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Sulfated *N*-linked carbohydrate chains in porcine thyroglobulin

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N-linked carbohydrate chains of porcine thyroglobulin were released by the hydrazinolysis procedure. The resulting mixture of oligosaccharide-alditols was fractionated by high-voltage paper electrophoresis, the acidic fractions were further separated by high-performance liquid chromatography on Lichrosorb-NH_2, and analyzed by 500-MHz ^{1}H-NMR spectroscopy and, partially, by permethylation analysis. Of the acidic oligosaccharide-alditols, the following sulfated carbohydrate chains could be identified: NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3[(SO_3Na→3)Galβ1→4GlcNAcβ1→2-Manα1→6]Manβ1→4GlcNAcβ1→4[Fucα1→6]GlcNAc-ol and NeuAcα2→6Galβ1→4(SO_3Na→6)$_{0-1}$GlcNAcβ1→2-Manα1→3[NeuAcα2→6Galβ1→4(SO_3Na→6)$_{1.0}$GlcNAcβ1→2Manα1→6]Manβ1→4GlcNAcβ1→4[Fucα1→6]GlcNAc-ol. The sulfated structural elements for porcine thyroglobulin form novel details of *N*-linked carbohydrate chains. They contribute to the fine structure of these oligosaccharides and are another type of expression of microheterogeneity.

Thyroglobulin; Sulfated *N*-linked carbohydrate; (Porcine)

1. INTRODUCTION

Thyroglobulin, the major iodinated glycoprotein synthesized in the thyroid gland, is the polypeptide precursor of the thyroid hormones. Analysis of the carbohydrate chains of thyroglobulin (molecular mass ~660 kDa) from several species has demonstrated that mainly two types of chains occur, generally called unit-A type (oligomannose type) and unit-B type (*N*-acetyllactosamine type) [1–10]. For human thyroglobulin also unit-C type (mucin type) [4] and unit-D type (proteoglycan type) [11] chains have been indicated. In many cases α1⟶3Gal residues can form part of the unit-B type chains [12–14].

Concerning the *N*-linked oligosaccharide chains of porcine thyroglobulin [5,6], the literature data indicate the presence of $Man_{5-9}GlcNAc_2$ structures, whereby the trimming of Man residues seems to take place randomly [7]. For the *N*-acetyllactosamine type a series of partially sialylated di- (75%) and tri- (25%) antennary structures with an α1⟶6-linked Fuc residue at the Asn-bound GlcNAc unit have been reported [8]. In addition, a terminal Galα1⟶3Galβ1⟶4 element in the Manα1⟶6 branch of a monosialylated (α2⟶6-linked NeuAc; Manα1⟶3 branch) diantennary structure has been shown to occur [14].

The recent finding of phosphate [10,15–17] and, especially, sulfate [18] groups in thyroglobulins from different biological origins prompted us to report data on the structural identification of sulfated *N*-linked unit-B type carbohydrate chains in porcine thyroglobulin.

2. MATERIALS AND METHODS

2.1. *Porcine thyroglobulin*

Porcine thyroglobulin was obtained from Sigma. SDS-polyacrylamide gel electrophoresis gave the usual pattern of two main bands, together with three faint bands of lower molecular mass [19,20]. Its sugar composition (Fuc/Man/Gal/GlcNAc/

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NeuAc = 0.4:3.0:1.2:2.6:0.4) and carbohydrate content (7%) are in accordance with literature data [1,2,21].

2.2. *Preparation of oligosaccharide-alditols*

The hydrazinolysis procedure on porcine thyroglobulin (six 125-mg portions) followed by high-voltage paper electrophoresis was carried out essentially as described [22,23]. The oligosaccharide-alditols were recovered from the paper by elution with water, yielding one neutral and three acidic fractions. The acidic fractions were further subfractionated by HPLC on Lichrosorb-NH_2 using elution systems of acetonitrile/15–30 mM KH_2PO_4-K_2HPO_4, pH 5.2 [24], and monitored at 205 nm. Fractions were desalted on Bio-Gel P-2 (200–400 mesh) using water as eluent and refractive index detection.

2.3. *Monosaccharide analysis*

Carbohydrate samples were subjected to the methanolysis procedure and analyzed by GLC on CP Sil 5, as described [25].

2.4. *Methylation analysis*

Methylation analysis was carried out essentially as described [26]. Permethylated material was hydrolyzed with 4 M trifluoroacetic acid, the obtained mixtures of partially methylated monosaccharides were reduced with NaB^2H_4 in water, and the partially methylated alditol acetates analyzed by GLC-MS.

2.5. *500-MHz ^{1}H-NMR spectroscopy*

Oligosaccharide-alditols were repeatedly exchanged in ^2H_2O (99.96 atom% ^{2}H) with intermediate lyophilization. Resolution-enhanced 500 MHz ^{1}H-NMR spectra were recorded in ^2H_2O at 27°C on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands). Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone (δ = 2.225 ppm) [27].

3. RESULTS

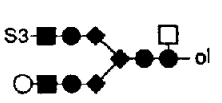
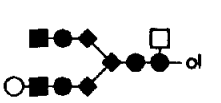
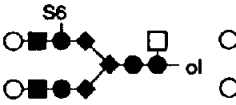
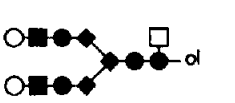
In the framework of our NMR studies on sulfated mono- [28] and oligo- [29,30] saccharides, porcine thyroglobulin was investigated for the presence of sulfated *N*-linked carbohydrate chains. The hydrazinolysis procedure in combination with high-voltage paper electrophoresis yielded one neutral (N) and three acidic (A1, A2, A3) fractions in the molar ratio of 27:35:28:10, respectively. As A2 and A3 turned out to contain the searched material, attention will only be paid to these fractions in this paper.

Subfractionation of A2, isolated from the 'double-negatively-charged' region of the paper electropherogram, on Lichrosorb-NH_2 yielded a series of peaks, of which A2b is of interest. The ^{1}H-NMR spectrum of A2b shows the presence of one major compound. The NMR features of this oligosaccharide-alditol, presented in table 1, resemble those of reference compound IgM, which is a classical diantennary structure terminated with β1⟶4-linked Gal in the Manα1⟶6 branch and α2⟶6-linked NeuAc in the Manα1⟶3 branch and having an α1⟶6-linked L-fucose residue at the Asn-bound *N*-acetylglucosamine [31]. However, of the structural-reporter-group signals the Gal-**6**′ H-1 doublet had shifted downfield from δ = 4.470 ppm to δ = 4.587 ppm. Furthermore, in contrast to the spectrum of IgM, in that of A2b two additional signals can be observed in the structural-reporter-group region, namely, a doublet of doublets at δ = 4.339 ppm (J values of 3.2 Hz and 10.0 Hz) and a doublet of doublets at δ = 4.294 ppm (J values of 3.2 Hz and ~1 Hz). The latter resonance has the typical shape of a Gal H-4 signal. By selective ^{1}H-decoupling of the signal at δ = 4.339 ppm, connected signals at δ = 4.294 ppm and δ = 3.684 ppm are found by difference spectroscopy. In the same way, selective ^{1}H-decoupling of the H-1 signal of Gal-**6**′ (δ = 4.587 ppm) and difference spectroscopy identified the resonance at δ = 3.684 ppm as Gal-**6**′ H-2. When a similar experiment is carried out for the H-1 signal of Gal-**6**, the corresponding H-2 resonance is found at δ = 3.534 ppm. In conclusion, the signals at δ = 4.339 ppm and δ = 4.294 ppm can be attributed to Gal-**6**′ H-3 and H-4, respectively.

Since the major compound in subfraction A2b is a monosialylated structure, an additional acidic substitution has to be present. In view of the literature data on acidic carbohydrate chains, it is obvious to suppose that either a phosphate or a sulfate group is involved [10,15–18]. The most obvious positions of attachment are Gal-**6**′ C-3 or C-4, as can be concluded from the downfield positions of Gal-**6**′ H-3 and H-4. Because of the absence of ^{31}P couplings on these signals, the occurrence of phosphate can be excluded. In the case of sulfate, the chemical shift values at δ = 4.339 ppm and δ = 4.294 ppm were compared to those of sulfated monosaccharide references. In table 2, the ^{1}H-NMR data of the methyl glycosides of α-D-galactopyranose, β-D-galactopyranose, 3-*O*-SO_3Na-α-D-galactopyranose and 4-*O*-SO_3Na-α-D-galactopyranose [28] are summarized. Going from methyl α-D-galactopyranoside to methyl

Table 1

^{1}H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of fractions A2b and A3c obtained from porcine thyroglobulin, together with those of reference compounds IgM and PT

Reporter group	Residue[a]	Chemical shifts[b] in[c]			
		A2b	IgM	A3c[d]	PT
					
H1	GlcNAc-2	4.713	4.714	n.d.	4.715
	Man-3	4.778	4.760	n.d.	4.786
	Man-4	5.136	5.136	5.139	5.135
	Man-4′	4.924	4.924	4.942	4.942
	GlcNAc-5	4.607	4.605	4.602	4.605
	GlcNAc-5′	4.580[e]	4.581	4.643	4.605
	Gal-6	4.444	4.445	4.445	4.443
	Gal-6′	4.587[e]	4.470	4.486	4.443
	Fuc	4.898	4.896	4.896	4.896
H-2	GlcNAc-1-ol	4.213	4.220	4.212	4.219
	Man-3	4.255	4.258	4.258	4.260
	Man-4	4.198	4.197	4.205	4.198
	Man-4′	4.108	4.110	4.112	4.114
H-3a	NeuAc	1.718	1.720	1.719[f]	1.718[f]
H-3e	NeuAc	2.669	2.668	2.671[f]	2.669/2.673
H-3	Gal-6′	4.339	n.d.	n.d.	n.d.
H-4	Gal-6′	4.294	n.d.	n.d.	n.d.
H-5	Fuc	4.072	4.071	4.073	4.072
H-6	GlcNAc-5′	n.d.	n.d.	4.434	n.d.
H-6′	GlcNAc-5′	n.d.	n.d.	4.314	n.d.
CH_3	Fuc	1.223	1.224	1.225	1.224
NAc	GlcNAc-1-ol	2.056	2.057	2.056	2.056
	GlcNAc-2	2.089	2.088	2.089	2.090
	GlcNAc-5	2.070	2.071	2.072	2.071
	GlcNAc-5′	2.048	2.048	2.066	2.065
	NeuAc	2.030	2.031	2.030[f]	2.030[f]

[a] For numbering of the monosaccharide residues, see text

[b] Chemical shifts are given for neutral solutions at 27°C, in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O, acquired at 500 MHz (but were actually measured relative to internal acetone: δ = 2.225 ppm)

[c] Structures are represented by short-hand notation [27]: ●, GlcNAc; ◆, Man; ■, Gal; ○, NeuAcα2⟶6; □, Fuc; S, sulfate

[d] For reasons of simplicity, the δ values for GlcNAc-5/5′ H-1 and Gal-6/6′ H-1 have been grouped for a sulfate group at GlcNAc-5′

[e] Assignments may have to be interchanged

[f] Signal stems from two NeuAc residues

3-*O*-SO_3Na-α-D-galactopyranoside, similar downfield shifts for H-3 and H-4 ($\Delta\delta$ = +0.666 ppm and $\Delta\delta$ = +0.356 ppm, respectively) were observed, as going from methyl β-D-galactopyranoside to Gal-6′ in subfraction A2b (H-3, $\Delta\delta$ = +0.695 ppm; H-4, $\Delta\delta$ = +0.372 ppm). In the case of the 4-*O*-sulfated analog quite a different NMR peak pattern was observed. It has to be noted that similar positions for Gal H-3 and H-4 have recently been reported for a terminal 3-sulfated Gal-β1⟶4 residue in an *O*-linked chain [32]. Summarizing, the terminal Gal residue in the Manα1⟶6 antenna (Gal-6′) in A2b is sulfated at position C-3, leading to the following structure:

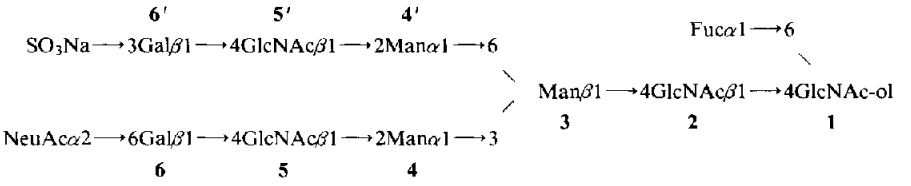


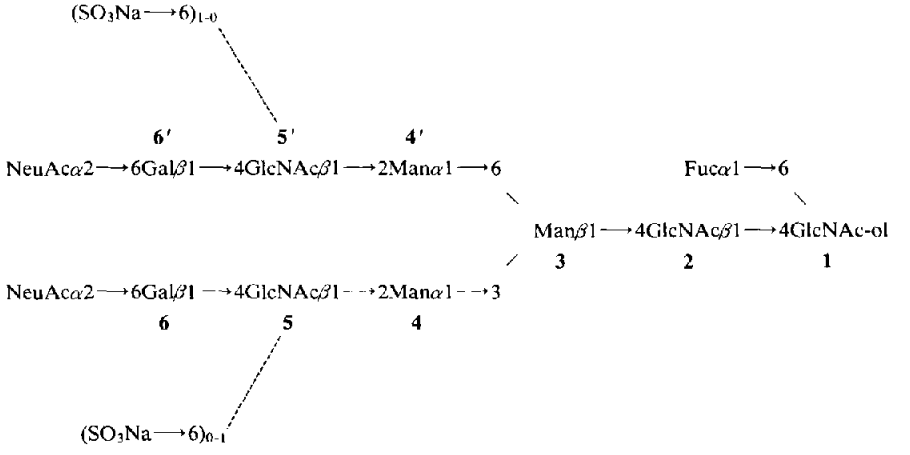
Table 2

^{1}H-NMR data for the methyl glycosides of α-D-galactopyranose, β-D-galactopyranose, 3-*O*-SO_3Na-α-D-galactopyranose and 4-*O*-SO_3Na-α-D-galactopyranose [28]

Protons	Chemical shifts[a] in			
	α-D-Gal	3-*O*-SO_3Na-α-D-Gal	4-*O*-SO_3Na-α-D-Gal	β-D-Gal
H-1	4.837	4.899	4.867	4.315
H-2	3.819	3.982	3.855	3.501
H-3	3.811	4.477	3.941	3.644
H-4	3.968	4.324	4.713	3.922
H-5	3.897	3.935	4.030	3.695
H-6	3.741	3.751	3.810	3.751
H-6′	3.752	3.759	3.755	3.796
OMe	3.414	3.430	3.425	3.573

[a] Chemical shifts are given at 27°C, in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O, acquired at 500 MHz (but were actually measured relative to internal acetone: δ = 2.225 ppm)

Subfractionation of fraction A3, isolated from the 'triple-negatively-charged' region of the paper electropherogram, on Lichrosorb-NH_2 resulted in several peaks, of which only fraction A3c is of interest. The ^{1}H-NMR spectrum of subfraction A3c shows a nearly pure compound. Comparison of the various resonances in the anomeric region of the spectrum as well as in the region δ < 2.8 ppm, with the spectral features of a classical α2→6 disialylated diantennary carbohydrate chain with an α1→6-linked Fuc residue at the Asn-bound GlcNAc unit, denoted PT, shows that the basic structure of A3c is similar to PT. The intensities of the GlcNAc-**5**/**5′** and Gal-**6**/**6′** H-1 doublets in A3c have only half the intensities of those of the corresponding signals in PT. But additionally, for GlcNAc as well as for Gal, a more downfield doublet is observed, namely, at δ = 4.643 ppm and at δ = 4.486 ppm, respectively. This means that in one of the antennae a structural element has changed, as compared to PT. Furthermore, two additional non-anomeric downfield signals, resonating at δ = 4.314 ppm (*J* values of 4.8 Hz and 11.0 Hz) and at δ = 4.434 ppm (*J* values of 1.2 Hz and 11.0 Hz) are observed. By selective irradiation it was found that both resonance patterns are coupled with each other. In view of earlier reports [29,33], such a typical coupled pattern belongs to H-6 and H-6′ of one of the monosaccharide residues, bearing a sulfate group at C-6. Methylation analysis of fraction A3c yielded a 4,6-disubstituted GlcNAc residue, indicating that GlcNAc-**5** or GlcNAc-**5′** bears a sulfate group at C-6. Combining the various data, the following two structures for A3c are possible:



Also the occurrence of a mixture of both structures cannot be excluded so far. The recently reported δ values of GlcNAc H-6 and H-6′ in the Galβ1 ⟶ 4(SO_3Na ⟶ 6)GlcNAc element, present in an *O*-linked chain, are in the same range as the values included here [34]. Recent studies on carbohydrate chains liberated from various ^{35}S-labeled mammalian cell lines suggested the occurrence of a NeuAcα2⟶6/3Galβ1⟶4(SO_3Na⟶6)GlcNAc element in *N*-linked oligosaccharides [35].

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