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Biosynthesis of Heparin

Effect of Detergent on the Microsomal Polymerization and Polymer Modification Processes*

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The formation of labeled heparin-precursor polysaccharide (*N*-acetylheparosan) from the nucleotide sugars, UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine, in a mouse mastocytoma microsomal fraction was abolished by the addition of 1% Triton X-100. In contrast, the detergent-treated microsomal preparation retained the ability to convert such preformed polysaccharide into sulfated products during incubation with 3'-phosphoadenylylsulfate (PAPS). However, as shown by ion-exchange chromatography of these products, the detegent treatment changed the kinetics of sulfation from the rapid, repetivive process characteristic of the unperturbed system to a slow, progressive sulfation, which involved all polysarccharide molecules simultaneously and yielded, ultimately, a more highly sulfated product. The detergent effect was attributed to solubilization of sulfotransferases from the microsomal membranes, along with other polymer-modifying enzymes and the polysaccharide substrate. The resulting product showed an apparently random distribution of *N*-acetyl and *N*-sulfate groups, instead of the predominantly block-wise arrangement achieved through membrane-associated biosynthesis. *O*-Sulfation occurred mainly at C2 of the iduronic acid units in the membrane-bound polysaccharide but at C6 of the glucosamine residues in the presence of detergent.

A capsular polysaccharide from *Escherichia coli* K5, previously found to have a structure identical to that of the nonsulfated heparin-precursor polysaccharide, was

Abbreviations: PAPS, 3'-phosphoadenylylsulfate; Hepes, 4-(2-hydroxy-ethyl)piperazineethanesulfonic acid; GlcUA, glucuronic acid.

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sulfated in the solubilized system in a fashion similar to that of the endogenous substrate, but was not accessible to the membrane-bound enzymes.

These findings suggest that the regulation of the polymer-modification process, and hence the structure of the final polysaccharide product, depends heavily on the organization of the enzymes and their proteoglycan substrate in the endoplasmic membranes of the cell.

The biosynthesis of heparin is a complex process which includes the assembly of polysaccharide chains on a protein core and subsequent modification of these polymers. Studies using microsomal fractions from mouse mastocytomas, with both endogenous and exogenous substrate polysaccharides, have shown the process to be rapid and highly ordered [1, 2]. Chain elongation proceeds by stepwise addition of D-glucuronic acid and *N*-acetyl-D-glucosamine residues from the respective UDP-sugars and is followed by an ordered sequence of polymer modification reactions, including *N*-deacetylation of *N*-acetylglucosamine residues, *N*-sulfation of unsubstituted amino groups, uronic acid C5 epimerization, and finally *O*-sulfation in at least three different positions [1-12]. In part, heparin biosynthesis is regulated by the strict substrate specificities of the enzymes involved [6, 10]. However, the ordered nature of the process presumably also requires an organized arrangement of the heparin precursor proteoglycan and the participating enzymes in the microsomal membranes.

In order to further establish the significance of an intact membrane organization for this complex polysaccharide synthesis, the process was studied after perturbation of membranous structures by addition of 1% by vol of the nonionic detergent, Triton X-100. The effects of this treatment on the polymerization, the kinetics of the polymer modification, and the structure of the final product confirm the necessity of an intact membrane organization for a normal functioning of the biosynthetic process.

Experimental Procedures

Materials

Hyaluronan, chondroitin 4-sulfate, and heparin reference standards were as described [8]. UDP-D-glucuronic acid, UDP-*N*-acetyl-D-glucosamine, and pronase were purchased from Sigma (St. Louis, MO, USA) and chondroitinase ABC from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Na[^{3}H]BH_4 (10 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U.K., 3′-phosphoadenylylsulfate (PAPS), the microsomal fraction from a mouse mastocytoma, and UDP-D-[^{3}H]glucuronic acid (5 Ci/mmol) were prepared as described [5]. UDP-D-[^{14}C]glucuronic acid was prepared like the ^{3}H-labeled compound except that [^{14}C]glucose was substituted for [^{3}H]glucose [5].

Capsular polysaccharide from *Escherichia coli* K5 (*N*-acetylheparosan) was prepared as described [13] and was radiolabeled by the following procedure. The polysaccharide (5 mg) was dissolved in 1 ml of borohydride-treated [14] 0.1 M Tris-HCl, pH 8.0, and 50 mCi of Na[^{3}H]BH_4 in 100 μl of 0.1 M NaOH was added. After incubation for 48 h at room temperature, unlabeled $NaBH_4$ (3 mg) was added, and the sample was left for another 24 h; the reaction mixture was then acidified to pH 4 with glacial acetic acid to destroy

$NaBH_4$ and adjusted to neutral pH with 4 M NaOH. The labeled polysaccharide was isolated by chromatography on a column (1 × 170 cm) of Sephadex G-15 (Pharmacia, Uppsala, Sweden), which was eluted with 0.2 M NH_4HCO_3. The labeled polymer was further separated from low molecular weight components on a column of Sephadex G-100, eluted with 1 M NaCl, and desalted by extensive dialysis against water. The specific activity of the product was 10^4 cpm/μg of uronic acid.

Methods

Uronic acid was determined by the method of Bitter and Muir [15], and protein was estimated by the procedure of Lowry *et al.* [16]. Radioactivity was measured by liquid scintillation spectroscopy as described previously [17]. Anion exchange chromatography and gel chromatography were performed as described in the Figure legends. High-pressure ion-exchange chromatography of disaccharides was carried out according to the method of Thunberg *et al.* [14], with ^{3}H-labeled internal-standard disaccharides prepared as described by Jacobsson *et al.* [18]. Depolymerization of polysaccharides with nitrous acid was carried out at pH 1.5 according to the procedure of Shively and Conrad [19], and the products were reduced with unlabeled $NaBH_4$ [14].

Polysaccharide chain formation was measured as labeled polymer formed by incubation of microsomes (5 mg of protein) with UDP-[^{14}C]glucuronic acid (10 μCi) and UDP-*N*-acetylglucosamine (0.25 μmol) in 0.5 ml of 0.05 M Hepes pH 7.4, containing 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 3.5 μM NaF. Triton X-100 was added to some samples at a final concentration of 1% (v/v). After incubation at 37°C for 30 min, the reaction mixtures were boiled for 3 min and digested with pronase as described [8]. Sodium hydroxide was added to a final concentration of 0.5 M, and, after 16 h at room temperature, the samples were neutralized and applied to columns (1 × 50 cm) of Sephadex G-50, which were eluted with 1 M NaCl. Labeled material eluted in the void volume was pooled and dialyzed extensively against water.

Modifications in the presence or absence of detergent of the polysaccharide synthesized on endogenous primers were examined as follows. Microsomal protein (40 mg), UDP-[^{14}C]glucuronic acid (40 μCi) and UDP-*N*-acetylglucosamine (2 μmol) were incubated in 4 ml of Hepes buffer, containing divalent cations and NaF as above, for 30 min at 37°C. After removal of a 250 μl aliquot, which was heat-inactivated, the remainder was divided into two equal portions. To one of these were added unlabeled UDP-glucuronic acid (10 μmol), PAPS (2 μmol), and 100 μl of water. The second half received 100 μl of 20% (v/v) Triton X-100 and, 5 min later, was transferred to a tube containing unlabeled UDP-glucuronic acid and PAPS as above. Incubation was continued at 37°C, and 250 μl aliquots were withdrawn after periods up to 1 h and heat-inactivated. Polymeric products were isolated by gel chromatography after pronase digestion and alkali treatment as described above.

Solubilization of the polysaccharide-modifying enzyme system by Triton X-100 was assessed as follows. A 1.9 ml reaction mixture identical to those used for studies of the modification reactions was incubated at 37°C for 30 min, and then 100 μl of 20% Triton X-100 and 10 μmol of unlabeled UDP-glucuronic acid were added. After continued incubation for 5 min, the mixture was cooled on ice and centrifuged in the cold at 100,000 × *g* for 60 min. The supernatant and pellet were separated, and the latter fraction was suspended in 2 ml of standard Hepes buffer containing divalent cations and

NaF. After addition of 2 μmol of PAPS to each fraction, incubation was continued for 60 min at 37°C. Polysaccharide products were isolated after proteolytic digestion and alkaline elimination as before.

Modification of exogenous polysaccharide substrate (*N*-acetylheparosan from *E. coli* K5) was examined as follows. The microsomal enzyme preparation (20 mg of protein) was preincubated in 1.85 ml of standard Hepes buffer with divalent cations and NaF for 30 min at 37°C. After addition of 100 μl of 20% Triton X-100 and 50 μl of a solution of ^{3}H-labeled *E. coli* polysaccharide (approximately 5×10^{6} cpm), incubation was continued for 5 min, and PAPS (2 μmol) was added to initiate modification reactions. Samples were removed at different times during continued incubation for 1 h, and the polysaccharide was recovered after pronase digestion and alkali treatment as described.

Results

The effects of the nonionic detergent, Triton X-100, on the synthesis of heparin in microsomes from a mouse mastocytoma have been examined in a series of experiments focused on: a) the formation of polysaccharide chains from UDP-glucuronic acid and UDP-*N*-acetylglucosamine on endogenous primer; b) the modification reactions initiated by addition of PAPS to incubation mixtures containing preformed polysaccharide; c) the distribution of polymer-modifying activity and of endogenous polysaccharide products between particulate and soluble fractions; d) the modification of exogenous polysaccharide substrate (*N*-acetylheparosan from *E. coli* K5); and e) the structure of polysaccharide products. Details of these experiments are now described.

Effect of Triton X-100 on Chain Elongation

Incubation of mastocytoma microsomes with UDP-[^{3}H]glucuronic acid and UDP-*N*-acetylglucosamine under standard reaction conditions, in the absence of detergent, yielded a nonsulfated polysaccharide $(\text{-GlcUA-GlcNAc-})_n$, which was partially deacetylated, as indicated by its elution position on DEAE-cellulose chromatography (Fig. 1A (top panel, zero time); *cf.* [4]). This finding is in accord with the results of several similar experiments described previously [4, 8, 9]. The total radioactivity incorporated into polymeric material in a typical experiment was 110 000 cpm/ml of incubation mixture. However, only small amounts of products were formed (18 000 cpm/ml) when Triton X-100 was included in the reaction mixture. Furthermore, this material was resistant to deamination by nitrous acid (pH 3.9; see [9]) but susceptible to digestion with chondroitinase ABC (results not shown) and thus would seem to be related to chondroitin sulfate rather than heparin.

Effect of Triton X-100 on Polymer Modification

The experimental system used to study the effect of Triton X-100 on the modification reactions has been described previously [4]. Briefly, polysaccharide chain elongation on endogenous primer was allowed to occur during a 30 min period in the presence of UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine. Unlabeled UDP-glucuronic acid was then added to quench further incorporation of radioactivity, and the modification reactions were initiated by addition of PAPS. The progression of the modifications

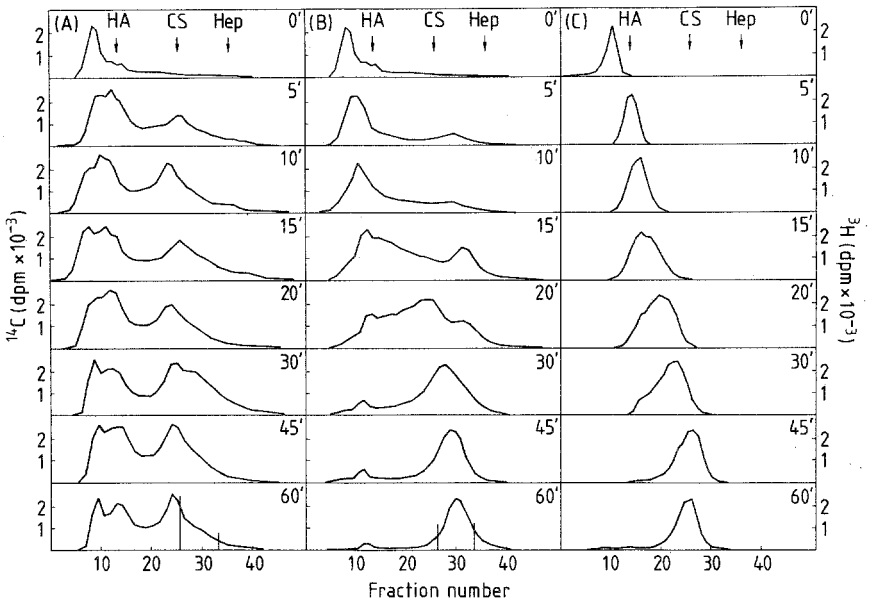

Figure 1. Anion-exchange chromatography of *N*-acetylheparosan after incubation for different periods of time with a mastocytoma microsomal enzyme preparation in the presence of PAPS.

The samples are biosynthetically-labeled, preformed microsomal polysaccharide, incubated in the absence (A), and presence (B) of Triton X-100, and (C) endgroup-labeled capsular polysaccharide from *E. coli* K5 incubated in the presence of Triton X-100. The isolated labeled polysaccharide samples and a mixture of standard hyaluronan (HA), chondroitin sulfate (CS), and heparin (Hep) were applied to a 5 ml column of DEAE-cellulose equilibrated with 0.05 M LiCl in 0.05 M sodium acetate buffer, pH 4.0. The column was rinsed with five volumes of the same buffer (not shown in the figures) and was then eluted with a linear gradient of 0.05-1.5 M LiCl in 0.05 M sodium acetate buffer, pH 4.0. Fractions of 3 ml were collected and analyzed for radioactivity and uronic acid (see arrows indicating peak elution positions of standard polysaccharides). For additional information see the text.

with time, in the presence or absence of Triton X-100 (final concentration, 1%), was monitored by chromatography of aliquots of the reaction mixtures on DEAE-cellulose (Fig. 1).

Analyses of control samples incubated in the absence of detergent (section A of Fig. 1) showed rapid sulfation of a portion of the labeled polysaccharide molecules. Sulfation was indicated by the appearance of a peak of labeled material which was eluted after the position of chondroitin sulfate. This peak was prominent already 5 min after the initiation of sulfation and reached its maximal size after about 10 min of incubation with PAPS. Similar observations in previous studies were interpreted as indicative of extensive sulfation affecting only a small proportion of the polysaccharide molecules at a time; *i.e.* maximally sulfated species are generated before modification of additional molecules occurs [4]. The retention of a fairly large fraction of essentially nonsulfated molecules throughout the 1 h incubation period probably reflects the continuous dilution of this pool by newly synthesized unlabeled polysaccharide (due to the addition of excess unlabeled UDP-glucuronic acid), rather than cessation of the sulfation process.

In the presence of Triton X-100, incubation for 5 min also yielded a highly sulfated product, which was eluted as a retarded peak on ion-exchange chromatography (section b in Fig. 1); however, this peak was smaller than the corresponding peak from the control sample. After 15-20 min incubation, the chromatographic pattern was dominated by molecular species of intermediate charge density which were eluted broadly between the starting material and the most retarded peak. Incubation for 1 h in the presence of detergent converted the starting material quantitatively into highly charged molecules, which emerged near the heparin standard. It should be noted that these modification products were eluted later than the analogous products synthesized in the absence of detergent. We conclude from these results that, in the presence of detergent, the preformed polysaccharide molecules were all modified at the same time in a gradual process which eventually resulted in maximal sulfation of all molecules. Thus, while the detergent changed the kinetics of the sulfation process radically, the ultimate extent of sulfation exceeded that obtained with the unperturbed system. The lack of residual nonsulfated material was presumably due to the inhibitory effect of the detergent on the polymerization reaction.

Analysis of Detergent-treated Reaction Mixtures for Solubilized Enzymes and Reaction Products

The reaction products in the soluble and particulate fractions of reaction mixtures containing detergent were compared to determine whether the modifying enzymes had been solubilized or whether the modification process in the particulate fraction had been otherwise disrupted. A reaction mixture containing preformed, labeled polysaccharide was incubated for 5 min in the presence of 1% Triton X-100 and was then centrifuged at 100 000 × *g* to separate soluble and particulate material (for details, see the Methods section). The presence of polymer-modifying enzymes in the two fractions was then assessed by continued incubation after addition of PAPS and unlabeled UDP-glucuronic acid. Analysis by gel chromatography on Sephadex G-50, following proteolytic digestion and alkali treatment of the fractions, showed that 80% of the labeled polysaccharide had been solubilized by the detergent treatment. Enzymatic activity was present in both the soluble and particulate fractions, since modification of preformed polysaccharide did occur in each fraction. However, the elution profiles on DEAE-cellulose chromatography were distinctly different. The pattern for the soluble fraction was similar to that of the detergent-treated sample shown in Fig. 1B, whereas the profile for the particulate fraction resembled that of the control sample shown in Fig. 1A. The presence of residual enzyme activity and of about 20% of the total labeled polysaccharide in the particulate fraction was consistent with the observation that a portion of the polysaccharide in the complete reaction mixture containing. Triton X-100 was modified as in the detergent-free control sample (see Fig. 1). It is concluded from this experiment that the altered modification pattern in detergent-treated samples was due to solubilization of the modifying enzymes and of their substrate.

Structural Analysis of Products Formed in the Presence and Absence of Triton X-100

The maximally-modified products formed in the presence and absence of detergent (fractions 26-33 in Fig. 1) were subjected to deaminative cleavage at pH 1.5 in order to assess the distribution of *N*-sulfated glucosamine residues. After reduction with borohydride, the resulting oligosaccharides were separated by chromatography on

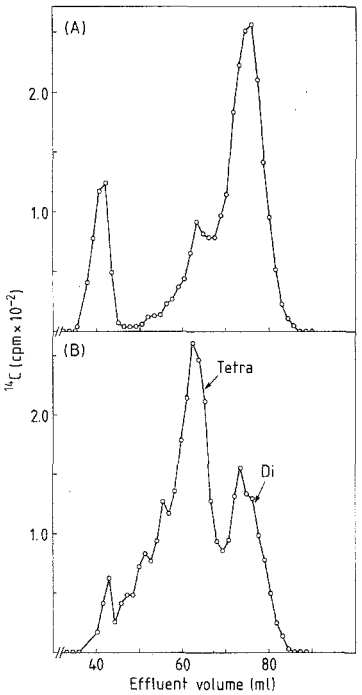

Figure 2. Gel chromatography of deamination products of microsomal polysaccharide incubated with PAPS in the absence (A) or presence (B) of Triton X-100.

Biosynthetically-labeled, preformed microsomal polysaccharide was incubated with mastocytoma microsomal fraction and PAPS in the absence or presence of detergent (see Fig. 1A and B, repectively). The retarded portions of the products obtained after incubation for 60 min (indicated by vertical lines in Fig. 1A and B, bottom panels) were desalted and subjected to deaminative cleavage at pH 1.5. After reduction, the samples were applied to a column (0.8 × 200 cm; excluded volume, V_0, 42 ml) of Sephadex G-25 superfine, which was eluted with 1 M NaCl. Fractions of 1.4 ml were collected and analyzed for radioactivity. The fractions between the vertical lines were pooled for further analyses of disaccharides (Fig. 3).

Sephadex G-25. As seen in Fig. 2, the product in the control sample yielded a major peak in the position of disaccharides, but some larger oligosaccharides were also seen as well as a prominent peak in the void volume. In contrast, the detergent-treated sample yielded a major peak at the elution position of tetrasaccharides and a smaller peak in the disaccharide region, in addition to large oligosaccharides and a small void volume component. These results indicate that most of the glucosamine residues in the control sample were *N*-sulfated, although a substantial proportion of *N*-acetylated block structure was also present. The finding that the detergent-treated sample contained only a small proportion of large molecules emerging in the void volume and that the major products were tetrasaccharides, derived from polymer sequences of alternating *N*-sulfated and *N*-acetylated disaccharide units, indicated that *N*-sulfation had occurred in a random fashion.

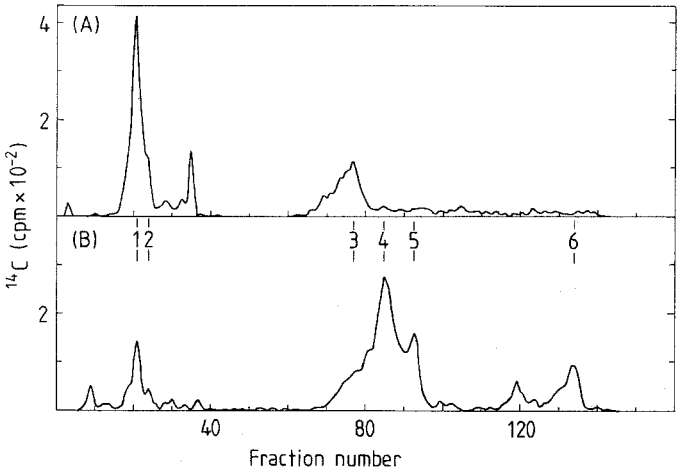

Figure 3. High performance ion-exchange chromatography of disaccharides isolated after incubation of microsomal polysaccharide with PAPS in the absence (A) or presence (B) of Triton X-100.

The disaccharide fractions obtained after gel chromatography of the deaminated incubation products (see Fig. 2) were desalted by gel chromatography on Sephadex G-15 in 0.2 M NH_4HCO_3 and were then lyophilized and analysed by high performance ion-exchange chromatography on a column of Aminex A-25. The elution positions of the following ^{3}H-labeled internal disaccharide standards are indicated by arrows; 1, glucuronosyl-anhydromannitol; 2, iduronosyl-anhydromannitol; 3, iduronosyl-(2-*O*-sulfate)-anhydromannitol; 4, glucuronosyl-anhydromannitol (6-*O*-sulfate); 5, iduronosyl-anhydromannitol(6-*O*-sulfate); and 6, iduronosyl(2-*O*-sulfate)-anhydromannitol(6-*O*-sulfate).

Further characterization of the reaction products was carried out by high-performance ion-exchange chromatography of the disaccharide fractions isolated after nitrous acid treatment and reduction with borohydride. Analysis of a control sample (Fig. 3A), labeled with ^{14}C in the uronic acid moieties, showed glucuronosyl-anhydromannitol and iduronosyl(2-sulfate)-anhydromannitol to be the major disaccharide components, along with smaller amounts of iduronosyl-anhydromannitol (indicated by the shoulder on the glucuronosyl-anhydromannitol peak). The pattern for the detergent-treated sample (Fig. 3B) differed substantially from the control profile and was dominated by the monosulfated disaccharides. Glucuronosyl-anhydromannitol(6-sulfate) was the major component. A significant amount of the disulfated disaccharide, iduronosyl(2-sulfate)-anhydromannitol(6-sulfate), was also present, as were the non-sulfated disaccharides, but the proportion of the latter was much lower than in the control sample.

Effect of Triton X-100 on Modification of N-Acetylheparosan from E. coli K5

A preparation of the capsular polysaccharide from a strain of *E. coli* K5 (*N*-acetylheparosan), which hade been end-labeled by reduction with Na [^{3}H]BH_4, was used as an exogenous substrate in experiments similar to those described above. After incubation with PAPS in the absence of detergent, no change in the charge density of the polysaccharide was observed, as indicated by chromatography on DEAE-cellulose. It thus appeared that the exogenous polysaccharide was restricted from entering the membrane space where the modifying enzymes were located. In contrast, the

presence of Triton X-100 in the reaction mixture resulted in extensive modification of the added polysaccharide, and, after incubation for 1 h, the product was eluted from DEAE-cellulose in a position close to that of the chondroitin sulfate standard (Fig. 1C). The conversion of bacterial *N*-acetylheparosan to a sulfated species resembled the processing of the endogenous polymer under the same conditions. The entire labeled polysaccharide pool was gradually modified, as evidenced by the continuous shift of the polysaccharide peak towards more retarded positions on ion-exchange chromatography. It may be noted that complete characterization of the modified polysaccharide in terms of the sulfate substitution pattern was not feasible, since the polysaccharide was only labeled in the reducing terminal residue of the chain.

Discussion

The biosynthesis of a proteoglycan is a multi-step process which requires the coordinated action of a large number of different enzymes in many steps. The assembly of a heparin proteoglycan involves at least 12 separate reactions, not counting the formation of the polypeptide core. Most of these reactions appear to take place in the Golgi apparatus but little is known regarding the organization of the corresponding enzymes in the functional biosynthetic apparatus; in fact, we can only state with some confidence that most of the enzymes and their macromolecular proteoglycan substrates are membrane-bound. In the present investigation we have approached this problem by studying the effects of the nonionic detergent, Triton X-100, on a cell-free heparin-synthesizing system. Both polysaccharide formation and polymer modification were assessed and related to the corresponding processes in the unperturbed system.

The glucuronosyl- and *N*-acetylglucosaminyltransferases involved in the formation of the nonsulfated heparin-precursor polysaccharide, *N*-acetylheparosan, are readily solubilized by treatment with detergent under appropriate conditions and may then be assayed with oligosaccharides of suitable structure as sugar acceptors [1, 20]. In such a system, the oligosaccharides are elongated essentially by one monosaccharide unit only, even if the reaction mixtures contain both UDP-sugars required for chain formation. In contrast, the appropriate oligosaccharides serve as primers for more extensive polymerization when added to an intact mastocytoma microsomal fraction, showing that a membrane-bound, nascent proteoglycan is not an obligatory initiator of such a reaction [20]. Conversely, in the present study polymerization on endogenous primer was abolished in the presence of detergent. Taken together, these findings suggest that the appropriate positioning of the glucuronosyl- and *N*-acetylglucosaminyltransferases in the endoplasmic membrane is of critical importance to the concerted action of these enzymes and is required for efficient formation of a polysaccharide chain.

The effects of membrane perturbation on the polymer-modification reactions appear to be more intricate. Both the proteoglycan substrate and the enzymes involved in the modification process were largely solubilized by the detergent. Ion-exchange chromatography of the products formed following the addition of PAPS to the solubilized system indicated that the overall extent of sulfation was not diminished but, in fact, greater than in the corresponding control incubations. However, the kinetics of the sulfation process were drastically changed, from the rapid, stepwise reactions displayed by the unperturbed system to simultaneously progressing, slow sulfation of all solubilized substrate molecules.

The polysaccharide obtained with intact microsomes contained extended sequences of disaccharide units that were either exclusively *N*-sulfated or *N*-acetylated, indicative of a non-random replacement of *N*-acetyl groups with *N*-sulfate groups. The apparent preponderance of iduronosyl 2-*O*-sulfate over glucosaminyl 6-*O*-sulfate groups may be due to the order of the various sulfation reactions, since 2-*O*-sulfation occurs before 6-*O*-sulfation, and, in the cell-free system, only a limited fraction of the substrate molecules pass through the entire sequence of modification reactions [6]. In contrast, the product sulfated by the solubilized system displayed a seemingly random distribution of *N*-acetyl and *N*-sulfate groups, with a large proportion of alternating *N*-acetylated and *N*-sulfated disaccharide units. Further, the products contained appreciably more 6-*O*-sulfate than 2-*O*-sulfate groups. These findings suggest that the kinetics and regulation of the polymer modification process depend heavily on the organization of the enzymes in the intracellular membranes of the cell and on the mode of transport of the proteoglycan substrate between these enzymes [2].

It is interesting to note that a bacterial polysaccharide with a structure similar to that of *N*-acetylheparosan (i.e., the initial heparin-precursor polysaccharide) could be substituted for the endogenous microsomal polysaccharide as a substrate in the solubilized enzyme system. The bacterial polysaccharide has been previously found to serve as a substrate in the *N*-acetylglycosaminyl deacetylase reaction which initiates polymer modification [21] and is apparently also an efficient acceptor of sulfate groups in subsequent sulfotransferase reactions.

During biosynthetic polymer-modification the polysaccharide chains of the heparin proteoglycan are tailored into various sequences designed for interations with a number of proteins (see [2, 22, 23]). Such interactions are likely to be reflected in many of the various biological activities attributed to heparin. The interacting sequence best characterized so far is the antithrombin-binding region, which is a pentasaccharide segment composed of three glucosamine units and one residue each of glucuronic acid and iduronic acid. The antithrombin-binding properties of this region depend on the presence of at least four sulfate groups in defined positions, one of which is unique to this particular region of the heparin chain and is located at C3 of the internal glucosamine residue [7, 24, 25]. It is difficult at present to assess precisely how the formation of a specific structure such as the antithrombin-binding region may be influenced by the organization of the polymer-modifying enzymes in the membranes. In fact, it is conceivable that these enzymes operate partly at random, within the limits dictated by substrate specificity. However, an altogether randomized mechanism appears unlikely in view of recent observations concerning the distribution of the antithrombin-binding regions in a small fraction of the polysaccharide chains only [2, 26, 27]. Indeed, a restricted distribution of key enzymes, such as the glucosaminyl 3-*O*-sulfotransferase, in the Golgi membrane may be of profound importance in allocating specific structures along the polysaccharide chains [2].

Acknowledgements

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