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MINI-REVIEW

Organization of glycosaminoglycan sulfation in the biosynthesis of proteochondroitin sulfate and proteodermatan sulfate

JEREMIAH E. SILBERT

Department of Veterans Affairs Medical Center, Bedford, 200 Springs Road, Bedford, MA 01730, USA and Department of Medicine, Harvard Medical School, USA

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Although the intermediates for sulfation of proteochondroitin and proteodermatan have been known for several decades, organizational aspects of this formation have not been clearly defined. Work in several laboratories, including our own, have indicated a pattern which strongly suggests that sulfation ordinarily takes place together with glycosaminoglycan polymerization in the same Golgi sites, and with close relationship to aspects of polymer elongation, polymer modification and polymer termination. The organization of sulfation together with polymerization may be a major factor controlling the location, type, and degree of sulfation, which in turn may direct specific functions of these proteoglycans.

Keywords: glycosaminoglycan, proteoglycan, chondroitin, dermatan, sulfate

Introduction

The sulfated glycosaminoglycans chondroitin/dermatan sulfate, heparin, heparan sulfate, and keratan sulfate are covalently linked to a wide range of core protein families. In addition they have considerable variation and heterogeneity in size and number of chains per core protein and considerable heterogeneity in position and degree of sulfation. This class of compounds shares in common a linear polymer structure which possesses repeat variably sulfated disaccharide units composed of a hexosamine alternating with another sugar. The functions of the proteoglycans may be as varied as their structures, with either the core protein or the highly anionic sulfated glycosaminoglycan structures directing these functions. It is likely that the core protein is involved in directing the nascent molecule to the appropriate Golgi sites for glycosaminoglycan formation and for channelling the transport and presentation of the completed proteoglycan to specific intracellular granule, cell surface, or matrix localities. It also appears likely that the fine structure of the highly anionic glycosaminoglycans may be of paramount importance in function at each location where the proteoglycans are presented.

The best example of a specific function of a glycosaminoglycan structure is that of the subset of heparin and heparan sulfate that binds to and activates antithrombin. In this case a specific pentasaccharide structure containing specific sulfation sites and specific uronic acid epimerization sites is essential [1, 2]. This structure serves as a prototype of how a glycosaminoglycan can direct function, and suggests that the locations and types of sulfate substituents on other glycosaminoglycans may be the determinants for other functions including cell-cell, cell-matrix, or cell-receptor interactions. Assuming that this may be the case, a sulfation process which is completely random seems unlikely. Thus there should be some biosynthetic mechanisms whereby sulfation is directed to specific types and sites on a glycosaminoglycan chain. However, no placement of sulfates in a programmed fashion on specific disaccharides in glycosaminoglycans has been determined, nor has the organization of sulfation relative to ongoing polymerization been completely defined. For this reason,

there has been some interest over a number of years in determining the control and specific localization of sulfation in relation to the polymerization of the glycosaminoglycans that contain these substituents.

Most of the experimentation concerning the relationship of sulfation to polymerization of glycosaminoglycans has been with chondroitin sulfate, which has a simpler structure than that of heparin and heparan sulfate. Sulfate localization in chondroitin sulfate is complicated, however, by the presence of GalNAc 4-S (S = sulfate) and GalNAc 6-S in the same glycosaminoglycan chains [3], by the presence of occasional GalNAc 4,6-diS and GlcA 2-S [4], and by the appearance of variable amounts of IdA and IdA 2-S [5] when dermatan sulfate is formed. The information concerning which GalNAc residues in the chain contain these variations in sulfation has been limited because of an inability to sequence the glycosaminoglycans. However, some data has been obtained regarding low sulfation of the first GalNAc residue at the reducing end [6] and there has been some detailed analysis of sulfation at the terminal non-reducing ends of glycosaminoglycans [7–9].

Recently chondroitin 6-sulfotransferase from chick embryo chondrocytes [10] and chicken serum [11] has been purified to homogeneity, and has subsequently been cloned [12]. This enzyme has also been shown to participate in the 6-sulfation of galactose in keratan sulfate, since the two activities copurify [10–12], and chondroitin and keratan are mutually competitive for sulfation with this enzyme [11].

Sulfation independent of chondroitin polymerization

Approximately 40 years ago Lipmann and his co-workers [13] identified 3′-phospho adenosine 5′ phosphosulfate (PAPS) as an 'active sulfate' and described its biosynthesis from ATP and inorganic sulfate [14]. In general the sulfate is derived directly from ingested sulfate or by metabolism of sulfated substances, but there is an indication that some might also be provided by oxidation of cysteine or methionine sulfhydryl groups [15, 16]. Subsequent to the characterization of PAPS, there was a flurry of activity concerning the use of $PAP^{35}S$ to demonstrate sulfation of chondroitin as well as other glycosaminoglycans [17]. Although a sulfated UDP-GalNAc (UDP-GalNAc-4-O-sulfate) was soon described [18], it was established that sulfate was incorporated directly from PAPS into chondroitin at the glycoconjugate level and not from the sulfated sugar nucleotide as precursor [19]. The earliest work concerned the use of soluble sulfotransferase systems to provide for the addition of sulfate onto occasional non-sulfated GalNAc residues of added soluble chondroitin or chondroitin sulfate. It later became apparent that microsomal systems containing the sulfotransferases worked more effectively, and were capable of incorporating sulfate from PAPS into preformed microsomal endogenous chondroitin/chondroitin sulfate [20]. Use of exogenous chondroitin oligosaccharides with these microsomal systems also proved to be particularly effective for examination of sulfotransferase activity [21, 22], while chondroitin was found to be somewhat less effective as an acceptor [23]. The addition of Triton X-100 or other detergents resulted in a greatly increased incorporation into added chondroitin or added oligosaccharides, and into one species of proteochondroitin [21, 22, 24, 25]. Exogenous proteochondroitin was also found to be a highly efficient acceptor, but the addition of detergent (Triton) was an absolute requirement for incorporation of any sulfate [23].

In the earlier work with sulfating enzymes, it was possible that infrequently some of the incorporation was onto already sulfated residues to form di-sulfated disaccharides. However, techniques were not available to confirm this. In all of these cases, the amount of sulfate that was incorporated was minor compared to the amount of GalNAc already present as part of the chondroitin sulfate. The characterization of incorporation to form GalNAc 4-S, GalNAc 6-S, GalNAc 4,6-diS, GlcA 2-S or IdA 2-S, and localization and distribution of the sulfated moieties in a chondroitin chain remained difficult if not impossible until a series of chondroitin sulfate-degrading bacterial enzymes and their products were characterized [26]. These enzymes and the methodologies that were developed [4, 27] for separation and identification of products have been invaluable for almost all of the subsequent characterizations related to positioning and specificities of sulfate substituents.

Even when non-sulfated chondroitin or non-sulfated chondroitin oligosaccharides were used as exogenous substrates with microsomal or with soluble sulfotransferase systems, the amounts of sulfate incorporated were small in comparison with the available sites for sulfation. Thus it was generally assumed that the incorporation mainly represented single random sulfate residues. However, when microsomal sulfotransferase systems were used for formation of chondroitin 6-sulfate and chondroitin 4-sulfate, close to 100% of the GalNAc residues in a percentage of endogenous nascent proteochondroitin could be sulfated in an 'all or nothing' pattern, while essentially no sulfate was incorporated into some of the nascent acceptors [28–30]. Moreover, under the proper conditions a modified 'all or nothing' pattern was also demonstrated for sulfation of exogenous proteochondroitin, chondroitin, or chondroitin oligosaccharide substrates [24, 25]. Even with soluble sulfotransferase, some of the acceptors were sulfated to a degree of 50% or more, while the rest remained unsulfated. Exogenous hexasaccharides were shown to accept sulfate on two or three GalNAc residues rather than on a single GalNAc residue even though the vast majority or added hexasaccharide

did not become sulfated at all [24, 25]. This indicated that the sulfotransferase enzymes preferentially continued sulfation on an individual chain or oligosaccharide once sulfation was initiated on that acceptor. These experiments also indicated that the microsomal preparation was more effective in this regard than was the soluble system.

In cell free biosynthesis with microsomal systems, the location of sulfation (4-sulfate or 6-sulfate) was found to be independent of the source or type of exogenous proteochondroitin, and only dependent upon whether the substrates had access to 4-sulfotransferase or 6-sulfotransferase [26]. There was also a pH effect, so that the sulfate incorporated into endogenous proteochondroitin in a cartilage microsomal system was 30–40% 4-sulfate and 60–70% 6-sulfate when incubations were at pH 6.5, while with incubations at pH 7.8 it was 100% 6-sulfate [31].

Sulfation linked to chondroitin polymerization

The results of [^{35}S]sulfate incorporation into endogenous receptors were in contrast to the sulfate incorporation observed when biosynthetically active microsomal systems were used together with the appropriate radioactively labelled sugar nucleotides (UDP-GlcA and UDP-GalNAc) to obtain prior or simultaneous chondroitin synthesis. In the absence of PAPS these incubations led to *de novo* formation of chondroitin glycosaminoglycan chains attached to endogenous core protein [32–35], demonstrating conclusively that sulfation was not necessary for polymerization to take place. When PAPS was added subsequent [28] to the polymerization in these microsomal systems, sulfation occurred to the same degree that was seen when sulfation accompanied the polymerization [28, 36]. Thus highly efficient sulfation of newly formed chondroitin could occur after completion of polymerization. The presence of PAPS during polymerization appeared to decrease the amount of polymer synthesized [30], although the final size of the proteoglycans or glycosaminoglycans was similar whether or not sulfation was taking place [28–30, 36]. The high efficiency in the percentage of individual sulfated chondroitin chains at the site of synthesis, was in contrast to the lesser efficiency in the percentage of individual sulfated exogenous chondroitin chains, supporting the concept of a similar subcellular site for sulfation and polymerization.

Some insight into the relationship of sulfation with polymerization has been obtained from experiments that have used xylosides such as methylumbelliferyl-xyloside or p-nitrophenyl-xyloside. When these xylosides were added to cultures of cells that produce proteochondroitin sulfate, chondroitin synthesis was stimulated on these substitute acceptors [37–39]. However, the degree of sulfation of the xyloside-linked chondroitin was less than the sulfation concurrently seen in the native proteochondroitin [40], indicating that the uncoupling of the nascent chondroitin from the normal membrane-bound nascent proteochondroitin decreased the efficiency of sulfation. This in turn implies that during synthesis, nascent membrane-attached proteochondroitin may be presented to membrane-attached sulfotransferases. Moreover, the efficiency of sulfation in cultured cells grown under conditions of sulfate deprivation has been shown to be lower for xyloside-linked chondroitin than for the native proteochondroitin [40].

The capacity to obtain essentially complete sulfation subsequent to polymerization in biosynthesis with cell free systems does not preclude the possibility that, *in vivo*, sulfation ordinarily occurs during polymerization rather than after polymerization is complete. If sulfation ordinarily takes place subsequent to polymerization, this would indicate that the subcellular location for sulfation could be separate from the location of glycosaminoglycan polymerization. Alternatively, if sulfation in intact membranes takes place during the polymerization process rather than following its completion, this would require the juxtapositioning of the polymerizing and sulfating enzymes in the same subcellular compartment. Early work concerning the sulfation of endogenous chondroitin receptors suggested that the latter situation was in fact the case. By use of microsomal systems to obtain the addition of a single radioactively labelled sugar from a single sugar nucleotide (either UDP-GlcA or UDP-GalNAc), it was shown that there were essentially no endogenous non-sulfated chondroitin chains or non-sulfated chondroitin oligosaccharides present in the endogenous proteoglycans at the microsomal site of synthesis [31]. Thus sulfate incorporation from PAP^{35}S into endogenous preformed glycosaminoglycan was into an occasional non-sulfated residue in chondroitin sulfate that was already highly sulfated. Nevertheless when PAP^{35}S was incubated with the microsomal system in the absence of glycosaminoglycan polymerization, small amounts of sulfate-labelled endogenous chondroitin oligosaccharides and endogenous short chain chondroitin sulfate was seen. This suggested that sulfation had already taken place on proteochondroitin oligosaccharides and short proteochondroitin glycosaminoglycans before they had been completely polymerized to the full size glycosaminoglycan chains. However it remained possible that these small sulfated chondroitin oligosaccharides and short chain sulfated chondroitin glycosaminoglycans could have represented occasional uncompleted glycosaminoglycan stubs occurring on completed proteoglycans that were not destined to undergo further polymerization.

To resolve this question, incubations of the microsomal system together with labelled sugar nucleotides and PAPS were conducted for short time periods at 10 °C in order to obtain proteochondroitin/chondroitin sulfate while it was actively growing [41]. The results demonstrated that some sulfation of the nascent chondroitin took place

under these conditions. The degree of sulfation increased with the size of the nascent chains, but even the smallest protein-linked oligosaccharides were partially sulfated. This confirmed the hypothesis that the sulfation was taking place during the active polymerization process but with sulfation lagging somewhat behind the polymerization. It also demonstrated that the enzymes of polymerization and sulfation were in juxtaposition in the membranes so that sulfation could follow shortly behind the incorporation of sugars into the growing chondroitin polymer. By subfractionation of microsomal preparations, it was shown that polymerization of chondroitin takes place in the Golgi [42], and more recently it has been shown that the enzymes of sulfation and polymerization are in the same medial and trans Golgi compartments [43].

The model of sulfation proceeding during the polymerization process fits well with the 'all or nothing' sulfation patterns that are seen. However, partial sulfation in intact cells has been produced under conditions of limiting concentrations of sulfate [44–47], as well as the presence of chlorate to limit production of PAPS [47–49], the presence of monensin to block PAPS transport to the sulfation site [50, 51], or the presence of xylosides which uncouple the chondroitin synthesis from its proteochondroitin membrane attachment [38, 40]. As might be anticipated, a pattern of random undersulfation generally has been found [40, 45, 49, 50], although there also have been reports of an 'all or nothing' pattern when limiting concentrations of sulfate were used [44].

Interactive control of sulfation and polymerization

Considering that there may be a relationship between chondroitin polymerization and sulfation, one might expect to find that the presence or absence of sulfate might affect the incorporation of sugars and that the sulfation itself might be related to the growing end of the chondroitin chain. This has indeed been shown. The presence of a 4-sulfate on a non-reducing terminal GalNAc abolishes the incorporation of GlcA from UDPGlcA onto the GalNAc [7]. The presence of 4-sulfate on a preterminal GalNAc with a terminal GlcA has a similar effect in abolishing incorporation of GalNAc onto the GlcA [52]. Thus the incorporation of a 4-sulfate in these positions could be considered as a mechanism to limit glycosaminoglycan chain size. In contrast, 6-sulfate has no effect [7], so that a polymer could continue to grow even if the terminal GalNAc were 6-sulfated. A distinct enzyme has been found in a variety of tissues from different vertebrate species that is capable of adding a 6-sulfate to a terminal GalNAc 4-S [53], which can then be 4-desulfated by another enzyme [54], resulting in a terminal GalNAc 6-S capable of allowing further polymerization to take place. These enzymes could serve a 'salvage' purpose, but it is not known whether or not this takes place *in vivo*.

Somewhat against the 'salvage' hypothesis as a major factor in biosynthesis is the data that has been obtained concerning the capabilities for addition of sulfate to terminal or pre-terminal GalNAc. Thus it has been demonstrated [23] with a chondroitin 6-sulfate-producing microsomal systems that no 6-sulfate will be added to exogenous chondroitin pentasaccharide containing GalNAc at its non-reducing end, and that there is only minimal addition of 4-sulfate to this pentasaccharide using a different chondroitin 4-sulfating microsomal system. However, there is good addition of 6-sulfate or 4-sulfate to a penultimate GalNAc of a chondroitin hexasaccharide. In favour of the 'salvage' hypothesis is the presence of terminal GalNAc 6-S in commercial chondroitin 6-sulfate and in endogenous microsomal chondroitin sulfate. This has been demonstrated [7] by incorporation of radioactively labelled GlcA with a microsomal system followed by identification of labelled GlcA-GalNAc 6-S after degradation of the products by use of chondroitin lyase. If terminal GalNAc 6-S cannot be formed directly, it would have to be derived either by removal of terminal GlcA by means of a glucuronidase or derived by 6-sulfation of a 4-sulfated terminal GalNAc residue followed by removal of the 4-sulfate. Moreover, by use of terminal sulfatases and glycosidases it has been shown that embryonic cartilage from both chick and rat contain significant proportions of terminal GalNAc 4-S and GalNAc 4,6-diS [8, 9]. Essentially identical findings have recently been reported with rat chondrosarcoma aggrecan [55].

When polymerization was produced with microsomal preparations and sugar nucleotides in the absence of PAPS or with PAPS added subsequent to polymerization, the size of the glycosaminoglycans formed were indistinguishable from the size of the glycosaminoglycans formed in the same system when PAPS was present during polymerization [28, 36]. It should be noted, however, that this absence of size control by sulfation could be an artifact of the microsomal system, and that sulfation of proteoglycans in intact cells might be an active determinant in the limitation of polymerization. In this regard, it has been suggested that undersulfation of chick embryo cartilage proteochondroitin by incubation of cartilage explants in low sulfate may result in altered chain lengths [44]. In contrast, it has been reported that cells incubated with chlorate in order to inhibit the formation of PAPS [49] or incubated under low sulfate conditions [46] produced undersulfated glycosaminoglycans that had the same length as those produced under normal sulfating conditions. Furthermore, the chain length of the undersulfated cartilage chondroitin sulfate of brachymorphic mice was shown to be essentially the same as that of normal mice [56].

Sulfation control of glycosaminoglycan modification

Another interaction of sulfation with the chondroitin polymer concerns the formation of dermatan, which is derived from chondroitin by epimerization of varying amounts of GlcA residues to IdA residues subsequent to formation of the chondroitin glycosaminoglycan [57]. It was shown with microsomal systems that the presence of PAPS resulted in an increase of epimerization of newly formed chondroitin, so that a higher percentage of dermatan disaccharides was found. Experiments confirming and extending these result were performed with cultured human skin fibroblasts grown under conditions of sulfate deprivation [46, 58]. Fibroblasts from some individuals showed a marked decrease in sulfation with a concomitant decrease in epimerization of GlcA to IdA. Moreover, only the IdA-containing disaccharide units were found to be sulfated, while the GlcA-containing disaccharides remained non-sulfated. The order of this relationship has not been completely established, but it is probable that epimerization to form the dermatan portions of the glycosaminoglycan occurs first [59] accompanied by a facilitated sulfation of the epimerized disaccharides. This has not been conclusively demonstrated, and it is still possible that sulfation precedes and facilitates the epimerization rather than the reverse. In any event it is clear that there is an important relationship between these two activities.

Sulfation of the linkage oligosaccharide

Varying degrees of 4- and/or 6-sulfation of Gal in linkage region GlcA-Gal-Gal-Xyl-Ser have been found in proteochondroitin sulfate from sources including whale cartilage [60], shark cartilage [61], and rat chondrosarcoma [6, 62, 63]. The amounts of linkage region Gal found to be sulfated was generally small. It is of note that no sulfation of Gal in the linkage region of proteoheparan/heparin sulfate has been described. This has led to the suggestion that the presence or absence of these substituents may function as a signal to sort the nascent proteoglycans (all containing the same GlcA-Gal-Gal-Xyl-Ser linkage region) to the appropriate sites for chondroitin or heparan synthesis. However it is of note that only 4-sulfation and no 6-sulfation of Gal has been found in the proteochondroitin 4-sulfate of rat chondrosarcoma, while 6-sulfation of Gal is found where there is a predominance of proteochondroitin 6-sulfate. Since the enzyme for 6-sulfation of Gal in keratan is the same as the enzyme for 6-sulfation of GalNAc in chondroitin [11], it is likely that the 6-sulfation of Gal in the linkage region may also be catalysed by this same 6-sulfotransferase. We believe this to be the case, since we have recently found that the purified chondroitin/keratan 6-sulfotransferase is capable of sulfating linkage region oligosaccharide [64]. Moreover, Gal transferase activities are found in early cis Golgi density gradient subfractions while 6-sulfotransferase activity is found in later medial-trans Golgi subfractions together with the chondroitin-polymerizing enzymes [43, 65]. This would indicate that sulfation of the linkage region Gal might occur only after the nascent proteoglycan has already been directed to the specific site for glycosaminoglycan polymerization and sulfation. If so, then sulfation of the linkage region could not be a signal for sorting nascent proteoglycans to select for synthesis of chondroitin versus heparan.

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