Biosynthesis of Chondroitin Sulfate

ORGANIZATION OF SULFATION*

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The potential relationship of an intact membrane organization for the synthesis of chondroitin and chondroitin 4-sulfate was examined after modification of a mouse mast cell microsomal system with the nonionic detergent, Triton X-100. The results indicated that Triton X-100 had no effect on the rate of polymerization but had a slight effect on the size of glycosaminoglycan chains. An "all or nothing" pattern of sulfation of newly formed chondroitin was obtained in both the presence and the absence of Triton X-100, and this pattern did not change whether sulfation was initiated concurrent with or subsequent to polymerization. Sulfation of exogenous [14C]chondroitin and exogenous proteo[H]chondroitin by the microsomal system required Triton X-100 but still produced an all or nothing pattern rather than a random sulfation pattern. When a 100,000 × g supernatant fraction was utilized for sulfation of [14C]chondroitin or proteo[H]chondroitin, Triton X-100 was not needed, and a partial sulfation pattern was obtained. However, it was similar to the all or nothing pattern in that it still produced two populations, with some chains nonsulfated and others approximately 50% sulfated. When chondroitin hexasaccharide was used with 3'-phosphoadenylphosphosphate[38S]sulfate, multiple GalNAc residues of the individual hexasaccharides were found to be sulfated. This was relatively independent of Triton X-100 or the concentration of the hexasaccharide acceptors. With soluble enzyme, sulfation of multiple GalNAc residues on the individual hexasaccharide molecules was even greater, so that trisulfated products were found. These results suggest that efficient sulfation of chondroitin is related to enzyme-substrate interaction more than to membrane organization.

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Sulfating mastocyte cell system that appears to produce a single species of proteochondroitin (7). Our results indicated that, in contrast to polymerization, efficient sulfation appeared to depend more upon enzyme-substrate interaction than membrane organization.

MATERIALS AND METHODS

UDP-[14C]GlcA, UDP-[3H]GalNAc, and PAPS were purchased from Du Pont-New England Nuclear. Nonradioactive UDP-GlcA, UDP-GalNAc, and PAPS were purchased from Sigma. Chondroitin 6-sulfate, chondroitin 4-sulfate, chondroitin ABC lyase, and various disaccharides plus nonsulfated hexasaccharide standards (3, 22) were purchased from Miles (Elkhart, IN). Chondroitin hexasaccharide was obtained as previously described (11, 12).

A microsomal fraction sedimenting between 10,000 x g and 105,000 x g was prepared from 2 ml (packed cells) of cultured P-815Y mastocytoma cells as previously described (7). Microsomal preparations as well as 105,000 x g supernatants, were stored at 20 °C until utilized.

Reaction mixtures containing 0.05 M MES buffer, pH 6.5, 0.015 M MsoCl2, 0.2 mM UDP-[14C]GlcA (350 x 10^6 cpm/μmol) plus 0.2 mM UDP-GalNAc or 0.2 mM UDP-[3H]GalNAc (980 x 10^6 cpm/μmol) and 0.2 mM UDP-GlcA and 12 μl of mouse mast cell microsomal preparation as a total volume of 45 μl were incubated in the absence or presence of Triton X-100 (1%) and PAPS (3.0 mM) at 37 °C for varying periods of time. Some incubations were first performed with labeled sugar nucleotides for 2 h. Then a 100-fold excess of cold sugar nucleotides was added to dilute the label, and PAPS was added. Aliquots from reaction mixtures that contained PAPS from the beginning, as well as from reactions that only had PAPS added after the initial 2 h incubation, were incubated for various time intervals, spotted on Whatman No. 1 paper, and chromatographed in ethanol/0.5 M ammonium acetate, pH 7.8 (5:2 overnight). Following chromatography, the origins were eluted with 0.5 mM sodium hydroxide at room temperature overnight to obtain the [14C]glycosaminoglycan or [3H]glycosaminoglycan chains. After neutralization with acetic acid, each eluate was lyophilized and then desalted on a Sephadex G-25 column. Aliquots of [14C]glycosaminoglycans obtained from incubations performed in the absence of Triton X-100 together with [3H]glycosaminoglycans obtained from incubations performed in the presence of Triton X-100 were co-chromatographed on Sepharose CL-4B (0.7 x 45 cm) columns or DEAE-cellulose (DE52) (0.7 x 5 cm) along with standards. The Sepharose CL-4B columns were eluted with 0.5 M ammonium bicarbonate. The DEAE-cellulose columns were eluted with a logarithmic gradient of 0.06–1.0 M LiCl. Aliquots from the fractions were assayed for radioactivity and uronic acid (23).

Other aliquots of the [14C]glycosaminoglycans or [3H]glycosaminoglycans were desulfated by treatment with chondroitin ABC lyase for 2 h, with 0.15 unit in 1 ml of 0.05-1.0 M NH4OH (2:3:1) overnight. Following chromatography, the origins were eluted with 0.5 M ammonium bicarbonate as ammonium acetate, pH 7.8 (5:2 overnight). Following chromatography, the origins were eluted with 0.5 M ammonium bicarbonate as ammonium acetate, pH 7.8 (5:2 overnight). Following chromatography, the origins were eluted with 0.5 M ammonium bicarbonate as ammonium acetate, pH 7.8 (5:2 overnight). Following chromatography, the origins were eluted with 0.5 M ammonium bicarbonate as ammonium acetate, pH 7.8 (5:2 overnight).

Sulfation of Chondroitin: [14C]chondroitin in the absence and presence of Triton X-100. Aliquots of [14C]chondroitin formed at varying time intervals in the absence (O) or presence (△) of 1% Triton X-100 were assayed as described under "Materials and Methods."
Chondroitin 4-sulfate standards were co-chromatographed on a DEAE-cellulose gradient elution of the [14C]chondroitin and [3H]chondroitin sulfate in the presence of Triton X-100 and PAPS added during polymerization. When the sulfated peak (fractions 68-76) was degraded by chondroitin ABC lyase, the products were 53% [14C]ADi-4S and 50% [3H]ADi-4S, 50% [3H]ADi-0S. Sulfation of exogenous [14C]chondroitin and proteo[3H]chondroitin was added to the microsomal preparation was examined by incubations with PAPS for 2 h in the presence of Triton X-100. As previously described (21), little or no sulfation took place on these exogenous macromolecules in the absence of Triton X-100. DEAE-cellulose chromatography of [14C]chondroitin sulfate together with the [3H]chondroitin sulfate derived from proteo[3H]chondroitin was shown in Fig. 4A. The total sulfation was 18-20% by chondroitin ABC lyase, considerably less than the 58% (Table I) on endogenous nascent chondroitin. However, the products still had a similar all or nothing pattern. When the sulfated peak (fractions 68-76) was degraded by chondroitin ABC lyase, the products were 95% [14C]ADi-4S, 5% [14C]ADi-0S and 95% [3H]ADi-4S, 5% [3H]ADi-0S.

We questioned whether this all or nothing sulfation pattern of exogenous [14C]chondroitin and proteo[3H]chondroitin was due to the membranous nature of the microsomal system. Therefore, we performed incubations (no Triton X-100) with soluble (100,000 x g supernatant) enzyme to see whether or not the organized sulfation would be lost, resulting in a heterogenous mixture of partially sulfated chains. The total sulfation was 10-15% by chondroitin ABC lyase. DEAE-cellulose chromatography of [14C]chondroitin sulfate together with the [3H]chondroitin sulfate derived from proteo[3H]chondroitin sulfate is shown in Fig. 4B. Surprisingly, the organized sulfation persisted, with two discrete populations, although the sulfated chains were only partially sulfated. When the sulfated peak (fractions 54-68) was degraded by chondroitin ABC lyase, the products were 47% [14C]ADi-4S, 53% [14C]ADi-0S and 50% [3H]ADi-4S, 50% [3H]ADi-0S.

A. 120' 56' 46 54 66 74 82 90 100

B. 120' 56' 46 54 66 74 82 90 100

C. 120' 56' 46 54 66 74 82 90 100

Fig. 3. DEAE-cellulose chromatograms of [14C]chondroitin and [3H]chondroitin sulfate formed in the absence of Triton X-100 and PAPS. Aliquots of [14C]chondroitin and [3H]chondroitin sulfate formed at various time intervals in the absence of Triton X-100 (1) and aliquots of [14C]chondroitin and [3H]chondroitin sulfate formed at various intervals in the presence of Triton X-100 (0) together with hyaluronic acid and chondroitin 4-sulfate standards were co-chromatographed on a DEAE-cellulose column (0.7 x 0.5 cm) with a logarithmic gradient of 0.05-1.0 M LiCl as described under "Materials and Methods." Fractions of 0.5 ml were collected and assayed for radioactivity and uronic acids. The cpm have been adjusted to reflect equal aliquots of the reactions. A, [14C]chondroitin and [3H]chondroitin formed in the absence of PAPS. B, [14C]chondroitin sulfate and [3H]chondroitin sulfate formed in the presence of PAPS added during polymerization. C, [14C]chondroitin sulfate and [3H]chondroitin sulfate formed in the presence of PAPS added subsequent to polymerization.

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>with Triton X-100</th>
<th>without Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[14C]</td>
<td>[3H]</td>
</tr>
<tr>
<td></td>
<td>ADi-4S</td>
<td>ADi-0S</td>
</tr>
<tr>
<td>A. 120'</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>B. 5'</td>
<td>18</td>
<td>82</td>
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<td>15'</td>
<td>26</td>
<td>74</td>
</tr>
<tr>
<td>120'</td>
<td>43</td>
<td>57</td>
</tr>
<tr>
<td>C. 15'</td>
<td>41</td>
<td>59</td>
</tr>
<tr>
<td>120'</td>
<td>87</td>
<td>43</td>
</tr>
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</table>

Sulfation of Chondroitin
Sulfation of Chondroitin

FIG. 4. DEAE-cellulose chromatograms of sulfated exogenous \[^{14}C\]chondroitin and sulfated \[^{3}H\]chondroitin obtained from sulfated exogenous proteo\[^{3}H\]chondroitin. Aliquots of sulfated exogenous \[^{14}C\]chondroitin (A) and sulfated \[^{3}H\]chondroitin (C) derived from sulfated exogenous proteo\[^{3}H\]chondroitin together with hyaluronic acid and chondroitin 4-sulfate standards were cochromatographed on a DEAE-cellulose column and assayed as in Fig. 3. Upper panel, sulfation by microsomal system; Triton X-100 present. Lower panel, sulfation by 100,000 \(x\) g supernatant; no Triton X-100.

TABLE II

<table>
<thead>
<tr>
<th>Hexasaccharide conc. (mM)</th>
<th>Triton X-100 (−)</th>
<th>Triton X-100 (+)</th>
<th>γGalNAc residues</th>
<th>GalNAc residues sulfated</th>
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<tbody>
<tr>
<td>Microsomal</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3.0</td>
<td>2250</td>
<td>9600</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>11</td>
<td>51</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>0.003</td>
<td>11</td>
<td>51</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
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</tr>
<tr>
<td>3.0</td>
<td>430</td>
<td>0.08</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>0.003</td>
<td>2.7</td>
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</table>

We next examined the pattern of chondroitin hexasaccharide sulfation. Previously we showed that incubation of a high concentration of hexasaccharide (0.7 mM) with the microsomal preparation resulted in sulfation of less than 1% of the available GalNAc residues. The percent of GalNAc residues sulfated could be greatly increased (up to 13%) by lowering the hexasaccharide concentration and by the addition of Triton X-100 (21). It was not determined whether the sulfation was of single or multiple GalNAc residues on individual hexasaccharides. Thus, one might expect that sulfation of multiple residues might be enhanced by the increased sulfating efficiency with low hexasaccharide concentration and with Triton X-100. We repeated such incubations with an even more extreme concentration difference (1000-fold) (Table II) in order to examine such products. DEAE-cellulose chromatograms of these \(^{35}S\)-hexasaccharides are shown in Fig. 5 together with standards of nonsulfated hexasaccharide and trisulfated hexasaccharide. Surprisingly, even at high hexasaccharide concentrations, most of the \(^{35}S\)-hexasaccharide was found to be disulfated with only a minor portion of monosulfated hexasaccharide. Lowering the hexasaccharide concentration had only a slight effect on the products, resulting in a small amount of trisulfated hexasaccharide. Even though the presence of Triton X-100 increased total sulfation 4-5-fold, there was no change in the percent of disulfated hexasaccharide relative to monosulfated hexasaccharide at high hexasaccharide concentration, and only a moderate increment of trisulfated hexasaccharide at low hexasaccharide concentration. Thus, the sulfation of multiple residues rather than a single residue in the hexasaccharide was mainly independent of hexasaccharide concentration and Triton X-100.

We questioned whether the sulfation of multiple residues was related to the particulate nature of the microsomal sulfating system. Therefore, we also performed incubations of hexasaccharide with soluble (supernatant) enzyme. In these experiments there was considerably less sulfation than with the microsomal system (Table II). Examination of the \(^{35}S\)-hexasaccharide products (Fig. 6) however, indicated a much higher proportion of trisulfated hexasaccharide even at the high hexasaccharide concentrations. Thus, the supernatant enzyme was significantly more effective at sulfating multiple residues than was the particulate system.

DISCUSSION

Detergents have been reported to have multiple and divergent effects upon the cell-free biosynthesis of glycoconjugates.
There are reports (25, 26) that the synthesis of macromolecules may be facilitated, or disrupted, or products may be modified. These detergent effects could presumably be exerted by solubilization of enzymes and or endogenous substrates or by altering membranes which in turn might affect permeability or transport and/or movement of substrates and products. Thus, depending upon detergent, conditions, and the membrane systems, the effects could be on many facets of membrane organization and/or enzyme-substrate interaction.

Previously described effects of detergents on glycosaminoglycan synthesis and sulfation have been variable depending upon the source of the microsomal enzyme, acceptor, and the type of glycosaminoglycan synthesized. However, the effects have not been examined extensively. It has been reported that the microsomal synthesis and sulfation of heparin is disrupted by detergents, so that heparin glycosaminoglycan is not produced, and sulfation is modified (27). In contrast, there are reports that in microsomal systems, neutral detergents stimulate the addition of single sugars to exogenous substrates (13) and stimulate sulfation of exogenous substrates (18, 20, 21). The effects of Triton X-100 on true polymerization of chondroitin rather than on the addition of one or a few sugars has not been previously described.

Our experiments utilizing sugar nucleotides clearly demonstrated that Triton X-100 had no effect on the rate of chondroitin synthesis in the mast cell microsomal system (Fig. 1), indicating that in this system the particulate nascent proteoglycan primer and the particulate glycosyl transferases were already in juxtaposition for efficient synthesis and that Triton X-100 did not effectively alter such a positioning of the substrates and enzymes. Triton X-100 did, however, have a slight effect on the size of the products (Fig. 2), suggesting that there might have been subtle changes in the architecture of the synthesizing site.

We had previously shown that the addition of Triton X-100 to this mast cell microsomal system (and to a chich cartilage microsomal system) did not change the amount of sulfate incorporated into nascent microsomal proteochondroitin (21). However, it was not clear whether the individual glycosaminoglycan chains would still maintain their characteristic all or nothing distribution of the sulfated residues in the presence of the Triton X-100. Contrary to our expectations, results have now demonstrated that the all or nothing sulfation pattern was undisturbed (Fig. 3B). This was true even when sulfation was subsequent to polymerization (Fig. 3C), suggesting that Triton X-100 did not separate the nascent microsomal proteochondroitin from the membrane-bound sulfotransferase. In contrast to the sulfation of endogenous chondroitin, the presence of Triton X-100 resulted in an increased incorporation of sulfate into exogenous acceptor. We had previously suggested that this was primarily due to a facilitation of acceptor movement into juxtaposition with the microsomal sulfation site rather than due to solubilization of the sulfotransferase (21). One might expect, however, that the detergent would also affect the organization of sulfation so that the all or nothing pattern might be lost. Surprisingly, an identical all or nothing pattern of sulfation was observed with the exogenous proteochondroitin and chondroitin (Fig. 4A) in a fashion similar to that of the endogenous substrate. Moreover, use of a 100,000 × g supernatant enzyme also resulted in an organized sulfation of exogenous proteochondroitin and chondroitin. Two distinct populations were seen, with some chains nonsulfated and others approximately 50% sulfated. Thus, exo-substrate interaction, rather than membrane organization would appear to have the greatest influence on the products. It should be noted, however, that none of the exogenous macromolecular substrates attained full sulfation, so that the microsomal system still appeared to play some role in the organization of sulfation.

Experiments with hexasaccharide indicated sulfation of more than one GalNAc residue on individual hexasaccharides at both low (much below the $K_m$) and high (much above the $K_m$) concentrations with both the microsomal and the soluble systems. These findings are in disagreement with statements by others (18) that sulfation of multiple GalNAc residues can only be attained with low concentrations of oligosaccharide substrates. Although Triton X-100 stimulated the total incorporation of sulfate, the degree of sulfation of individual hexasaccharide molecules was independent of this detergent. These results indicated that sulfation in the chondroitin sulfate synthesizing system depended more on enzyme-substrate interaction than on membrane organization. Thus, once the substrate attaches to the site of sulfation, it is not released until it is completely or nearly completely sulfated. Sulfation of a large substrate might be unidirectional, so that attachment of the enzyme to the middle of an exogenous chain might result in the partial sulfation that was seen with the supernatant enzyme.

The results obtained with this system are considerably different from those described with a heparin-synthesizing system (27). In that case, Triton X-100 was reported to inhibit polymerization completely and to disrupt the all or nothing pattern of sulfation.

REFERENCES