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Charge distribution and calcium affinity of sulfated α-L-galactans from ascidians. Comparison between linear and highly branched polymers

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Abstract

Calcium binding and charge distribution on highly branched and linear sulfated L-galactans from ascidians have been studied using a metallochromic indicator and conductimetric titrations. The distance between charged groups of the linear and highly branched galactans does not vary despite their marked differences in sulfate/total sugar molar ratios. These results indicate that the sulfated L-galactose units are concentrated in the central polysaccharide core and not intercalated among non-sulfated units. This inference is consistent with the chemical studies of these galactans. Surprisingly, calcium affinity increases with increasing amounts of non-sulfated sugar branches in the molecule. Thus, calcium binding in these polymers is not a simple function of availability of anion binding sites but a more complex calcium–polysaccharide interaction.

1. Introduction

The ability of sulfated polysaccharides to complex divalent cations, especially calcium, is an important biological function of this class of polymer. For example, proteoglycans (and their constituent glycosaminoglycan chains) perform an important role in the regulation of endochondral ossification since local degradation or disaggregation of proteoglycan initiates calcification of epiphyseal cartilages [1–4]. Studies of the charge distribution on polysaccharides are also important in order to understand the biological function of these molecules. An abundance of sulfate ester substitution in polysaccharides may increase their water binding capacity and may therefore contribute to the resilience of connective tissues [5]. In

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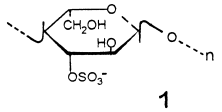
addition, sulfate esters in these polymers are important for the binding of a variety of other molecules, such as growth factors [6,7] and plasma proteins [8,9].

During recent years we have studied sulfated polysaccharides in different invertebrate connective-like tissues. The main purpose of such studies is to compare these polysaccharides with the well-known glycosaminoglycans that occur in vertebrate tissues and to relate their structure with physicochemical and biological properties. We observed that sulfated polysaccharides are abundant in several connective-like tissues from invertebrates [10–18]. In particular, high-molecular-mass, sulfated α-L-galactans, composed mainly of 3-sulfated and 4-linked units, are present in the tunics of all species of ascidians (Chordata-Tunicata) studied so far [12,15,16,19,20]. Moreover, a marked structural variation is observed among these sulfated galactans depending on the species. For example, the sulfated α-L-galactan from *Herdmania monus* is a linear polysaccharide composed of monosulfated sugar units (**1**). In contrast, the sulfated galactans from *Styela plicata* and *Ascidian nigra* are highly branched polymers, containing non-sulfated L-galactopyranosyl and D-glucopyranosyl residues at non-reducing ends (**2** and **3**). The biological significance of structural diversity among sulfated galactans from different species of ascidians remains unclear.



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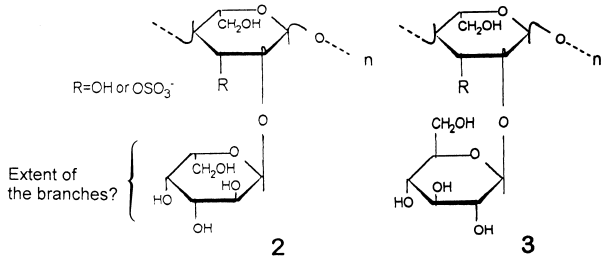

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Recently we studied charge distributions of a polysaccharide [21] based on data from conductimetric titration interpreted using a polyelectrolyte theory [22,23]. Our results showed that the theory can be applied to branched polysaccharides [21]. In addition, we determined calcium binding using a metallochromic indicator and distinguished between calcium affinities for sulfate and carboxyl groups. In this report, similar methodologies have been extended to sulfated polysaccharides extracted from various species of Tunicates, and highly branched and linear sulfated L-galactans are compared.

2. Experimental

Sulfated polysaccharides.—Sulfated L-galactans were extracted from the tunic of ascidians by papain digestion and purified by procedures previously described [11]. Purification procedures involve ion-exchange and gel-filtration column chromatography as described previously [11,12].

Chemical analysis of the polysaccharides.—Total hexose was measured by the method of Dubois et al. [24]. After acid hydrolysis of the polysaccharide (4.0 *M* trifluoroacetic acid for 6 h at 100°C), total hexosamine was measured by a modified Elson–Morgan reaction [25], and sulfate by the $BaCl_2$–gelatin method [26]. The percentages of hexoses and hexosamine in the hydrolyzates were estimated by gas–liquid chromatography of the corresponding alditol acetates [27] and by paper chromatography in *n*-butyl alcohol–pyridine–water (3:2:1,v/v) for 48 h. The sugars were located on the paper chromatograms by silver nitrate staining and quantitated by densitometry.

Conductimetric titration.—Conductimetric titrations were performed as previously described [21]. In these experiments increasing volumes of 0.1 *M* KOH or LiOH were added to a 5 mL soln of polysaccharide (~5 mequiv/L, acid form, pH ~2.9), and the conductivity was measured with a CD21 Digimed conductimeter equipped with platinized electrodes.

Data from the conductimetric titrations were interpreted using the polyelectrolyte theory proposed by Manning [22,23] and described in a previous publication [21]. Accordingly, from conductivity values (σ) obtained at the end point corresponding to neutralization of the polymer by KOH or LiOH, a parameter known as the transport factor (f) can be calculated. The transport factor is related to linear charge density in the polymer (ξ) and from this value the distance between charged groups (b) can be calculated.

Calcium titration.—Calcium titrations were performed with the use of tetramethylmurexide (TMM) as a metallochromic indicator, purified and generously donated by Professor R. Kohn

(Bratislava University, Slovakia). TMM is a tetramethyl derivative from Murexide: 5-[(hexahydro-2,4,6-trioxo-5-pyrimidinyl)imino]-2,4,6(1H,3H,5H)-pyrimidinetrione monoammonium salt [28]. The method is a slight modification of that proposed by Kohn and Furda [29], as described elsewhere [30,31]. The titration experiments were run at 17°C in 10 m*M* NaCl solns, containing polysaccharide (1 to 5 mequiv/L), 30 μM TMM and increasing amounts of $CaCl_2$. The absorbances at 490 and 530 nm were measured on a double-beam spectrophotometer (Hitachi, model U-2000).

In aqueous soln, TMM binds Ca^{2+} reversibly in a 1:1 stoichiometry, and when calcium is complexed it undergoes a shift from 530 to 490 nm in the peak of its absorption spectrum [28]. Thus, the thermodynamics of complex formation and the spectral properties of the free and bound TMM allow the determination of free and bound calcium in a given soln, as described previously [21].

The binding data are plotted according to the usual Scatchard diagram;

$$[Ca]_b/[Ca]_f \text{ versus } [Ca]_b$$

where $[Ca]_b$ = concentration of bound calcium and $[Ca]_f$ = concentration of free calcium.

3. Results and discussion

Linear and highly branched sulfated L-*galactans from ascidians*.—The sulfated α-L-galactan from *H. monus* is a linear polysaccharide, composed of 3-sulfated and 4-linked galactose units (**1**). The sulfated galactans from *S. plicata* and *A. nigra* have a more complex structure. In addition to the core composed of 4-linked units, they contain large proportions of non-reducing L-galactopyranoside and D-glucopyranoside end units when compared with total sugar units, indicative of highly branched polymers (**2** and **3**). Concerning these latter galactans, the exact proportions of sulfated galactose units in the central core and the extent of branching remain unknown, due to complexity of NMR spectra and of methylation analysis [12,15,16,19]. Based on the proportions of 2,3,4,6-tetra-*O*-methyl hexose formed after methylation of the native polysaccharide, we estimated the amount of non-reducing end units on the sulfated L-galactans of *H. monus*, *S. plicata* and *A. nigra* as 5, 12 and 40%, respectively, of the total sugar units [12,15,16,19]. Finally, these polysaccharides also show other differences in their chemical composition. Sulfated L-galactan from *H. monus* contains exclusively L-galactose, *S. plicata* galactan contains non-sulfated L-galactose and D-glucose branches, and *A. nigra* L-galactan contains traces of mannose and aminohexose in addition to these branches (Table 1).

The distance between sulfate groups does not vary from the linear to the highly branched sulfated L-*galactans*.—Conductimetric titrations of anionic polymers have been used to determine their ionic properties. Typically, polymers containing both strong (sulfate) and weak (carboxyl) groups show mixed behavior in the titration curve. A sharp decrease in conductivity with neutralization, attributed to the sulfate groups, is followed by a slow increase in this parameter, characteristic of carboxyl groups, up to the point where complete neutralization of the polymer occurs. Conductimetric titrations of both linear and highly branched sulfated L-galactans show curves compatible with the presence of sulfate groups only (Fig. 1).

These experimental data (shown in Fig. 1) and the calculations described in ref. [21] were used to determine the transport factor, the linear charge density, and the distance between charged groups of the various sulfated L-galactans (Table 2). The

Table 1
Chemical composition and specific optical rotation of sulfated polysaccharides from different species of ascidians

Species	Composition (molar ratios)					$[\alpha]_D^{20\,°C}$
	L-Gal	D-Glc	Man	HexN	Sulfate/total sugar	
H. monus [a]	1.00	< 0.05	< 0.05	< 0.05	1.07	−125
S. plicata [b]	0.82	0.13	< 0.05	< 0.05	0.50	−132
A. nigra [b]	0.67	0.13	0.09	0.11	0.17	−100

[a] See also Santos et al. (ref. [16]).
[b] See also Pavão et al. (ref. [15]).

charge density and the distance between charged groups of the linear and highly branched galactans do not vary despite their marked differences in sulfate/total sugar molar ratios (Table 1). Therefore, the presence of large proportions of non-sulfated galactose and glucose end groups does not modify the anionic charge densities of these polysaccharides. These results indicate that the sulfated L-galactose residues are in fact concentrated in the central polysaccharide core and not intercalated among non-sulfated units. This inference is in agreement with our previous chemical studies [15,16,19].

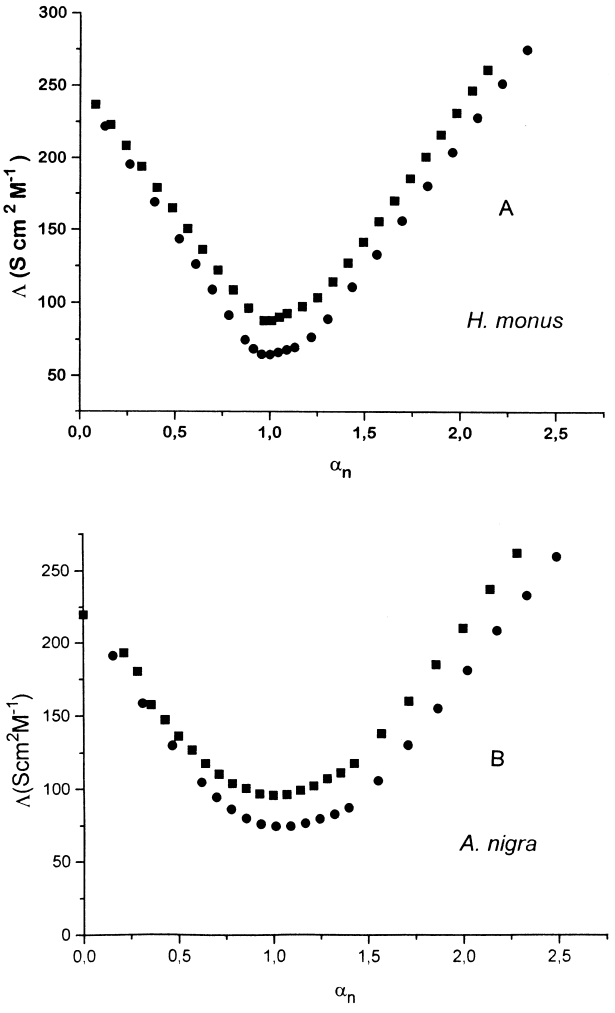

Fig. 1. Conductimetric titrations of sulfated α-L-galactans from *H. monus* (A) and from *A. nigra* (B). The conductimetric titrations of the polysaccharides (acid form) were performed as described under Methods, with increasing amounts of KOH (■) or LiOH (●). The degree of neutralization (α_n) is the concentration of KOH or LiOH divided by the concentration of the polymer (Cp); both as equiv/L.

Distances between charged groups for the galactans and for other acidic polysaccharides are listed in Table 2. The measured distance of 5.0 Å between charged groups of *H. monus* is close to that for vertebrate chondroitin 6-sulfate, which has one charged group per residue; hyaluronic acid has one charged group every alternate residue and an inter-charge distance of 11.1 Å. A fucosylated chondroitin sulfate from sea cucumber [32], with sulfated fucose branches in addition to one charge per residue in the backbone, gives a value of 2.79 Å between charges. The values of 4.8 and 4.9 Å for *A. nigra* and *S. plicata* galactans, therefore, indicate that every residue in the backbone bears a charged substituent. For the *A. nigra* branched galactan, the ratio of sulfate to sugar is only ~0.17. If all the residues in the backbone are sulfated it follows that more than 80% of the monosaccharide residues are in branches, with an average of four residues per branch.

Binding of calcium to linear and highly branched sulfated L-*galactans*.—Scatchard plots of calcium binding to the various sulfated L-galactans (Fig. 2) indicate the presence of a single binding site, as expected since these polysaccharides contain only sulfate groups. The apparent association constants (K) estimated by TMM complexation can be obtained from the Scatchard plots as $[Ca]_b/[Ca]_f \times [Ca]_b$, and are shown in Table 3. Among ascidian L-galactan, sulfate groups have approximately the same range of calcium affinity observed in vertebrate chondroitin sulfate. In con-

Table 2
Transport factor (f), linear charge density (ξ) and distance between charged groups (b) of the various acidic polysaccharides

Polysaccharide	f	ξ	b (Å)
Sulfated L-galactan from *H. monus*	0.598	1.46	5.0
Sulfated L-galactan from *S. plicata*	0.588	1.48	4.9
Sulfated L-galactan from *A. nigra*	0.584	1.49	4.8
Fucosylated chondroitin sulfate from sea cucumber [a]	0.340	2.56	2.79
Chondroitin 6-sulfate from shark cartilage [a]	0.620	1.40	5.10
Hyaluronic acid from umbilical cord [a]	0.940	0.64	11.10

[a] See Ruggiero et al. (ref. [21])

Table 3
Apparent association constants (K) as M^{-1} for various polysaccharides

Polysaccharides	Anionic groups	
	Sulfate	Carboxyl
Sulfated L-galactan from *H. monus*	4270 ± 280	
Sulfated L-galactan from *S. plicata*	5240 ± 420	
Sulfated L-galactan from *A. nigra*	7490 ± 710	
Fucosylated chondroitin sulfate from sea cucumber [a]	15000 ± 1200	1000 ± 130
Chondroitin 6-sulfate from shark cartilage [a]	5100 ± 100	1100 ± 300
Hyaluronic acid [a]		1400 ± 100

[a] See Ruggiero et al. (ref. [21])

trast, the sulfate groups of heparin [33] and of a fucosylated chondroitin sulfate [21] show a ~3–5-fold higher affinity for calcium.

There are differences between the calcium affinities in sulfated L-galactans from different ascidian species. Surprisingly, calcium affinity increases with increasing amounts of non-sulfated sugar branches in the molecule. Thus, sulfated L-galactan from *A. nigra* has the highest calcium affinity of the three (Table 3) despite its lower level of sulfate content (Table 1) and higher amount of branches (**2**,**3**).

At present we have no explanation for this unexpected result. Nevertheless, it indicates that calcium binding is not a simple function of availability of anion binding sites but may be a more complex calcium–polysaccharide interaction. Binding of calcium to sulfated polysaccarides has been studied in glycosaminoglycans [33] and it has been shown that sulfate groups are capable of binding calcium with a stronger affinity than expected for simple salt formation. Calcium affinity of the three compounds studied increases as the mean distance between charged groups decreases, which may indicate that calcium binding requires more than one sulfate group for each calcium atom, as was reported to be the case for glycosaminoglycans [33].

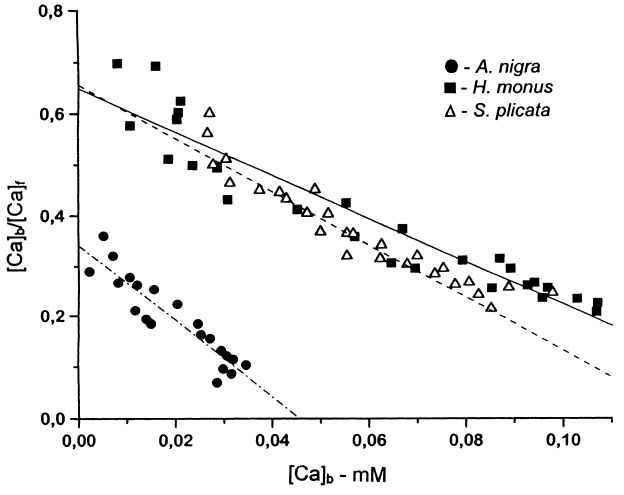

Fig. 2. Scatchard plots of calcium binding to sulfated α-L-galactan from *H. monus* (■), *S. plicata* (△) and *A. nigra* (●). Calcium binding was performed as described in Methods. The free calcium concentration ($[Ca]_f$) and the concentration of calcium complexed with the polysaccharide ($[Ca]_b$) were calculated as described in ref. [21] and the binding data were plotted as usual Scatchard diagram.

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References

[1] M.A. Logan, *J. Biol. Chem.*, 110 (1935) 375–389.
[2] M. Takagi, R.T. Parmley, and F.R. Denys, *J. Histochem. Cytochem.*, 31 (1983) 1089–1100.
[3] N.C. Blumenthal, A.S. Posner, L.D. Silverman, and L.C. Rosenberg, *Calcif. Tissue Int.*, 27 (1979) 75–82.
[4] C.C. Chen and A.L. Boskey, *Calcif. Tissue Int.*, 37 (1985) 395–400.
[5] V.C. Hascall and G.K. Hascall, in E.D. Hay (Ed.), *Cell Biology of Extracellular Matrix* Plenum, New York, 1981, pp. 39–63.
[6] M. Maccarana, B. Casu, and U. Lindahl, *J. Biol. Chem.*, 268 (1993) 23898–23905.

[7] A. Walker, J.E. Turnbull, and J.T. Gallagher, *J. Biol. Chem.*, 269 (1994) 931–935.
[8] C.S. Alves and P.A.S. Mourão, *Atherosclerosis*, 73 (1988) 113–124.
[9] A.M.F. Tovar and P.A.S. Mourão, *Atherosclerosis*, 126 (1996) 185–195.
[10] R.M. Albano and P.A.S. Mourão, *Biochim. Biophys. Acta*, 760 (1983) 192–196.
[11] R.M. Albano and P.A.S. Mourão, *J. Biol. Chem.*, 261 (1986) 758–765.
[12] P.A.S. Mourão and A.S. Perlin, *Eur. J. Biochem.*, 166 (1987) 431–436.
[13] P.A.S. Mourão and I.G. Bastos, *Eur. J. Biochem.*, 166 (1987) 639–645.
[14] R.P. Vieira and P.A.S. Mourão, *J. Biol. Chem.*, 263 (1988) 18176–18183.
[15] M.S.G. Pavão, R.M. Albano, A.M. Lawson, and P.A.S. Mourão, *J. Biol. Chem.*, 264 (1989) 9972–9979.
[16] J.A. Santos, B. Mulloy, and P.A.S. Mourão, *Eur. J. Biochem.*, 204 (1992) 669–677.
[17] R.P. Vieira, C. Pedrosa, and P.A.S. Mourão, *Biochemistry*, 32 (1993) 2254–2262.
[18] M.S.G. Pavão, M.A. Rodrigues, and P.A.S. Mourão, *Biochim. Biophys. Acta*, 1199 (1994) 229–237.
[19] R.M. Albano, M.S.G. Pavão, P.A.S. Mourão, and B. Mulloy, *Carbohydr. Res.*, 208 (1990) 163–174.
[20] M.S.G. Pavão, P.A.S. Mourão, and B. Mulloy, *Carbohydr. Res.* 208 (1990) 153–161.
[21] J. Ruggiero, R.P. Vieira, and P.A.S. Mourão, *Carbohydr. Res.*, 256 (1994) 275–287.
[22] G.S. Manning, *J. Phys. Chem.*, 79 (1975) 282–285.
[23] G.S. Manning, in E. Sélègni (Ed.), *Polyelectrolytes*, Reidel, Dordrecht, The Netherlands, 1974, pp. 9–37.
[24] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–354.
[25] C.J. Rondle and W.T.J. Morgan, *Biochem. J.*, 61 (1955) 586–589.
[26] H. Saito, T. Yamagata, and S. Suzuki, *J. Biol. Chem.*, 243 (1968) 1542–1563.
[27] H.W. Kircher, *Anal. Chem.*, 32 (1960) 1103–1106.
[28] S.T. Ohnishi, *Anal. Biochem.*, 85 (1978) 165–179.
[29] R. Kohn and I. Furda, *Collect. Czech. Chem. Commun.*, 32 (1967) 1925–1937.
[30] J. Ruggiero, G. Manzini, and F. Quadrifoglio, *Biopolymers*, 26 (1987) 1975–1979.
[31] G. Manzini, L.E. Xodo, F. Fogolari, and F. Quadrifoglio, *Biopolymers*, 30 (1990) 325–333.
[32] P.A.S. Mourão, M.S. Pereira, M.S.G. Pavão, B. Mulloy, D.M. Tollefsen, M.C. Mowinckel, and U. Abildgaard, *J. Biol. Chem.*, 271 (1996) 23973–23984.
[33] G.K. Hunter, K.S. Wong, and J.J. Kim, *Arch. Biochem. Biophys.*, 260 (1988) 161–167.