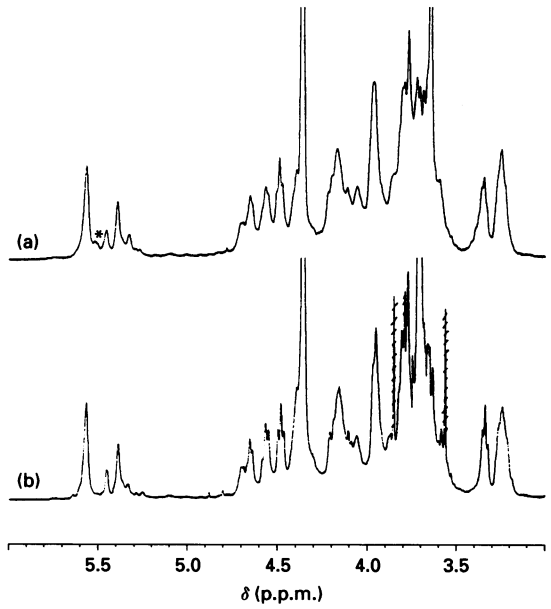

Figure 11 ^{1}H-NMR-spectroscopic analysis of polysaccharide samples

^{1}H-NMR spectra (500 MHz, ^2H_2O) of (**a**) subfraction 4 of compound Bb-2 with high affinity for AT and (**b**) subfraction 1, with low affinity for AT. This weak signal indicated by an asterisk in (**a**) is from H-1 of $GlcNSO_3$(3,6-di-OSO_3) residues. Cancelled signals in (**b**) are from buffer components.

by reduction with NaB^3H_4 and separated by gel chromatography. A fraction containing predominantly labelled deca- and dodeca-saccharides, along with smaller molecules, was recovered and further separated by affinity chromatography on AT–Sepharose (see the Experimental section; results not shown). Isolated high-affinity oligosaccharides were desalted and converted into disaccharides by HNO_2/NaB^3H_4 treatment. Anion-exchange HPLC of the products gave a pattern (Figure 8b) largely similar to that pertaining to the high-affinity fraction 5 of compound Bb-2 (Figure 8a; labelled disaccharides derived from intact polysaccharide, without previous enrichment of AT-binding oligosaccharides); however, the content of GlcA-aMan_R(3,6-di-OSO_3) was significantly higher, amounting to approx. 13% (Figure 8b) (compared with 3–4% in Figure 8a) of the total disaccharides.

Further structural analysis of fractions 1 and 4 of compound Bb-2, which had low and relatively high affinity for AT respectively, was performed by NMR spectroscopy. As illustrated in Figure 11, the two ^{1}H-NMR spectra are remarkably similar, except for the weak signal at 5.53 p.p.m. (labelled with an asterisk), due to H-1 of $GlcNSO_3$(3,6-di-OSO_3) residues [10].

DISCUSSION

The functional properties ascribed to the putative AT-binding region in heparin were conclusively confirmed by elaborate chemical synthesis of the pentasaccharide structure implicated in the interaction [8]. In particular, the synthetic pentasaccharide expressed exceedingly high anti-(Factor Xa) activity, which was believed to reflect a conformational change in the AT molecule similar to that induced by intact heparin [24]. Previous, mostly unsuccessful, attempts to generate functional heparin analogues by chemical modification of various naturally occurring polysaccharides [9] generally utilized compounds with carbohydrate backbones unrelated to that of heparin. The products described in the present study were derived from the *E. coli* K5 capsular polysaccharide and thus would mimic the backbone structure of heparin, except for the lack of IdoA units. In a previous report we demonstrated that substitution of glucosaminyl *N*-sulphate for *N*-acetyl groups followed by O-sulphation in various, only partly defined, positions yielded products that displayed affinity for AT as well as appreciable anticoagulant activity [10]. Of the two compounds, Bb-2 and Bc-2, selected for more detailed analysis, the latter species was more heavily sulphated and showed a larger proportion of AT-binding components (Figure 2). Judging from the affinity chromatograms, these components, from either source, bound AT with an affinity intermediate between those of HA- and LA-heparin. The high anti-(Factor Xa) activities suggested a specific interaction with AT; other anticoagulant tests often used in screening of heparin analogues are less informative, as they are influenced in a non-specific manner by the polyanion character of the saccharide [25].

The mode of interaction between AT and a high-affinity subfraction of compound Bb-2 was studied using spectroscopic techniques. The saccharide induced a conformational change in the proteinase inhibitor, as revealed by near-UV CD and UV-absorption difference spectroscopy, highly similar to that caused by HA-heparin (Figures 4 and 5). The binding strength (K_d approx. 200 nM) determined by fluorescence titration was about 10-fold lower than that of HA-heparin but about 50-fold higher than that of LA-heparin [22]. Somewhat weaker binding was determined for unfractionated compound Bc-2. In view of the apparent functional similarity between HA-heparin and the modified AT-binding bacterial polysaccharide, it was considered important to obtain information on the structure of the AT-binding region in the latter polymer. Particular attention was paid to the occurrence of the 3-O-sulphated GlcN residue previously identified as a marker component of the AT-binding region in heparin (reviewed in ref. [24]).

Preliminary evidence suggesting a 3-O-sulphated AT-binding region in compound Bb-2 was obtained by compositional analysis of subfractions recovered after affinity chromatography on AT–Sepharose [10]. The content of a disaccharide deamination product tentatively identified as GlcA-[^{3}H]aMan_R(3,6-di-OSO_3) [hence corresponding to a -GlcA-$GlcNSO_3$(3,6-di-OSO_3)- sequence in the intact polysaccharide] was thus found to increase with increasing affinity of the parent polysaccharide fraction for AT. As we lacked other di-O-sulphated GlcA-aManR disaccharide standards, it was important to obtain further proof of the identity of the putative marker disaccharide. In the present work the structure was conclusively verified by demonstrating the formation of ^{3}H-labelled aMan_R(3,6-di-OSO_3) monosaccharide on digestion of the disaccharide with β-D-glucuronidase. Assuming M_r values of 36000 for the intact polysaccharide and 600 for the average disaccharide unit, the yield of GlcA-aMan_R(3,6-di-OSO_3) (about 4% of the total disaccharide units) would correspond to 2.4 3-O-sulphated GlcN residues per chain. Any additional GlcN 3-O-sulphate groups in the major di-O-sulphated disaccharide fraction (Di-diS-a) would have escaped detection. The total content of GlcN 3-*O*-sulphate groups would thus equal or exceed the number of AT-binding regions (about two per chain) calculated from fluorescence-titration data. Similarly, the estimated content of 3-*O*-sulphate groups in compound Bc-2 (5% or more of the total disaccharide units, based on analysis of partially desulphated disaccharides) would be sufficient to account for the single AT-binding site present in each 13000-M_r polysaccharide chain. To confirm further the association of GlcN 3-*O*-sulphate groups with the AT-binding regions in the modified bacterial polysaccharide, AT-binding oligosaccharides were isolated after partial depolymerization of

compound Bb-2 fraction 4. Compositional analysis of a recovered high-affinity oligosaccharide fraction revealed a striking enrichment of GlcA-aMan$_R$(3,6-di-OSO$_3$) (13% of total disaccharide units) over undegraded material (cf. 3–4% in fraction 5). Although this value is still rather low to account for one GlcN 3-*O*-sulphate group in each oligosaccharide (17% being the expected value for a dodecasaccharide with a single 3,6-di-O-sulphated disaccharide unit), it seems reasonable to conclude that most of the AT-binding sites in compound Bb-2 contain a -GlcA-GlcNSO$_3$(3,6-di-OSO$_3$)- sequence corresponding to units B and C in Figure 1. Moreover, as the modified bacterial polysaccharide is predominantly 6-O-sulphated, it presumably reproduces the entire non-reducing-terminal trisaccharide sequence A–C of the AT-binding region in heparin (Figure 1). This sequence has the structure -GlcNR′(6-OSO$_3$)-GlcA-GlcNSO$_3$(3-OSO$_3$; 6-OR″), where R′ is -COCH$_3$ or -SO$_3$ and R″ is -H or -SO$_3$ [6]. Previous studies suggest that it has an essential role in initiating the heparin–AT binding that is prerequisite to subsequent interaction involving the reducing-terminal disaccharide unit [8,26–28].

Compound Bc-2 also contains sufficient amounts of 3,6-di-O-sulphated GlcN units to satisfy the requirements for an AT-binding sequence in each high-affinity polysaccharide molecule. However, the majority of these residues were recovered in trisulphated disaccharides after deamination, and it therefore seems likely that the adjacent GlcA, unit B, would be largely either 2-O- or 3-O-sulphated. Such sulphation was reported to decrease the anti-(Factor Xa) activity of the synthetic pentasaccharide [8], and might in fact explain the somewhat weaker binding to AT of compound Bc-2 (K_d approx. 1 μM) than that of compound Bb-2 fraction 5 (K_d approx. 200 nM). The reducing-terminal disaccharide unit of the AT-binding region in heparin (units D–E in Figure 1) has the same structure, -IdoA(2-OSO$_3$)-GlcNSO$_3$(6-OSO$_3$)-, as the predominant disaccharide component of heparin. In contrast with the trisaccharide sequence A–C, which has a rigid shape [28], the disaccharide unit D–E, because of the presence of the IdoA unit, is considered to be conformationally flexible and this property is believed to be of importance in the final stage of the interaction between heparin and AT [8,29]. The chemical procedures used to modify the K5 polysaccharide would not be expected to involve C-5 epimerization of GlcA to IdoA units (except perhaps at the non-reducing termini of fragments generated by cleavage during hydrazinolysis [30]), and the structural analysis of the products have revealed only traces of IdoA-containing disaccharide units. We therefore conclude that unit D of the AT-binding regions of compound Bb-2 (and presumably of compound Bc-2) is GlcA rather than IdoA, as implied in Figure 1. This difference probably explains the lower overall affinities of the *E. coli* K5-derived compounds for AT than HA-heparin; in fact, a larger difference would be predicted from available literature data based on the AT-binding properties of synthetic pentasaccharides [8]. It is conceivable that the interaction is promoted by the presence of a sulphate substituent on the GlcA unit D.

The present results demonstrate that products obtained by chemical modification of the *E. coli* K5 capsular polysaccharide have AT-binding characteristics and anticoagulant activities comparable with those of heparin. It seems likely that these properties, compared with the relatively modest activities displayed by previously described heparin analogues, depend on the basic structural similarity between heparin and this particular bacterial polysaccharide. However, it is remarkable that a saccharide sequence of such specific structure as the AT-binding region may be generated (or simulated) using chemical methods that are intrinsically random. The biological activity can probably be further increased by appropriate refinement of the modification procedure. These possibilities point to novel practically feasible routes for the generation of heparin-like compounds with various pharmacologically relevant biological activities.

The technical assistance of Helena Grundberg and the expert NMR analysis performed by Marco Guerrini are gratefully acknowledged. This work was supported by grants from The Swedish Medical Research Council (grants 2309 and 4212), the Swedish Research Council for Engineering Sciences, Italfarmaco S.p.A. (Milan, Italy) and Polysackaridforskning AB (Uppsala, Sweden).

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Received 25 January 1995; accepted 17 March 1995