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Conformational changes and anticoagulant activity of chondroitin sulfate following its *O*-sulfonation [1]

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Abstract

Chondroitin sulfate from bovine tracheal cartilage, with the basic structure (4-*O*-sulfo-D-Gal*p*NAcβ1 → 4-D-Glc*p*A)$_n$, was chemically modified by *O*-sulfonation. Depending on the reaction conditions, the products showed a different degree of *O*-sulfonation. A fully *O*-sulfonated chondroitin sulfate, having no free hydroxyl groups, and a sulfo ester group:disaccharide unit ratio of 4.0 was prepared. This chondroitin sulfate derivative was shown by ^{1}H NMR spectroscopy to have a uronate residue with an altered conformation. Usually, the uronate residue in chondroitin sulfate resides in the $^4C_1$ form. Fully *O*-sulfonated chondroitin sulfate had an uronate residue in the $^1C_4$ form at 30 °C, similar to the preferred conformation of the 2-*O*-sulfo-iduronate residue most commonly found in heparin. The $^2S_0$ form of the uronate residue was also found in fully *O*-sulfonated chondroitin sulfate at 60 °C. The anti-factor IIa activity of fully *O*-sulfonated chondroitin sulfate was 40 units/mg. This value is similar to the activities reported for various low-molecular-weight heparins, and substantially higher than those previously reported for partially *O*-sulfonated chondroitin sulfates having an average sulfate group/disaccharide unit of 2.5 to 3.3. The anti-factor Xa activity of the fully *O*-sulfonated chondroitin sulfate was 12 units/mg. This value is considerably lower than the activities reported for various low-molecular-weight heparins, consistent with the critical importance of an antithrombin III pentasaccharide binding site for anti-factor Xa activity. These findings suggest that the conformational change of glucuronic acid residue in

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[1] Abbreviations: GAG(s), glycosaminoglycan(s); Glc*p*A, D-glucuronic acid; Glc*p*A2S, 2-*O*-sulfo-D-glucuronic acid; Glc*p*A3S, 3-*O*-sulfo-D-glucuronic acid; Gal*p*N, D-galactosamine; Ac, acetyl; PAGE, polyacrylamide gel electrophoresis; GPC, gel-permeation chromatography; TBA, tributylammonium; TBA · SO_3, tributylamine–sulfur trioxide; Py · SO_3, pyridine–sulfur trioxide; ATIII, antithrombin III; HCII, Heparin cofactor II; NHP, normal human plasma; 1D, one-dimensional; 2D, two-dimensional

chondroitin sulfate resulting from its full *O*-sulfonation can result in enhanced anticoagulant activity, particularly as measured by anti-factor IIa assay.

Keywords: Chemical oversulfonation; Chondroitin sulfate; Anticoagulant activity; Conformational change; ^{1}H NMR spectroscopy

1. Introduction

Chondroitin sulfates are families of structurally complex, sulfated, linear polysaccharides called glycosaminoglycans (GAGs) with alternating D-glucuronic acid (Glc*p*A) and *N*-acetylated D-galactosamine (Gal*p*NAc) residues [1]. Chondroitin sulfates contain, on average, one sulfate group per disaccharide unit at either the C-4 or C-6 positions of GalNAc [1]. This polysaccharide is part of proteoglycans found localized on cell surfaces and in the extracellular matrix and is important in cell–cell communication [1–3]. While chondroitin sulfates appear to be involved in maintaining hemostasis [4], chondroitin sulfates lack clinically relevant levels of anticoagulant activity, presumably the result of their structural differences from the clinical anticoagulant heparin, including their low level of sulfation [5].

Heparin has been the drug of choice in clinical, pre-surgical and post-surgical prophylaxis of thrombotic events [6]. However, because of its side effects, such as bleeding and other disadvantages, developing alternatives to heparin is an important research goal [7]. Recently, other polysaccharides and modified polysaccharides have been examined as potential heparin analogs in drug development [6,8]. Oversulfated chondroitin sulfates with two to three sulfate groups per disaccharide unit have been shown to exhibit enhanced antithrombotic activity [9]. These chemically prepared oversulfated chondroitin sulfates still contain glucuronic acid residues, making them both structurally and conformationally different from the iduronic acid residues found in heparin [10,11].

In this paper, chondroitin sulfate is completely *O*-sulfonated, and the solution conformations of its glucuronate residues are examined using ^{1}H NMR spectroscopy. The relationship between the conformation of the glucuronic acid residues and anticoagulant activity is discussed.

2. Results

Preparation and characterization of chemically oversulfated chondroitin sulfates.—Chemical *O*-sulfonation reactions of bovine tracheal cartilage chondroitin sulfate at different temperatures resulted in oversulfated chondroitin sulfates having different levels of sulfation. Gradient polyacrylamide gel electrophoresis (PAGE) analysis [12] (data not shown) of each sample was undertaken to determine molecular weight. In addition to showing a slight increase in molecular weight on *O*-sulfonation, the microheterogeneity of the sample of the partially *O*-sulfonated sample increases as shown by a reduction in clearly defined banding on gradient PAGE analysis. Gel-permeation chromatography (GPC) [13] also showed an expected small increase in molecular weight concomitant with increased level of sulfation. These results are consistent with the added mass of the *O*-sulfo groups as well as the stability of the glycosidic linkages in the polysaccharides under the reaction conditions. Sulfate analysis of chondroitin sulfate and oversulfated chondroitin sulfates prepared at 0 °C and at 40 °C are consistent with 1, 2.5–3.3 and 4 *O*-sulfo groups/disaccharide repeating unit.

A disaccharide compositional analysis of the partially and fully *O*-sulfonated chondroitin sulfate was attempted by exhaustive treatment chondroitin sulfate lyases ABC/ACII, followed by HPLC analysis, to confirm that *O*-sulfonation had taken place. The recoveries of the unsaturated disaccharides from partially oversulfated chondroitin sulfate were decreased depending on the sulfation degree. In the case of the fully *O*-sulfonated chondroitin sulfate sample, no unsaturated disaccharide products were detected (data not shown). This result was expected based on the known resistance of oversulfated domains to these enzymes [14].

IR spectra of the chondroitin sulfate and fully *O*-sulfonated chondroitin sulfate (data not shown) strongly suggest the conversion of hydroxy groups to axial *O*-sulfonate groups. The intensity of the absorbances at 1240 cm^{-1} and 820–850 cm^{-1} attributed to the stretching of S=O bond and C–O–S bonds, respectively, are dramatically increased by *O*-sulfonation. Similarly, the intensity of the bands at 2900, 1440, 1380 and 1100 cm^{-1}, attributed to the stretching and/or deformation vibration of C–O–H

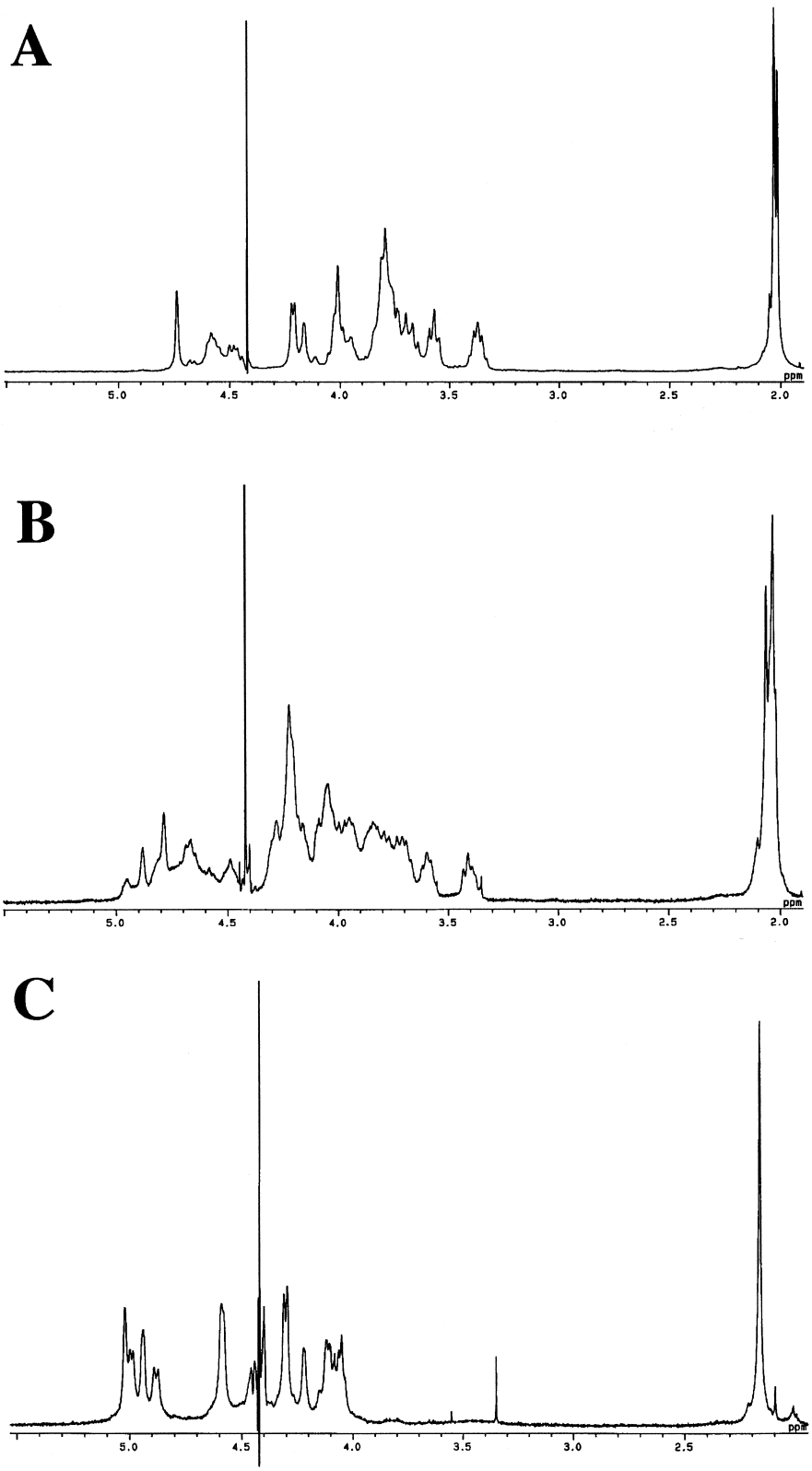

Fig. 1. One-dimensional ^{1}H NMR spectra of chondroitin sulfate and chemically *O*-sulfonated chondroitin sulfates measured at 303 K. (A) Intact bovine tracheal chondroitin sulfate; (B) partially *O*-sulfonated ($SO_3H/COOH = 3.2$) chondroitin sulfate prepared from bovine tracheal chondroitin sulfate; (C) fully *O*-sulfonated chondroitin sulfate ($SO_3H/COOH = 4.0$).

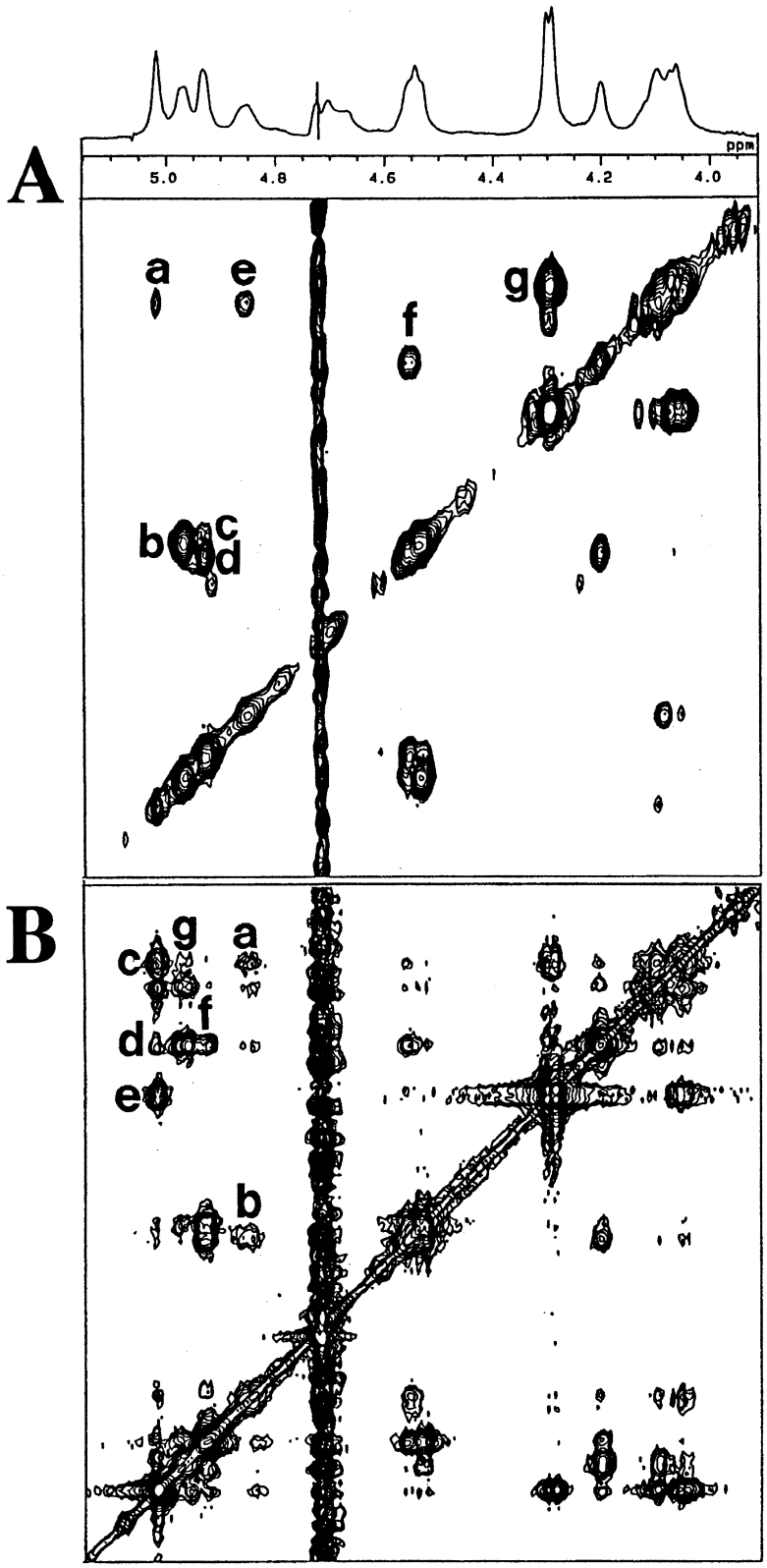

Fig. 2. Two-dimensional DQF-COSY and NOESY spectra of fully *O*-sulfonated chondroitin sulfate measured at 333 K. (A) DQF-COSY spectrum; (B) NOESY spectrum of fully *O*-sulfonated chondroitin sulfate. *Cross peaks (upper panels)*: (a) Gal*p*NAc H-3/H-4; (b) Glc*p*A H-1/H-2; (c) Glc*p*A H-2/H-3; (d) Glc*p*A H-3/H-4; (e) Gal*p*NAc H-1/H-2; (f) Glc*p*A H-4/H-5; (g) Gal*p*NAc H-5/H-6: (*lower panel*) (a) Gal*p*NAc H-1/H-3; (b) Gal*p*NAc H-1/Glc*p*A H-4; (c) Gal*p*NAc H-4/H-5; (d) Gal*p*NAc H-4/Glc*p*A H-5; (e) Gal *p*NAc H-4/H-6; (f) Glc*p*A H-3/H-5; (g) Glc*p*A H-1/Gal*p*NAc H-3.

Table 1
Chemical shifts (ppm) and coupling constants (Hz) of fully *O*-sulfonated chondroitin sulfate

Residue probe temperature	H-1 $J_{1,2}$	H-2 $J_{2,3}$	H-3 $J_{3,4}$	H-4 $J_{4,5}$	H-5 $J_{5,6}$	H-6	N–Ac
Gal*p*NAc							
333 K	4.88	4.11	4.11	5.02	4.05	4.30	2.17
	7.1	n.d.[a]	n.d	< 1.5	6.1		
303 K	4.86	4.10	4.10	5.02	4.06	4.29	2.16
	7.4	n.d.	n.d.	< 1.5	6.2		
Glc*p*A							
333 K	5.00	4.59	4.94	4.59	4.23	–	–
	5.9	n.d.	< 1.5	< 1.5			
303 K	4.97	4.53	4.94	4.55	4.20		
	< 1.5	< 1.5	< 1.5				

[a] Not determined.

bonds, are decreased in the spectrum of the fully *O*-sulfonated chondroitin sulfate.

Assignments of IR absorption bands at 1240 cm^{-1} [15], and 1430 cm^{-1} were based on reports by Casu et al. [16], and bands in the 820–850 cm^{-1} spectral region were attributed to C–O–S stretching based on the results of the work of Orr [17]. Multiple bands at about 800–820 cm^{-1} were tentatively ascribed to sulfate half-ester based on the report of Grant et al. [18], and the band at 800 cm^{-1} was ascribed to C–O–S stretching within predominantly axial 2-*O*- and 3-*O*-sulfo groups of glucuronate residues based on the work of Sanderson et al. [19].

A change in the optical rotation of chondroitin sulfate from $-30°$ to $-8°$ accompanies its full *O*-sulfonation. In the case of partially oversulfated samples, the difference of the optical rotation from the intact chondroitin sulfate was not so significant (data not shown). The magnitude and direction of this change is consistent with a significant change in the molecular conformation of these derivatives. These observations suggest that the most important factor in the optical rotational change is not the degree of *O*-sulfonation but rather the result of conformational change [18].

One-dimensional (1D) ^1H NMR spectra of chondroitin sulfate and *O*-sulfonated chondroitin sulfates prepared at 0 °C and 40 °C shown in Fig. 1. The spectra of the parent chondroitin sulfate showed a substantial level of structural heterogeneity resulting from the presence and/or absence of sulfation at the 4- and/or 6-positions of the Gal*p*NAc residue. Chemical *O*-sulfonation at 0 °C shows an expected [20,21] increase in the structural heterogeneity compared to the parent chondroitin sulfate. This increased heterogeneity results from the introduction of addi-

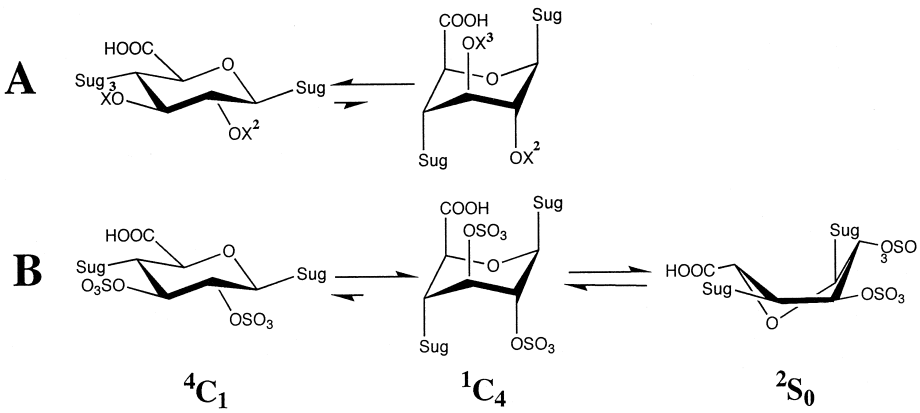

Fig. 3. Effect of the level of *O*-sulfonation on the conformation of the glucuronic acid residue in chondroitin sulfate. (A) The glucuronate residue of partially *O*-sulfonated chondroitin sulfate, where $X^2 = SO_3^-$ and $X^3 = H$ or $X^2 = H$ and $X^3 = SO_3^-$, resides primarily in the $^4C_1$ conformer. (B) The glucuronate residue of fully *O*-sulfonated chondroitin sulfate resides primarily in the $^1C_4$ conformer at 30 °C and in the $^2S_0$ conformer at 60 °C.

tional sulfate groups at the 4- and/or 6-positions of Gal*p*NAc as well as the 2- and/or 3-positions of Glc*p*A. Surprisingly, chemical *O*-sulfonation at 40 °C results in a considerably less complex 1D ^{1}H NMR spectrum, suggesting a reduced structural heterogeneity consistent with full *O*-sulfonation. Two-dimensional (2D) ^{1}H NMR experiments, DQF-COSY and NOESY spectra, of the fully *O*-sulfonated chondroitin sulfate, depicted in Fig. 2, clearly show the downfield shifts of ring protons attached to the *O*-sulfonated carbons, such as Glc*p*A H-2, H-3, and Gal*p*NAc H-4, H-6, and also affords sequence confirmation. The cross peaks detected in NOESY spectrum between Glc*p*A H-1 and Gal *p*NAc H-3, and Gal*p*NAc H-1 and Glc*p*A H-4 strongly suggest that the sequence and linkage positions of the fully *O*-sulfonated chondroitin sulfate are maintained.

The chemical shifts and coupling constants of ring protons of each sample are summarized in Table 1. Based on the Karplus equation, the coupling constant of each ring proton of glucuronate at 30 °C shows that the dihedral angles of vicinal protons of glucuronate are not at 180°, which is typically observed for a glucuronate residue. These data strongly suggest the glucuronate residue has undergone a conformational change from $^4C_1$ to $^1C_4$ promoted through the full *O*-sulfonation of this residue (Fig. 3). The coupling constant between H-1 and H-2 of glucuronate residue at 60 °C dramatically changes from < 1.5 to 5.9 Hz (Table 1). These observations strongly suggest that at 60 °C the conformation of the glucuronate residue, in the fully *O*-sulfonated chondroitin sulfate, has changed from $^1C_4$ to $^2S_0$ (Fig. 3).

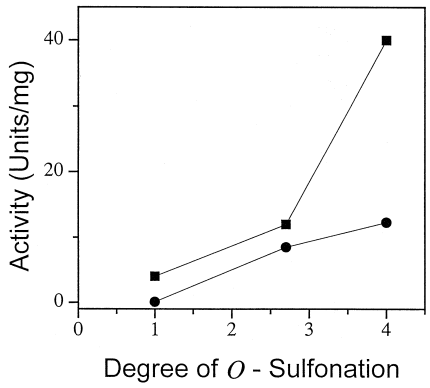

Fig. 4. Effect of the degree of *O*-sulfonation of chondroitin sulfate on the anticoagulant activity. Anti-factor IIa activity (■) and anti-factor Xa activity (●) in units/mg (determined based on a heparin standard curve) are plotted as a function degree of *O*-sulfonation (*O*-sulfonate groups/disaccharide repeating units).

Effect of oversulfated chondroitin sulfate on the inactivation of factor IIa and factor Xa by human plasma.—A correlationship between the sulfation level of chemically *O*-sulfonated chondroitin sulfates and their inactivation of factor IIa activity is shown in Fig. 4. The dependence of the anti-factor IIa anticoagulant activity is clear with a dramatic increase in activity observed for the fully *O*-sulfonated chondroitin sulfate. This dramatic increase on full *O*-sulfonation suggests that the anti-factor IIa activity is not merely the result increased overall charge and that some other structural change, such as a shift in conformation, might be responsible for the high activity observed for the fully *O*-sulfonated chondroitin sulfate derivative. While an increase in anti-factor Xa activity was also observed in the oversulfated chondroitin sulfate derivatives, the magnitude of this increase was considerably less. Thus, the increased anti-factor Xa activity may simply result from a nonspecific effect associated with the overall molecular charge.

3. Discussion

Oversulfated disaccharide sequences have been reported to account for a minor but important part of the structure of chondroitin sulfates derived from mammalian tissues [22,23]. Although chemical *O*-sulfonation of chondroitin sulfate has been previously reported [21], unmodified hydroxyl groups remained, affording a product of high structural heterogeneity as demonstrated from the complexity of both gradient PAGE analysis (data not shown) and the 1D NMR spectrum shown in Fig. 1B. Optimum conditions for complete *O*-sulfonation of chondroitin sulfate were determined to be 40 °C for 1 h with 15 equiv. of sulfonation reagent/mol of hydroxy group. Under these conditions, the molecular weight increased slightly, consistent with the mass of the added *O*-sulfo and the absence of breakdown of the glycosidic linkages (data not shown). Full *O*-sulfonation of chondroitin sulfate is demonstrated by both 1D and 2D ^{1}H NMR experiments (Figs. 1 and 2), by the sulfate analysis data, and by the failure of chondroitin lyases to act on this product.

The present results demonstrate that products obtained by chemical modification of chondroitin sulfate show anti-factor IIa activities (Fig. 4) comparable with the activities displayed by previously described heparin analogs [8] and various low-molecu-

lar-weight heparins [6]. Optical rotation measurements suggest a change in conformation, and a band at 800 cm^{-1} in the IR spectra suggest as an axial disposition of the 2- and 3-*O*-sulfo groups in the glucuronate residue (data not shown). NMR spectroscopy (Figs. 1 and 2) demonstrates the conformation of glucuronate residues of a fully *O*-sulfonated chondroitin sulfate derivative is altered from $^4C_1$ to $^1C_4$ at 30 °C (Fig. 3), possibly resulting from the repulsion of negatively charged sulfate groups. This conformational change corresponds to a substantial increase in anti-factor IIa activity (Fig. 4).

The $^1C_4$ conformation of the glucuronate residue in fully *O*-sulfonated chondroitin sulfate closely resembles the 2-*O*-sulfo-iduronate residue commonly found in heparin. Interestingly, the same magnitude of increase is not observed in anti-factor Xa activity. Indeed, while the anti-factor IIa activity of fully *O*-sulfonated chondroitin sulfate is comparable to that of low-molecular-weight heparins, the anti-factor Xa activity is less than 20% of that of low-molecular-weight heparins [6]. These results may be explained by the different protease inhibitors present in plasma that inhibit factor IIa and factor Xa. Factor IIa can be inhibited by both antithrombin III (ATIII) and heparin cofactor II (HCII), while factor Xa is only inhibited by ATIII. While ATIII is known to bind to a specific pentasaccharide sequence found within heparin's structure, HCII binds with considerably less specificity to oversulfated domains of heparin [24], dermatan sulfate, and chondroitin sulfates [25]. Thus, it is likely that the large enhancement of anti-factor IIa activity observed for fully *O*-sulfonated chondroitin sulfate is an HCII-mediated activity.

It is possible that the anticoagulant activity can be further increased by appropriate refinement of the modification procedure for *N*-deacetylation–*N*-desulfonation [26] of fully *O*-sulfonated chondroitin sulfate. These possibilities point to new practically feasible routes for the generation of heparin-like compounds with various pharmacologically relevant biological activities.

4. Experimental

Materials.—Chondroitin sulfate from bovine tracheal cartilage was kindly gifted from Shin-Nippon Yakugyo (Tokyo, Japan). Unsaturated disaccharides [2-acetamido-2-deoxy-3-*O*-(β-D-*threo*-4-hexenopyranosyluronic acid)-D-glucose (ΔDi-HA), 2-acetamido-2-deoxy-3-*O*-(β-D-*threo*-4-hexenopyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-*O*-(β-D-*threo*-4-hexenopyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-*O*-(β-D-*threo*-4-hexenopyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-*threo*-4-hexenopyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-S_B)], and chondroitin lyase ABC (Chase ABC, EC 4.2.2.4) and ACII *arthro* (Chase ACII, EC 4.2.2.5) were purchased from Seikagaku Kogyo (Tokyo, Japan). TSKgel NH_2-60 anion-exchange resin (particle size, 5 μm) for HPLC column packing was obtained from Tosoh (Tokyo, Japan). The Asahipak gel-permeation chromatography (GPC) HPLC column was from Asahikasei (Yokohama, Japan).

Preparation of chemically oversulfated chondroitin sulfate.—Chemical *O*-sulfonation to obtain oversulfated chondroitin sulfate was carried out under mild conditions with adducts of sulfur trioxide (SO_3) in aprotic solvents [27]. Fully *O*-sulfonated chondroitin sulfate was prepared from the tributylamine (TBA) salt, obtained from 100 mg of chondroitin sulfate, sodium salt by strong cation-exchange chromatography, and concentration by lyophilization. The resulting salt was dissolved in 0.8 mL of *N*,*N*-dimethylformamide (DMF) to which a required excess (15 mol/equivalent of available hydroxy group in chondroitin sulfate) of pyridine–sulfur trioxide complex had been added. After 1 h at 40 °C, the reaction was interrupted by addition of 1.6 mL of water, and the raw product was precipitated with 3 volumes of cold ethanol saturated with anhydrous sodium acetate, and then collected by centrifugation. The resulting fully *O*-sulfonated chondroitin sulfate was dissolved in water, dialyzed to remove salts, and lyophilized.

Estimation of molecular weight.—The weight average molecular weight of each chondroitin sulfate sample was estimated using gradient PAGE analysis [12]. Determinations were made in a 12–22% gradient mini-gel visualized with Alcian Blue by the method of Edens et al. [12]. The relative molecular weights of each chondroitin sulfate were confirmed by their elution position from a GPC–HPLC column eluted with 50 mM NaOAc, pH 7.4 at a flow rate of 1 mL/min, with detection at 206 nm [13].

HPLC conditions for disaccharide analysis.—The determination of unsaturated disaccharides prepared from intact and modified chondroitin sulfates was performed on chondroitin lyase-digested samples using HPLC [28]. The HPLC conditions were as follows: Injection size 10 to 20 μL (5 μg/mL), on a

TSKgel NH_2-60 column (4.6 mm i.d. × 250 mm length) eluted with 40 mM Tris–borate buffer (pH 7.5 adjusted with HCl) and 7 mM Na_2SO_4 in 54% acetonitrile at a flow rate of 0.5 mL/min. Post-column derivatization was used to detect each unsaturated disaccharide. NaOH solution (0.3 M) and 1% 2-cyanoacetamide solution were delivered to the eluate at a flow rate of 0.25 mL, each controlled by a reciprocated double plunger pump. The mixture was then introduced to a polytetrafluoroethylene (PTFE) reaction coil (0.5 mm i.d. × 10 m) thermostated at 105 °C by using a dry reaction aluminum heating block, then introduced to a PTFE cooling coil (0.25 mm i.d. × 2 m) in water bath. The effluent was monitored by a fluorometric detector (excitation, 346 nm; emission, 410 nm).

Sulfate analysis.—Chondroitin sulfate samples were prepared for the determination of sulfate by exhaustive dialysis against distilled water using MWCO 3500 tubing, lyophilization and drying for 2 days in a desiccator over phosphorus pentoxide. The determination of sulfate group was performed following combustion by HPLC using a conductivity detector, Tosoh model CM-8 (Tokyo, Japan).

IR spectroscopy.—For IR spectroscopy of solid samples, a Jasco model FTIR 230 (Tokyo, Japan) was used. A 100 μg portion of glycosaminoglycan was mixed with 500 μg of dried potassium bromide, pressed, and the resulting salt disc (3 mm diameter) was placed in the spectrometer.

Optical rotation measurements.—The same dried samples used for sulfate analysis were used to measure optical rotation. Samples were weighed and dissolved in distilled water at a concentration of 5 mg/mL, and their optical rotations were determined at the sodium D-line on a Jasco model DIP-140 spectropolarimeter (Tokyo, Japan).

^{1}H NMR spectroscopy.—^{1}H NMR spectroscopy was performed under the conditions described previously [29]. Briefly, oversulfated chondroitin sulfate (approximately 2 mg) was dissolved in 0.5 mL of deuterium oxide (99.9%) and freeze-dried repeatedly to remove exchangeable protons. The sample was kept in a desiccator over phosphorus pentoxide in vacuo overnight at room temperature. The thoroughly dried sample was then dissolved in 0.5 mL of deuterium oxide (99.96%) and passed through 0.45 μm syringe filter and transferred to a NMR tube (5.0 mm o.d. × 25 cm, pp-528; Wilmad Glass, Buena, NJ). 1D and 2D NMR experiments were performed on a JEOL GSX500A spectrometer equipped with 5-mm field gradient-tunable probe with standard JEOL software at 303 K for NOE spectra or 333 K for other experiments on 500 μL samples. The HOD signal was suppressed by presaturation during 3 or 1.5 s for 1D or 2D spectra, respectively. To obtain 2D spectra, 512 experiments resulting 1024 data points for a spectral width of 2000 Hz were measured, and the time-domain data were multiplied after zerofilling (data matrix size, 1 K × 1 K) with a shifted sine-bell window functions for 2D double-quantum-filtered (DQF)-COSY, NOESY or TOCSY experiments. An MLEV-17 mixing sequence of 100 ms was used for 2D TOCSY and NOESY experiments by using 150, 250 and 500 ms as the mixing time were performed.

Anti-factor Xa and anti-factor IIa activities.—Normal human plasma (NHP) was collected from healthy volunteers. Anti-factor Xa activity was determined using a Coatest LMW heparin/heparin kit (Chromogenix, Mölndal, Sweden). Briefly, chondroitin sulfate, oversulfated chondroitin sulfate derivatives, and LMW heparin standard were in diluted normal human plasma. Chromogenic Xa substrate S-2732 (Suc-Ile-Glu(γ-Piperidyl)-Gly-Arg-*p*NA) 2.9 mM in 50 mM Tris, 7.5 μM EDTA, pH 8.4 buffer (200 μL), was added to 25 μL of plasma containing sample and 200 μL of bovine Factor Xa (1.25 mL^{-1}). After mixing, the reaction was incubated for 8 min at 37 °C and 200 μL of 20% aqueous CH_3COOH was added. Residual factor Xa was then determined by measuring absorbance at 405 nm. Anti-factor IIa activity was determined by incubating 50 μL of chondroitin sulfate or oversulfated derivatives in 30 μL of NHP with 20 μL of human thrombin (1.2 NIH units/mL) at 37 °C for 30 s. Then, 50 μL (1.9 μmol/mL) of Chromogenic TH (ethylmalonyl-Pro-Arg-*p*-nitroanilide hydrochloride) was added, and the amidolytic thrombin activity was measured at 405 nm. Measurements were performed on an ACL 300 plus from Instrumentation (Lexington, MA) and calculated in comparison with USP Heparin Reference Standard (K-3) supplied by U.S. Pharmacopeial Convention (Rockville, MD) [26].

Acknowledgements

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References

[1] K. Kimata, M. Okayama, A. Oohira, and S. Suzuki, *Mol. Cell. Biochem.*, 1 (1973) 211–228.
[2] J.T. Gallagher, M. Lyon, and W.P. Steward, *Biochem. J.*, 236 (1986) 313–325.
[3] M.C. Bourin, A.K. Ohlin, D. Lane, J. Stenflo, and U. Lindahl, *J. Biol. Chem.*, 263 (1988) 8044–8052.
[4] H. Engelberg, *Pharmacol. Rev.*, 36 (1984) 91–109.
[5] P. Sie, F. Ofosu, F. Fernadez, M.R. Buchanan, M. Petitou, and B. Boneu, *Br. J. Haematol.*, 64 (1986) 707–774.
[6] R.J. Linhardt, T. Toida, in Z.B. Witczak and K.A. Nieforth (Eds.), *Carbohydrates as Drugs— Heparin Oligosaccharides— New Analogs Development and Applications*, Marcel Dekker, New York, 1997, pp 277–341.
[7] B. Casu, *Adv. Carbohydr. Chem. Biochem.*, 43 (1985) 51–134.
[8] N. Razi, E. Feyzi, I. Bjork, A. Naggi, B. Casu, and U. Lindahl, *Biochem. J.*, 309 (1995) 465–472.
[9] M.C. Bourin, A. Lundgren, E. Kerlund, and U. Lindahl, *J. Biol. Chem.*, 265 (1990) 15424–5431.
[10] G. Qiu, T. Toida, and T. Imanari, *Biol. Pharm. Bull.*, 102 (1997) 721–726.
[11] B. Casu, *Semin. Thromb. Haemost.*, 17S (1991) 9–14.
[12] R.E. Edens, A. Al-Hakim, J.M. Weiler, D.G. Rethwisch, J. Fareed, and R.J. Linhardt, *J. Pharm. Sci.*, 81 (1992) 823–827.
[13] T. Toida, H. Yoshida, H. Toyoda, I. Koshiishi, T. Imanari, R.E. Hileman, J.R. Fromm, and R.J. Linhardt, *Biochem. J.*, 322 (1997) 499–506.
[14] R.J. Linhardt, in A. Varki (Ed.), *Current Protocols in Molecular Biology*, Vol. 2, Wiley Interscience, Boston, 1992, pp 17.13.17–17.13.32.
[15] F. Cabassi, B. Casu, and A.A. Perlin, *Carbohydr. Res.*, 63 (1978) 1–11.
[16] B. Casu, G. Scovenna, A.J. Cifonelli, and A.A. Perlin, *Carbohydr. Res.*, 63 (1978) 13–27.
[17] S.F.D. Orr, *Biochim. Biophys. Acta*, 14 (1954) 173–181.
[18] D. Grant, W.F. Long, C.F. Moffat, and F.B. Williamson, *Biochem. J.*, 275 (1991) 193–197.
[19] P.N. Sanderson, T.N. Huckerby, I. Nieduszynski, and A. Nieduszynski, *Biochem. J.*, 243 (1987) 175–181.
[20] R.M. Maaroufi, J. Tapon-Bretandiere, J. Mardiguian, C. Sternberg, M.D. Dauztenberg, and A.M. Fischer, *Thromb. Res.*, 59 (1995) 174–758.
[21] C. Bartolucci, L. Cellai, M.A. Iannelli, D. Lamba, L. Liverani, G. Mascellani, and E. Perola, *Carbohydr. Res.*, 276 (1995) 401–408.
[22] R.J. Linhardt, A. Al-Hakim, L.S. Yu, Y.S. Kim, and J. Fareed, *Semin. Thromb. Haemost.*, 17S (1991) 15–22.
[23] R.J. Linhardt, A. Al-Hakim, J. Lin, D. Hoppensteadt, G. Mascellani, P. Bianchini, and J. Fareed, *Biochem. Pharmacol.*, 42 (1991) 1609–1691.
[24] Y.S. Kim and R.J. Linhardt, *Thromb. Res.*, 53 (1989) 57–71.
[25] R.J. Linhardt and R.E. Hileman, *Gen. Pharmacol.*, 26 (1995) 443–451.
[26] V.D. Nadkarni, T. Toida, C.L. van Gorp, R.L. Schubert, J.M. Weiler, K.P. Hansen, E.E.O. Caldwell, and R.J. Linhardt, *Carbohydr. Res.*, 290 (1996) 87–96.
[27] K. Nagasawa, H. Uchiyama, and N. Wajima, *Carbohydr. Res.*, 158 (1986) 183–190.
[28] H. Toyoda, H. Motoki, M. Tanikawa, K. Shinomiya, H. Akiyama, and T. Imanari, *J. Chromatogr.*, 565 (1991) 141–148.
[29] T. Toida, H. Toyoda, and T. Imanari, *Anal. Sci.*, 9 (1993) 53–58.