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Biosynthesis of heparin

Use of *Escherichia coli* K5 capsular polysaccharide as a model substrate in enzymic polymer-modification reactions

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A capsular polysaccharide from *Escherichia coli* K5 was previously found to have the same structure, $[-(4)\beta GlcA(1)\rightarrow(4)\alpha GlcNAc(1)-]_n$, as that of the non-sulphated precursor polysaccharide in heparin biosynthesis [Vann, Schmidt, Jann & Jann (1981) Eur. J. Biochem. **116**, 359–364]. The K5 polysaccharide was *N*-deacetylated (by hydrazinolysis) and *N*-sulphated, and was then incubated with detergent-solubilized enzymes from a heparin-producing mouse mastocytoma, in the presence of adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate ([^{35}S]PAPS). Structural analysis of the resulting ^{35}S-labelled polysaccharide revealed the formation of all the major disaccharide units found in heparin. The identification of 2-*O*-[^{35}S]sulphated IdoA (L-iduronic acid) as well as 6-*O*-[^{35}S]sulphated $GlcNSO_3$ units demonstrated that the modified K5 polysaccharide served as a substrate in the hexuronosyl C-5-epimerase and the major O-sulphotransferase reactions involved in the biosynthesis of heparin. The GlcA units of the native (*N*-acetylated) *E. coli* polysaccharide were attacked by the epimerase only when PAPS was present in the incubations, whereas those of the chemically *N*-sulphated polysaccharide were epimerized also in the absence of PAPS, in accord with the notion that *N*-sulphate groups are required for epimerization. With increasing concentrations of PAPS, the mono-*O*-sulphated disaccharide unit -IdoA(2-OSO_3)-$GlcNSO_3$- was progressively converted into the di-*O*-sulphated species -IdoA(2-OSO_3)-$GlcNSO_3$(6-OSO_3)-. A small proportion of the ^{35}S-labelled polysaccharide was found to bind with high affinity to the proteinase inhibitor antithrombin. This proportion increased with increasing concentration of PAPS up to a level corresponding to $\sim$ 1–2 % of the total incorporated ^{35}S. The solubilized enzymes thus catalysed all the reactions required for the generation of functional antithrombin-binding sites.

INTRODUCTION

The biosynthesis of heparin involves the formation of non-sulphated polysaccharide chains, covalently bound to a protein core, followed by various modifications of these polymers. The process has been studied extensively using microsomal fractions from mouse mastocytoma tissue (for references, see Lindahl *et al.*, 1986; Lindahl & Kjellén, 1987; Lindahl, 1989). Chain elongation occurs by alternating transfer of D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) from the corresponding UDP-sugars to the non-reducing end of the growing polymer. The resulting non-sulphated product, *N*-acetylheparosan, is subsequently modified through a series of reactions, including deacetylation of GlcNAc residues, sulphation of the resulting unsubstituted amino groups, C-5 epimerization of D-GlcA to L-iduronic acid (IdoA) units, and finally, *O*-sulphation at C2 of IdoA and at C-6 of glucosamine units. In addition, a minor proportion of the GlcA and glucosamine units become sulphated at C-2 (or C-3) and at C-3 respectively. Sulphation of GlcA units is concurrent with that of IdoA units (Kusche & Lindahl, 1990), whereas 3-*O*-sulphation of glucosamine units concludes the polymer-modification process (Kusche *et al.*, 1988).

The above modification process occurs in a stepwise manner, such that certain reactions must be completed before subsequent modifications can be initiated (Jacobsson & Lindahl, 1980). The presence of *N*-sulphate groups is of critical importance for the final structure, as they are required for substrate recognition in all subsequent modification reactions (Höök *et al.*, 1975; Jacobsson *et al.*, 1979*a*, 1984; Jacobsson & Lindahl, 1980). If all the modification reactions were to go to completion, the resulting product would be a polysaccharide exclusively composed of the trisulphated disaccharide unit -IdoA(2-OSO_3)-$GlcNSO_3$(6-OSO_3)-. However, the reactions are generally incomplete, and will therefore generate a mixture of disaccharide units at different stages of modification. Apart from the substrate-recognition properties of the enzymes involved, the factors in control of target selection in the various reactions are unknown. A detailed understanding of the selection process is important, as this mechanism provides the very basis for the elaboration of polysaccharide sequences of different structure, and is therefore responsible for the formation of defined regions with specific functional properties. One such region in the heparin molecule is the antithrombin-binding site, which is composed of a pentasaccharide sequence with the predominant structure -GlcNAc(6-OSO_3)-GlcA-$GlcNSO_3$(3,6-di-OSO_3)-IdoA(2-OSO_3)-$GlcNSO_3$-(6-OSO_3)-. The structure–function relationships elucidated for this region emphasize the need of control in biosynthetic polymer modification, since even subtle changes in the structure will result in a dramatic loss of the blood anticoagulant activity of the polysaccharide (Lindahl *et al.*, 1984; Atha *et al.*, 1985; Petitou *et al.*, 1988; Lindahl, 1989). A particularly noteworthy feature of this sequence is the presence of the 3-*O*-sulphate group, which is

Abbreviations used: HexA, unspecified hexuronic acid; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcNAc, 2-deoxy-2-acetamido-D-glucose (*N*-acetyl-D-glucosamine); PAPS, adenosine 3′-phosphate 5′-phosphosulphate; $aMan_R$, 2,5-anhydro-D-mannitol formed by reduction of terminal 2,5-anhydromannose residues with $NaBH_4$; -NSO_3, *N*-sulphate group; -OSO_3, *O*-sulphate, ester sulphate group (the locations of *O*-sulphate groups are indicated in parentheses).

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invariably present on the internal glucosamine residue, but is either absent or present in very small amounts in other parts of the heparin molecule (Kusche *et al.*, 1990). As mentioned above, the 3-*O*-sulphotransferase reaction concludes polymer modification and thus requires a sulphate acceptor sequence which differs from a functional antithrombin-binding region only by lacking the 3-*O*-sulphate group (Kusche *et al.*, 1988).

Vann *et al.* (1981) described a capsular polysaccharide isolated from *Escherichia coli* K5 that was composed of alternating GlcA and GlcNAc units joined in the same manner as in the non-sulphated heparin precursor polysaccharide. The bacterial polysaccharide served as a substrate in the *N*-acetylglucosaminyl deacetylase reaction (Navia *et al.*, 1983), which initiates polymer modification in heparin biosynthesis, and, furthermore, incorporated sulphate groups upon incubation with mouse mastocytoma enzymes in the presence of the sulphate donor adenosine 3′-phosphate 5′-phosphosulphate (PAPS) (Riesenfeld *et al.*, 1987). The present paper describes the use of chemically *N*-deacetylated/*N*-sulphated K5 polysaccharide as a substrate for enzymes that catalyse the late polymer-modification reactions, i.e. hexuronosyl C-5-epimerization and *O*-sulphation. The resulting polysaccharide was found to contain all the major disaccharide units recognized in heparin (and heparan sulphate). In addition, formation of the antithrombin-binding sequence was indicated by the finding that a significant fraction of this product bound with high affinity to antithrombin.

EXPERIMENTAL

Materials

A microsomal fraction was prepared from a transplantable mouse mastocytoma (Furth *et al.*, 1957) as described by Jacobsson *et al.* (1979*a*), and was used as enzyme source after detergent solubilization as described below. In some experiments the enzyme preparation was first fractionated on DEAE-cellulose, yielding an unretarded fraction which contained virtually all of the hexuronosyl C-5-epimerase, and a retarded fraction which contained *O*-sulphotransferases; the *N*-acetylglucosaminyl deacetylase and *N*-sulphotransferase activities appeared to be divided between the two fractions (H. Wlad, H. H. Hannesson, M. Kusche, L. Kjellén & U. Lindahl, unpublished work).

Capsular polysaccharide from *E. coli* K5 was kindly given by Dr. Willie F. Vann (Bureau of Biologics, Food and Drug Administration, Bethesda, MD, U.S.A.) and was modified by the following procedure. The polysaccharide (5 mg) was *N*-deacetylated through treatment with hydrazine/hydrazine sulphate at 100 °C for 2 h as described by Höök *et al.* (1982; see also Shaklee & Conrad, 1984 and Kusche *et al.*, 1988). After repeated evaporation to dryness in the presence of toluene the resulting *N*-deacetylated polysaccharide was desalted by passage through a column of Sephadex G-15 (1 cm × 100 cm), which was eluted with aq. 10% (v/v) ethanol. *N*-Sulphation was achieved by reaction with trimethylamine–sulphur trioxide complex as described by Levy & Petracek (1962). The *N*-sulphated polysaccharide was re-isolated by chromatography on Sephadex G-15 as described above.

Unlabelled PAPS was obtained from Sigma Chemical Co. Unlabelled hyaluronan, chondroitin sulphate and heparin standards were as described by Enerbäck *et al.* (1985). *N*-[^{3}H]Acetyl-labelling of the glycosaminoglycans was conducted as described by Höök *et al.* (1982). Additional reagents such as ^{35}S-labelled PAPS, radiolabelled reference hexuronic acid-2,5-anhydro-D-mannitol (HexA-aMan$_R$) disaccharides with or without *O*-sulphate groups at various positions and antithrombin covalently bound to Sepharose 4B were as described by Kusche *et al.* [1988; see also Jacobsson *et al.* (1979*b*) regarding non-sulphated disaccharide standards].

Sephadex G-15 was purchased from Pharmacia LKB Biotechnology, and DEAE-cellulose (DE-52) was from Whatman Biochemicals.

Methods

Incubations with mastocytoma microsomal enzymes. Native or chemically modified (*N*-deacetylated, *N*-sulphated; see under 'Materials') *E. coli* polysaccharide (250 μg/ml) was incubated with 0.05 M-Hepes, 10 mM-$MnCl_2$, 10 mM-$MgCl_2$, 5 mM-$CaCl_2$, 3.5 μM-NaF and 1% (v/v) Triton X-100, containing 10 mg of mastocytoma microsomal protein/ml, in the presence or absence of 2.0 mM-PAPS. The PAPS was added in four equal portions, one at the beginning of the incubations and three further additions at 20 min intervals. In control incubations, water was substituted for the PAPS. After 2 h of incubation at 37 °C the reactions were terminated by heating at 100 °C for 5 min. The samples were then centrifuged and the resulting pellet was washed with 0.7 ml of 0.05 M-LiCl in 0.05 M-acetate buffer, pH 4.0. The combined supernatants were mixed with ^{3}H-labelled standards of hyaluronan, chondroitin sulphate and heparin and applied to small columns (~ 1 ml) of DEAE-cellulose equilibrated with the above buffer. The columns were rinsed with 10 ml of the same buffer and were then eluted with a linear LiCl gradient as described below. Fractions (~ 1 ml) were collected and analysed for uronic acid and radioactivity.

^{35}S-Labelled glycosaminoglycan was generated in similar incubation mixtures containing 25 μg of *N*-deacetylated/*N*-sulphated K5 polysaccharide, 5 mg of microsomal protein and 100 μCi of [^{35}S]PAPS, with or without additional unlabelled PAPS (0.5, 1.0 or 2.0 mM final concns., added at the beginning of the incubations), in a total volume of 0.5 ml. In some incubations 50 μl of a partially purified *N*- and *O*-sulphotransferase preparation (retarded fraction on DEAE-cellulose chromatography; see under 'Materials'), essentially free of epimerase activity, was substituted for the crude microsomal fraction. Before centrifugation, 0.5 mg of heparin was added as a carrier. The pellet was rinsed with 0.5 ml of 2 M-Na_2SO_4, and the pooled supernatants were passed through a column (1 cm × 100 cm) of Sephadex G-15 equilibrated with 0.2 M-NH_4HCO_3. Labelled polysaccharide that was eluted at the void volume of the G-15 column was recovered and desalted by freeze-drying.

Analytical methods. Structural analysis of the bacterial K5 polysaccharide after chemical/enzymic modification was based on identification of HexA-aMan$_R$ disaccharides generated by deaminative cleavage with HNO_2. The procedures employed were essentially as described by Pejler *et al.* (1987) and Kusche *et al.* (1990). Briefly, *N*-sulphated polysaccharides were deaminated at pH 1.5, whereas *N*-acetylated species were *N*-deacetylated by hydrazinolysis before deamination at pH 3.9. Unlabelled degradation products were radiolabelled by reduction with NaB^3H_4, whereas ^{35}S-labelled products were reduced with unlabelled $NaBH_4$. The resulting labelled disaccharides were recovered by gel chromatography on a column (1 cm × 150 cm) of Sephadex G-15, equilibrated with 0.2 M-NH_4HCO_3, and were then desalted by freeze-drying. They were identified by anion-exchange h.p.l.c. using a Whatman Partisil-10 SAX column (Bienkowski & Conrad, 1985), as described in detail by Pejler *et al.* (1987) and Kusche *et al.* (1988).

Anion-exchange chromatography of polysaccharides was carried out on columns (1 ml) of DEAE-cellulose (DE-52); the polysaccharides were eluted using a linear salt gradient (total volume, 60 ml) extending from 0.05 to 1.5 M-LiCl in 0.05 M-

acetate buffer, pH 4.0 (see also the legend to Fig. 2). Affinity chromatography on antithrombin–Sepharose was performed as described by Thunberg *et al.* (1982), using a 3 ml column of the affinity matrix and continuous salt-gradient elution. High-voltage paper electrophoresis was performed on Whatman no. 3MM paper in 0.83 M-pyridine/0.5 M-acetic acid (pH 5.3; 80 V/cm). After drying, guide strips were analysed for ^3H radioactivity. The portions of the strips corresponding to non-sulphated HexA-[1-^{3}H]aMan$_R$ disaccharides were cut out, and the labelled components were eluted with water from the strips. Paper chromatography was carried out on the same paper, in ethyl acetate/acetic acid/water (3:1:1, by vol.).

Hexuronic acid was determined by the carbazole method (Bitter & Muir, 1962). Radioactivity was measured by liquid-scintillation counting using a Beckman model LS 3800 liquid-scintillation spectrometer, or a Flo-One β/CR radioactive-flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.) equipped with a 2.5 ml cell.

RESULTS

In a previous study Riesenfeld *et al.* (1987) showed that the *E. coli* K5 capsular polysaccharide could serve as a substrate in sulphation reactions involved in the biosynthesis of heparin. The resulting products were analysed by ion-exchange chromatography only, and no attempt was made to determine the location of incorporated sulphate groups. However, *N*-sulphation is known to precede the incorporation of *O*-sulphate groups (see the Introduction), and it was therefore assumed that the K5 polysaccharide in its native form, i.e. with *N*-acetylated GlcN units, would serve primarily as a substrate for the *N*-sulphotransferase. Since the present study was mainly concerned with the later polymer-modification reactions, we decided to by-pass the enzymic *N*-deacetylation/*N*-sulphation steps by means of the corresponding chemical modifications. The product was tested as an *O*-sulphotransferase substrate using [^{35}S]PAPS as a sulphate donor. Further, the ability of the K5 polysaccharide to serve as a substrate in the GlcA C-5-epimerase reaction was investigated, with special regard to the influence of *N*-substitution. Finally, attempts were made to assess the potential of the combined epimerization and *O*-sulphation reactions in generating the specific antithrombin-binding pentasaccharide sequence.

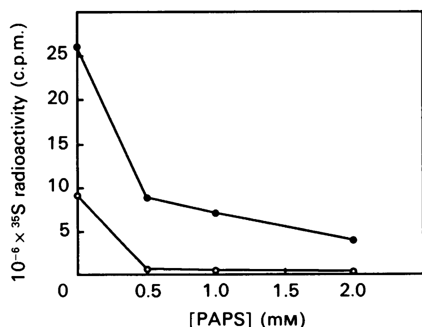

Fig. 1. Incorporation of [^{35}S]sulphate into *N*-deacetylated *N*-sulphated *E. coli* K5 polysaccharide and into endogenous microsomal polysaccharide

Mastocytoma microsomal fraction was incubated with 100 μCi of [^{35}S]PAPS in a total volume of 0.5 ml in the presence (●) or absence (○) of exogenous sulphate acceptor (25 μg). Unlabelled PAPS was added at the concentrations indicated. The resulting labelled polysaccharide was isolated and quantified by scintillation counting. For additional experimental details, see the Experimental section.

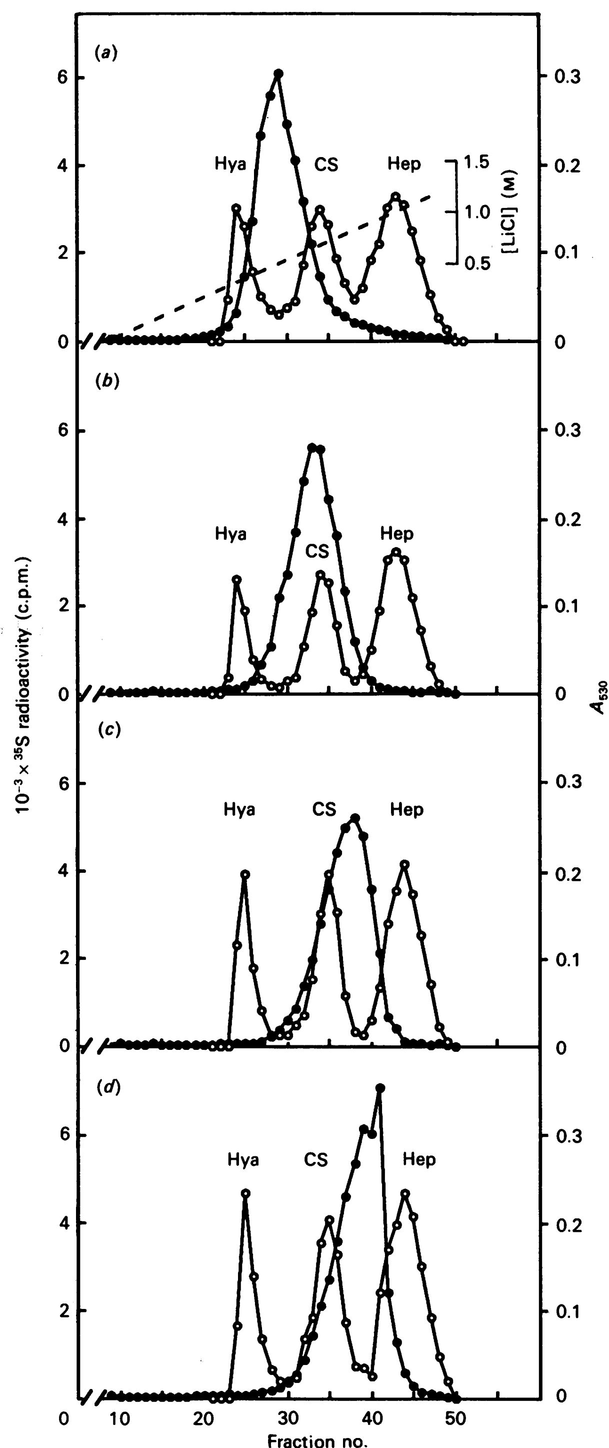

Fig. 2. Ion-exchange chromatography on DEAE-cellulose of ^{35}S-labelled *N*-deacetylated *N*-sulphated *E. coli* K5 polysaccharide

N-Deacetylated *N*-sulphated *E. coli* K5 polysaccharide was labelled by incubation with [^{35}S]PAPS as described in the legend to Fig. 1, in the presence of (*a*) no additional, (*b*) 0.5 mM (*c*) 1.0 mM and (*d*) 2.0 mM unlabelled PAPS. Samples (50×10^3 c.p.m. of ^{35}S) of the resulting products were mixed with unlabelled standards of hyaluronan (Hya), chondroitin sulphate (CS) and heparin (Hep) and were applied to columns ($\sim$ 1 ml) of DEAE-cellulose, equilibrated with 0.05 M-LiCl in 0.05 M-acetate buffer, pH 4.0. The columns were rinsed with 10 bed volumes of the same buffer and were then eluted with a linear gradient (----) from 0.05 M- to 1.5 M-LiCl in the above acetate buffer (see the Experimental section). Fractions ($\sim$ 1 ml) were collected and analysed for radioactivity (●) by scintillation counting and for uronic acid (○) by the carbazole method.

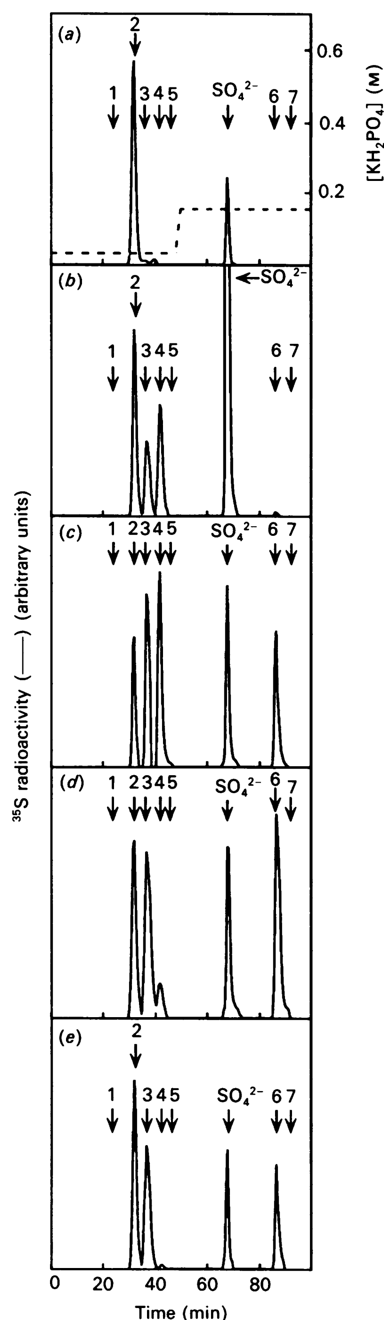

Fig. 3. Anion-exchange h.p.l.c. of disaccharide fractions obtained on deamination of ^{35}S-labelled *N*-deacetylated *N*-sulphated *E. coli* K5 polysaccharide

N-Deacetylated *N*-sulphated *E. coli* K5 polysaccharide was incubated with [^{35}S]PAPS in the presence of solubilized and partially purified mastocytoma microsomal enzymes, essentially free of hexuronosyl C-5 epimerase (*a*) or (*b–e*) with [^{35}S]PAPS in the presence of crude solubilized enzymes and no additional (*b*), 0.5 mM (*c*), 1.0 mM (*d*), or 2.0 mM (*e*) unlabelled PAPS. The labelled polysaccharides formed were isolated and degraded by HNO_2 (pH 1.5)/$NaBH_4$ treatment, and the resulting disaccharide fractions were isolated by gel chromatography. Samples (20×10^3 c.p.m.) of ^{35}S were applied to a Partisil-10 SAX column and eluted with KH_2PO_4 at different concentrations (0.030 M for monosulphated disaccharides, 0.154 M for disulphated disaccharides and inorganic sulphate), as indicated by the broken line. The elution positions of standard disaccharides are indicated by numbered arrows. Arrow 1, GlcA(2-OSO_3)-aMan$_R$; arrow 2, GlcA-aMan$_R$(6-OSO_3); arrow 3, IdoA-aMan$_R$(6-OSO_3); arrow 4, IdoA(2-OSO_3)-aMan$_R$; arrow 5, GlcA-aMan$_R$(3-OSO_3); arrow 6, IdoA(2-OSO_3)-aMan$_R$(6-OSO_3) and arrow 7, GlcA-aMan$_R$(3,6-di-OSO_3). SO_4^{2-}, inorganic sulphate.

Incorporation of *O*-sulphate groups

Effect on polyanionic properties. Chemically modified (*N*-deacetylated, *N*-sulphated) *E. coli* K5 polysaccharide was incubated with solubilized mastocytoma microsomal enzymes in the presence of ^{35}S-labelled PAPS and unlabelled PAPS at different concentrations, as described under 'Methods' above. The effects of [^{35}S]PAPS dilution on the incorporation of [^{35}S]sulphate into the modified bacterial polysaccharide are illustrated in Fig. 1. As expected, labelling of the exogenous sulphate acceptor decreased as unlabelled PAPS was included in the incubations. However, at 2 mM-PAPS the amount of ^{35}S incorporated was still $\sim 15\%$ of that obtained in the absence of any added unlabelled PAPS. Incubations of the microsomal fraction with [^{35}S]PAPS in the absence of both exogenous sulphate acceptor and unlabelled PAPS resulted in ^{35}S-labelling of endogenous preformed microsomal polysaccharide, corresponding to about one-third of that obtained in the presence of the bacterial polysaccharide. Addition of unlabelled PAPS to such incubations resulted in a much more pronounced decrease in ^{35}S incorporation, as compared with the analogous incubations with exogenous sulphate acceptor (Fig. 1). This difference is presumably due to the relatively small amounts of acceptor sites available in the endogenous microsomal polysaccharide.

The modest effect of PAPS dilution on the [^{35}S]sulphate incorporation into the exogenous acceptor would suggest a more extensive overall utilization of available acceptor sites with increasing PAPS concentration. This conclusion was confirmed by anion-exchange chromatography on DEAE-cellulose of ^{35}S-labelled polysaccharide generated at different PAPS concentrations (Fig. 2). The polysaccharide sulphated in the presence of [^{35}S]PAPS without the addition of unlabelled PAPS emerged in a position between those of the hyaluronan and chondroitin sulphate standards (Fig. 2*a*). With increasing concentration of PAPS the elution position of the entire labelled polysaccharide gradually became more retarded. The ^{35}S-labelled polysaccharide formed in the presence of 2 mM unlabelled PAPS thus was eluted before the heparin standard, but after chondroitin sulphate (Fig. 2*d*). It may be noted that not only the charge density but also the molecular size of the polysaccharide will influence the elution properties of a polyanion on ion-exchange chromatography. The bacterial polysaccharide used in these experiments had an M_r of approx. $(4–10) \times 10^3$, as compared with $(8–20) \times 10^3$ for the heparin standard used. Therefore the most highly sulphated bacterial polysaccharide could well have a sulphate content approaching that of heparin.

Composition of *O*-[^{35}S]sulphated disaccharide units. For structural analysis of the ^{35}S-labelled bacterial polysaccharide, the various incubation products were subjected to HNO_2 (pH 1.5)/$NaBH_4$ treatment, and the resulting labelled deamination products were isolated by gel chromatography on Sephadex G-15 (results not shown). The elution profiles, essentially similar for all preparations, showed a major disaccharide peak which corresponded to 70–90% of the total label, and in addition smaller peaks of tetra- and hexa-saccharides. The latter components, which were presumably at least partly due to so-called 'anomalous ring contraction' during the deamination reaction (see Shively & Conrad, 1976), were not analysed further.

The ^{35}S-labelled polysaccharide isolated from the incubation performed in the absence of added unlabelled PAPS contained almost exclusively mono-*O*-[^{35}S]sulphated disaccharide units. Analysis by anion-exchange h.p.l.c. of the sulphated HexA-aMan$_R$ disaccharides, recovered after HNO_2/$NaBH_4$ treatment

of the polysaccharide, thus showed prominent peaks of GlcA-aMan$_R$(6-OSO$_3$), IdoA-aMan$_R$(6-OSO$_3$), and IdoA(2-OSO$_3$)-aMan$_R$, but virtually no di-*O*-sulphated IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$) species (Fig. 3*b*). An analogous sample, derived from polysaccharide that had been incubated with fractionated microsomal enzymes lacking the hexuronosyl C-5 epimerase activity, yielded GlcA-aMan$_R$(6-OSO$_3$), but no significant amounts of the IdoA-containing disaccharides (Fig. 3*a*). This result demonstrates that the modified bacterial polysaccharide is a substrate for the epimerase and that the resulting IdoA units will provide targets for further modification, catalysed by the IdoA 2-*O*-sulphotransferase. Increasing the amounts of unlabelled PAPS in the incubations resulted in a progressive decrease in the amounts of IdoA(2-OSO$_3$)-aMan$_R$ detected, whereas the IdoA-aMan$_R$(6-OSO$_3$) component remained essentially unaffected (Figs. 3*c*–*e*). The polysaccharide obtained after incubation in the presence of 2 mM-PAPS was practically devoid of -IdoA(2-OSO$_3$)-GlcNSO$_3$- sequences. Instead, a prominent peak of IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$) was seen, corresponding to a -IdoA(2-OSO$_3$)-GlcNSO$_3$(6-OSO$_3$)- sequence in the intact polysaccharide. Increasing the PAPS concentration thus resulted in progressive 6-*O*-sulphation of -IdoA(2-OSO$_3$)-GlcNSO$_3$- units to yield the corresponding di-*O*-sulphated structure. This finding is in accord with previous conclusions regarding the order of addition of the various *O*-sulphate substituents in heparin (Jacobsson & Lindahl, 1980). No disaccharide units indicative of 'low-abundancy' sulphation reactions [GlcA(2/3-OSO$_3$)-aMan$_R$, GlcA(2/3-OSO$_3$)-aMan$_R$(6-OSO$_3$), GlcA-aMan$_R$(3-OSO$_3$), GlcA-aMan$_R$(3,6-di-OSO$_3$)] were detected (see also the Discussion section).

In addition to the various *O*-sulphated disaccharide units, the h.p.l.c. patterns showed significant amounts of inorganic [^{35}S]sulphate, presumably derived from *N*-[^{35}S]sulphate groups in the parent polysaccharide. It is recalled that the hydrazinolysis used to *N*-deacetylate the bacterial polysaccharide was interrupted after a reaction period of 2 h. These conditions, which were chosen to avoid undue depolymerization of the polysaccharide (Shaklee & Conrad, 1984), will not result in complete *N*-deacetylation (see also Höök *et al.*, 1982), and the labelled *N*-sulphate groups therefore had probably been introduced through a concerted enzymatic *N*-deacetylation/*N*-sulphation exchange mechanism (see Riesenfeld *et al.*, 1982).

Epimerization of D-glucuronic acid units

The identification of *O*-[^{35}S]sulphated disaccharide units, as described above, demonstrated that part of the GlcA units of the polysaccharide substrate had undergone C-5 epimerization along with the incorporation of *O*-sulphate groups. However, previous studies on the biosynthetic polymer-modification process have indicated that, whereas *N*-sulphation of the polysaccharide is prerequisite to the epimerization reaction, *O*-sulphation is not (Jacobsson *et al.*, 1979*a*, 1984; Jacobsson & Lindahl, 1980). We therefore chose a more direct approach to evaluate the K5 polysaccharide as a substrate in the C-5 epimerization reaction, based on paper chromatographic separation of non-sulphated GlcA-[^{3}H]aMan$_R$ and IdoA-[^{3}H]aMan$_R$ disaccharides generated through HNO$_2$/NaB^{3}H$_4$ treatment of incubation products. Native or chemically *N*-deacetylated/*N*-sulphated K5 polysaccharide was incubated with solubilized mastocytoma enzymes in the absence or presence of (unlabelled) PAPS as described under 'Methods' above and was then recovered by anion-exchange chromatography. Native K5 polysaccharide that had been incubated with 2 mM-PAPS (Fig. 4*b*) emerged significantly retarded in relation to the product of a control incubation lacking PAPS (Fig. 4*a*), indicating that the substrate had become *N*- and possibly *O*-sulphated. Deaminative cleavage of the

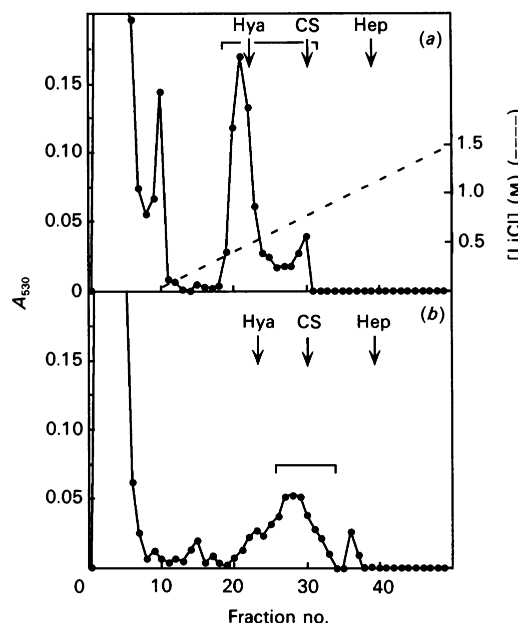

Fig. 4. Ion-exchange chromatography on DEAE-cellulose of products obtained by incubating native (*N*-acetylated) *E. coli* polysaccharide with a mastocytoma microsomal preparation

Bacterial polysaccharide (250 μg) was incubated with solubilized microsomal enzymes as described in the Experimental section. The incubations were performed in the absence (*a*) or in the presence (*b*) of 2.0 mM-PAPS. After incubation the mixtures were heated at 100 °C for 5 min and centrifuged (5 min, 14000 *g*), and the resulting supernatants were mixed with 20 × 10^3 c.p.m. each of ^{3}H-labelled standards of hyaluronan, chondroitin sulphate and heparin. The samples were then applied to columns (~ 1 ml) of DEAE-cellulose equilibrated with 0.05 M-LiCl in 0.05 M-acetate buffer, pH 4.0. The columns were rinsed with 10 bed volumes of the same buffer and were then eluted using a linear gradient (----) from 0.05 to 1.5 M-LiCl in the above acetate buffer as described in the Experimental section. Fractions (~ 1 ml) were collected and analysed for radioactivity by scintillation counting and for uronic acid by the carbazole method. The arrows on the top of the Figure indicate the peak elution positions of hyaluronan (Hya), chondroitin sulphate (CS) and heparin (Hep) standards. The carbazole-positive (discoloured) material in the break-through fractions was observed also in the absence of added K5 polysaccharide and thus originated from the microsomal preparation. Fractions were pooled as indicated by the horizontal bars and were then desalted by passage through a column of Sephadex G-15 eluted with aq. 10% (v/v) ethanol.

sulphated product by HNO$_2$ at pH 1.5, followed by reduction with NaB^{3}H$_4$, yielded disaccharides as the predominant labelled components (as demonstrated by gel chromatography on Sephadex G-15; results not shown), indicating that most of the GlcN residues had become *N*-sulphated. Further analysis by paper electrophoresis showed that ~ 70% of the ^{3}H-labelled disaccharides lacked *O*-sulphate groups whereas the remainder was mono-*O*-sulphated (results not shown). Paper chromatography of the non-sulphated fraction, isolated by preparative paper electrophoresis, gave 67% GlcA-[^{3}H]aMan$_R$ and 33% IdoA-[^{3}H]aMan$_R$ (Fig. 5*b*). Polysaccharide recovered from the control incubation (Fig. 4*a*) was *N*-deacetylated by hydrazinolysis and cleaved by treatment with HNO$_2$ at pH 3.9, and the ^{3}H-labelled disaccharide obtained by reduction with NaB^{3}H$_4$ was isolated by gel chromatography. As expected, paper electrophoresis showed the disaccharide to be exclusively non-sulphated (results not shown). Paper chromatography indicated GlcA-[^{3}H]aMan$_R$ as the only component present; no IdoA-[^{3}H]aMan$_R$

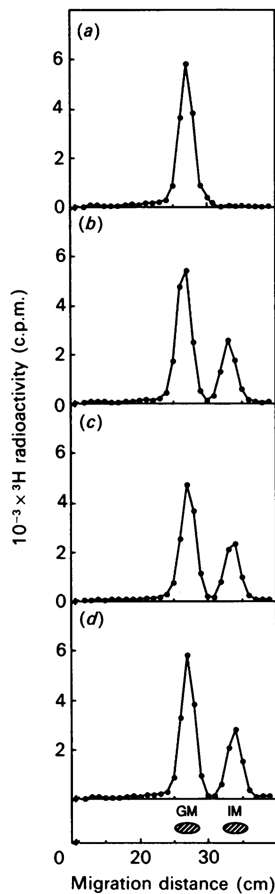

Fig. 5. Paper chromatography of HexA-[1-^{3}H]aMan$_R$ disaccharides generated by HNO_2/NaB^3H_4 treatment of K5 polysaccharide incubated with solubilized microsomal enzymes

Polysaccharide samples were incubated, recovered and processed to generate HexA-[^{3}H]aMan$_R$ disaccharides as described in the text. Non-sulphated disaccharides were isolated by preparative paper electrophoresis and further separated by paper chromatography as described in the Experimental section. The Figure shows paper chromatograms of disaccharides ($\sim 30 \times 10^3$ c.p.m.) derived from native K5 polysaccharide incubated in the absence (*a*) or presence (*b*) of PAPS and from chemically *N*-deacetylated/*N*-sulphated K5 polysaccharide incubated in the absence (*c*) or presence (*d*) of PAPS. The migration positions of standard [^{14}C]GlcA-aMan$_R$ (GM) and IdoA-[1-^{3}H]aMan$_R$ (IM) are indicated below the chromatograms.

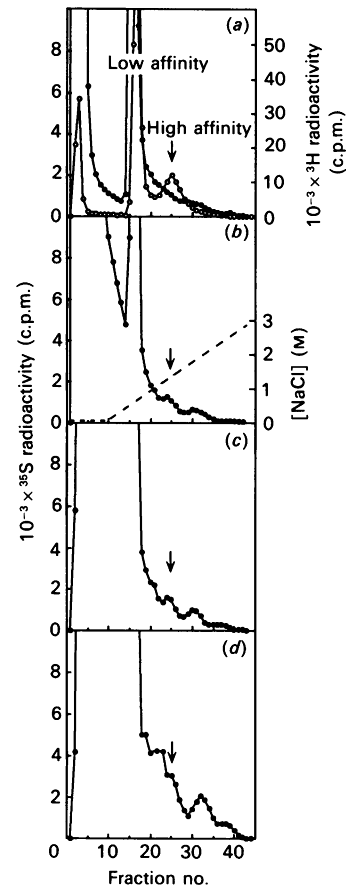

Fig. 6. Affinity chromatography on antithrombin–Sepharose of ^{35}S-labelled *N*-deacetylated *N*-sulphated *E. coli* K5

The chemically modified bacterial polysaccharide was incubated with solubilized mastocytoma microsomal enzymes in the presence of [^{35}S]PAPS, either in the absence of added unlabelled PAPS (*a*) or in the presence of 0.5 mM (*b*), 1.0 mM (*c*) or 2.0 mM (*d*) unlabelled PAPS. Samples of the resulting ^{35}S-labelled polysaccharides (500×10^3 c.p.m.) were mixed with ^{3}H-labelled heparin (500×10^3 c.p.m.) and applied to a column (~ 3 ml) of antithrombin–Sepharose. Elution was performed with a linear gradient, (beginning at fraction no. 11) extending from 0.05 M- to 3.0 M-NaCl (----) in 0.05 M-Tris/HCl, pH 7.4 (shown in *b*). Effluent fractions (2.2 ml) were collected and analysed for ^{35}S (●) and for ^{3}H (○) radioactivity. The elution pattern for standard heparin, showing distinct fractions with high or low affinity for antithrombin, was similar for all samples and is only shown in (*a*). The peak elution positions of standard high-affinity heparin is indicated by arrows.

was detected (Fig. 5*a*). Paper chromatography of the corresponding disaccharide fractions derived from chemically *N*-deacetylated/*N*-sulphated K5 polysaccharide again showed GlcA-[^{3}H]aMan$_R$ and IdoA-[^{3}H]aMan$_R$ in ratios of $\sim 2{:}1$, but now regardless of whether PAPS had been absent (Fig. 5*c*) or present (Fig. 5*d*) during the incubation.

Formation of the antithrombin-binding region

The labelled products obtained by incubating the chemically modified *E. coli* K5 polysaccharide with [^{35}S]PAPS, in the presence of mastocytoma microsomal enzymes, were analysed by affinity chromatography on immobilized antithrombin (Fig. 6). The ^{35}S-labelled product generated in the absence of added unlabelled PAPS showed predominantly low or no apparent affinity for the proteinase inhibitor, trailing into the high-affinity region, but with no distinct high-affinity component (Fig. 6*a*). However, such components were observed when unlabelled PAPS had been added to the incubations, the relative proportion of high-affinity material increasing with increasing PAPS concn. (Figs. 6*b*–6*d*). Of the product recovered from the incubation containing 2 mM-PAPS, about 1% of the total label emerged as a high-affinity component (Fig. 6*d*). Control incubations, at various PAPS concentrations, lacking any exogenous sulphate acceptor, failed to produce any significant labelled high-affinity material (results not shown), thus demonstrating that the generation of such components was indeed due to modification of the added bacterial polysaccharide.

DISCUSSION

The process of polymer modification in heparin biosynthesis has mainly been studied in relation to the endogenous polysaccharide generated by incubating a mastocytoma microsomal fraction with the appropriate UDP-sugars. Addition of PAPS to

such a system initiates the series of sulphation and epimerization reactions that are required to form a heparin-like product. Studies on the kinetics of polymer modification showed that the process is rapid, being completed in less than 30 s for an individual polysaccharide chain and then resumed with new sets of substrate molecules (Höök *et al.*, 1975). Both the enzymes catalysing this process and their proteoglycan (see Lidholt *et al.*, 1988) substrates are membrane-bound and appear to interact in a stepwise fashion, as demonstrated by the generation of a number of distinct intermediary species (Höök *et al.*, 1975; Jacobsson *et al.*, 1979*a*; Jacobsson & Lindahl, 1980). Disruption of the microsomal membranes by the addition of detergent (Triton X-100), after formation of the polysaccharide chains, results in loss of order during subsequent polymer modification (Riesenfeld *et al.*, 1987). Instead of the rapid, stepwise process typical of the intact microsomal system, the solubilized substrate is modified in a slow, progressive fashion, which simultaneously involves all the polysaccharide molecules available. Moreover, exogenous substrates, such as the *E. coli* K5 polysaccharide, which are barely attacked by the membrane-bound enzymes, will be efficiently processed in the solubilized system.

The present study was undertaken to explore the potential of the bacterial polysaccharide, after the appropriate chemical modification, to serve as a substrate for the enzymes catalysing the late polymer-modification reactions in heparin biosynthesis. The three major reactions, i.e. C-5 epimerization of GlcA to IdoA units, 2-*O*-sulphation of the latter units, and 6-*O*-sulphation of $GlcNSO_3$ residues, were readily demonstrated with the novel substrate. Moreover, the results clearly indicate that the substrate specificities of the *O*-sulphotransferases are in accord with the order of incorporation of the differently positioned *O*-sulphate groups, as deduced from the structures of the biosynthetic intermediates generated by the intact microsomal system (Jacobsson & Lindahl, 1980). In particular, it is noted that an IdoA unit will not serve as a substrate for 2-*O*-sulphation if the adjacent (at C-1) GlcN substituent is already 6-*O*-sulphated, whereas the reverse order of substitution is readily demonstrated. Additional examples of modifications that are inhibitory to already overdue reactions 'upstream' in the biosynthetic scheme, while compatible with, or even prerequisite to, later reactions are found in reviews by Lindahl & Kjellén (1987) and Lindahl (1989).

In addition to the major substituents referred to above, heparin and heparan sulphate contain low-abundancy *O*-sulphate groups that have been ascribed important functional roles. The 3-*O*-sulphate group which occurs on one out of ~ 50 $GlcNSO_3$ units in commercial heparin is prerequisite to the antithrombin-dependent anticoagulant activity of the polysaccharide and serves as a marker group for the specific antithrombin-binding region (see the Introduction). The formation of functional antithrombin-binding regions, albeit in low amount, was clearly suggested by the finding that a minor proportion of the *N*-deacetylated *N*-sulphated bacterial polysaccharide had acquired high affinity for antithrombin through the incubation with PAPS in the presence of mastocytoma microsomal enzymes. The minimal enzymic modifications required to form such a region would involve epimerization of a GlcA unit followed by 6-*O*-sulphation and 3-*O*-sulphation of two separate GlcN residues, located at defined positions in relation to the IdoA epimerization product (Kusche *et al.*, 1988). Considering that both the epimerization and the 6-*O*-sulphation reactions will occur partly at random in the solubilized system employed, whereas the 3-*O*-sulphotransferase will require a specific recognition sequence created through the other two reactions, it is not surprising that the final binding regions will be sparsely distributed. Hence, no 3-*O*-sulphated disaccharide unit could be detected in the total *O*-[^{35}S]sulphated bacterial polysaccharide; the amounts of labelled high-affinity component were insufficient for structural analysis. The increasing proportion of functional binding region with increasing concentrations of PAPS in the incubations can presumably be rationalized in terms of the probability of introducing two *O*-sulphate groups within the same trisaccharide sequence.

Another low-abundancy *O*-sulphate group with interesting potential functional implications is the 2-*O*- (or 3-*O*-) sulphate substituent on GlcA units, which accounts for ~ 1 % of the disaccharide units in commercial heparin preparations (Bienkowski & Conrad, 1985; Kusche *et al.*, 1990). Sulphated GlcA residues, interspersed between *N*-sulphated GlcN units, were found to be accumulated in a nuclear fraction of heparan sulphate in cultured hepatocytes, and it was proposed that these components may be somehow involved in the regulation of cell growth (Fedarko & Conrad, 1986; Ishihara *et al.*, 1987). The GlcA *O*-sulphotransferase reaction was recently demonstrated with the intact mastocytoma microsomal system, by incubating radiolabelled UDP-GlcA with UDP-GlcNAc and PAPS. *O*-Sulphation of GlcA units was found to be concomitant with that of IdoA units. The most highly sulphated polysaccharide fractions obtained contained -GlcA(2/3-OSO_3)-$GlcNSO_3$- sequences which accounted for 7–10 % of the total disaccharide units (Kusche & Lindahl, 1990). By contrast, no sulphated GlcA units were detected in the present study, on analysis of the *N*-deacetylated/*N*-sulphated bacterial polysaccharide after incubation with PAPS and solubilized mastocytoma microsomal enzymes. While the reason for this discrepancy is unknown, it is conceivable that the sulphotransferase which catalyses the sulphation of GlcA units is selectively inactivated during solubilization of the microsomal preparation. Alternatively, the enzyme may require a specific saccharide sequence for substrate recognition, the assembly of which depends on the more ordered kinetics of polymer modification obtained with the particulate membrane-bound system.

The bacterial polysaccharide also provides a substrate for the GlcA C-5 epimerase that is required to generate IdoA units. The formation of IdoA was evidenced by the identification of ^{35}S-labelled mono- and di-*O*-sulphated IdoA-aMan disaccharides and of the non-sulphated disaccharide labelled by reduction with NaB^3H_4. The presence of *N*-sulphate groups was found to be an absolute requirement for C-5 epimerization, in agreement with previous findings (Jacobsson *et al.*, 1979*a*, 1984; Jensen *et al.*, 1983). The *N*-sulphate groups were introduced either by chemical modification of the K5 polysaccharide or by enzymic *N*-deacetylation/*N*-sulphation during incubation of the native polysaccharide in the presence of PAPS. Epimerase action on -$GlcNSO_3$-GlcA-Glc-NSO_3- sequences, obtained by either the chemical or enzymic route, resulted in conversion of about one-third of the GlcA into IdoA units. The epimerization reaction is known to be freely reversible (Jacobsson *et al.*, 1979*a*, 1984; Jensen *et al.*, 1983), and it seems likely that the GlcA/IdoA ratio of ~ 2:1 reflects the equilibrium of the reaction. It may be noted that epimerization (in either direction) appears to be precluded by *O*-sulphation of either the potential target HexA or an adjacent GlcN unit (Jacobsson *et al.*, 1984; Kusche & Lindahl, 1990).

The results of the present study suggest that the *E. coli* K5 polysaccharide may be useful as a starting material in designing specific substrates for enzymes involved in heparin biosynthesis. Other possibilities relate to the clinical applicability of the heparin preparations currently available. Heparins are heterogeneous with regard to inherent biological activities, pharmacological properties, and clinical side effects [for reviews, see several articles in Lane & Lindahl (1989) and in Ofosu *et al.* (1989)]. This variability is presumably due to the heterogeneity in structure

that is typical of heparin (and heparan sulphate). The artificial segregation of the *N*-sulphation, GlcA C-5 epimerization and *O*-sulphation reactions, as outlined in the present paper, would seem to offer interesting prospects for tailoring saccharide sequences into products with selective biological activities and/or clinical properties.

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