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Primary structure of the acidic carbohydrate chain of hemocyanin from *Panulirus interruptus*

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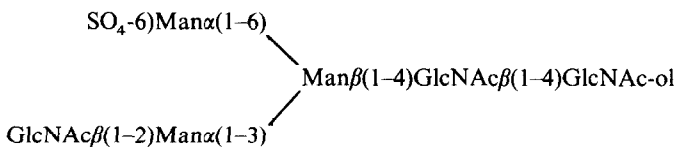
The *N*-glycosidic carbohydrate chains of hemocyanin from the spiny lobster *Panulirus interruptus* were liberated by hydrazinolysis of a pronase digest and subsequently reduced. Separation of the mixture of oligosaccharide-alditols by high-voltage paper electrophoresis resulted in a neutral (90%) and an acidic (10%) fraction. 500-MHz ^{1}H-NMR spectroscopy of the acidic fraction revealed a single component with the following novel structure:



Hemocyanin; *N*-linked carbohydrate; Sulfate; *(Panulirus interruptus)*

1. INTRODUCTION

Hemocyanins are high-M_r copper-containing oxygen-transport proteins, which are found in the hemolymph of some arthropods and molluscs. It has been shown that interesting species-specific differences in carbohydrate chains occur among the hemocyanins in both phyla [1-5]. The hemocyanin from the spiny lobster *Panulirus interruptus* is an *N*-glycoprotein, having a carbohydrate content of 1.5% with Man and GlcNAc as the only constituting monosaccharides [5]. Recently, the primary structures of the neutral carbohydrate chains were established to be Manα(1-6)[GlcNAcβ(1-2)Manα(1-3)]Manβ(1-4)GlcNAcβ(1-4)GlcNAc and Manα(1-6)[Manα(1-3)]Manα(1-6)[Manα(1-3)]Manβ(1-4)GlcNAcβ(1-4)GlcNAc [5]. Here, the structural characterization of the acidic carbohydrate material, representing 10% of the carbohydrate part, is described.

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Abbreviations: Man, D-mannopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; GlcNAc-ol, 2-acetamido-2-deoxy-D-glucitol

2. MATERIALS AND METHODS

The preparation and isolation of the acidic carbohydrate fraction from a hydrazinolysate of the

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pronase digest of *P. interruptus* hemocyanin have been described in [5].

Methyl α-D-mannopyranoside 6-*O*-sulfate was synthesized according to [6]. Briefly, methyl α-D-mannopyranoside (194 mg, 1 mmol) was dissolved in 5 ml dry pyridine. After cooling to 5°C, 73 μl chlorosulfonic acid (1.1 mmol) in 300 μl dry chloroform was added. The mixture was stirred for 30 min at 5°C, and then for 2 h at 25°C. After the addition of 2 ml water, the solvent was evaporated to dryness. The crude material was purified on a silica column (2 × 7 cm), using a mixture of dichloromethane:methanol (5:3, v/v) as eluting system. The final product was converted into the corresponding sodium salt by neutralization with NaOH.

For ^{1}H-NMR analysis, samples were repeatedly exchanged in ^2H_2O (99.96 atom% ^{2}H, Aldrich) with intermediate lyophilization. ^{1}H-NMR spectra were recorded on a Bruker AM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier-transform mode at a probe temperature of 27°C. One-dimensional spin lock difference spectroscopy was performed according to [7], in combination with the MLEV-17 composite pulse decoupling cycle [8], using a spin lock time of 160 ms. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [9]. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone in ^2H_2O (δ 2.225) [10].

3. RESULTS AND DISCUSSION

Hydrazinolysis of a pronase digest of hemocyanin from the spiny lobster *P. interruptus* resulted in complete liberation of the *N*-linked carbohydrate chains [5]. After re-*N*-acetylation and reduction, the mixture of oligosaccharide-alditols was fractionated by high-voltage paper electrophoresis, yielding 10% acidic material, which chromatographed as one peak, denoted **A**. In view of the sugar analysis of the native glycoprotein, showing only Man and GlcNAc as monosaccharides, no sugar constituent can be held responsible for the acidic nature of fraction **A**.

To elucidate the primary structure of the oligosaccharide-alditol present in fraction **A**, a 500-MHz ^{1}H-NMR spectrum was recorded (fig.1A). Relevant ^{1}H-NMR data of **A**, together with those of Manα(1-6)[GlcNAcβ(1-2)Manα(1-3)]Manβ(1-4)GlcNAcβ(1-4)GlcNAc-ol, being the previously reported structure in the neutral fraction **c** [5], are presented in table 1. The equal intensity of the anomeric proton signals in the ^{1}H-NMR spectrum of **A** points to the presence of a single compound. When comparing the ^{1}H-NMR data of **A** and **c** (table 1), it is evident that all structural-reporter groups which are present in the spectrum of **c** also occur in that of **A**, having essentially the same chemical shifts. This means that the compound in fraction **A** and **c** must have the structural element GlcNAcβ(1-2)Manα(1-3)[Manα(1-6)]Manβ(1-4)GlcNAcβ(1-4)GlcNAc-ol in common. However, the spectrum of **A** shows two additional downfield signals, resonating at δ 4.354 and δ 4.2. The doublet of doublets at δ 4.354 has a large coupling constant of 11.6 Hz, which can only correspond to a geminal coupling of H-6 and H-6′ of Man, or GlcNAc. To identify this monosaccharide residue, one-dimensional spin lock difference spectroscopy was performed. For this purpose a selective 180° pulse was given to the signal at δ 4.354 and the difference spectrum revealed the complete set of proton signals from one sugar unit (fig.1B). This residue could be identified as Man-4′ from the H-2 signal at δ 3.979, and from the (less distinct) H-1 signal at δ 4.911. The unknown signal at δ 4.2 belongs to H-6′ of Man-4′. The appearance of the Man-4′ H-6 and H-6′ at downfield positions as found for **A**, in comparison to **c**, must be attributed to the presence of an acidic substituent at C-6. In view of the literature data on acidic carbohydrate chains, it is reasonable to propose that either a phosphate or a sulfate group is involved. It has been demonstrated that 6-*O*-phosphorylation of a terminal α-D-Man residue causes a downfield shift of H-6 and H-6′ to δ 4.061 in Manα(1-6)Man-R [11] and a downfield shift of H-6 to δ 4.096 in Manα(1-2)Man, whereby a ^{1}H-^{31}P coupling (6.5 Hz) is evident [12]. However, in the ^{1}H-NMR spectrum of **A**, the H-6 (and H-6′) are shifted to even more downfield positions than observed for H-6 (and H-6′) in terminal 6-*O*-phosphorylated α-D-mannose residues [11,12]. Furthermore, no ^{1}H-^{31}P coupling is present on the Man-4′ H-6 and H-6′ signals. Therefore, a sulfate

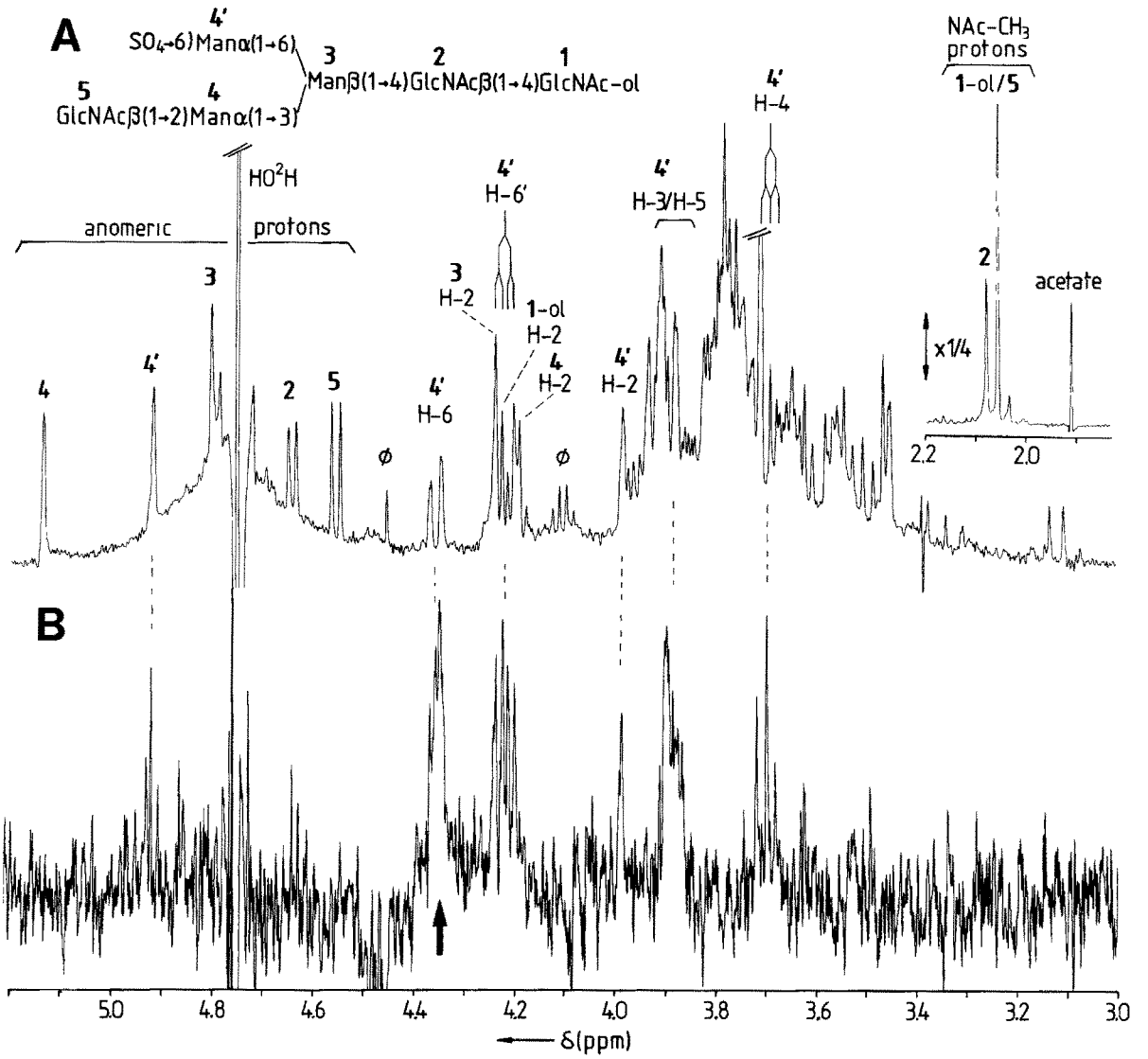


Fig.1. (A) Structural-reporter group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum (^2H_2O at 27°C) of oligosaccharide-[1-^{2}H]alditol fraction **A** from *P. interruptus* hemocyanin. The numbers in the spectrum refer to the corresponding residues in the structure. The relative intensity scale of the *N*-acetyl region differs from that of the other part of the spectrum as indicated. ϕ denotes impurity. (B) Spin lock difference spectrum, with a selective 180° pulse (indicated by the arrow) on the H-6 signal of Man-4'.

group should be present. To confirm this suggestion, the ^{1}H-NMR spectra of the methyl α-D-mannopyranoside and methyl α-D-mannopyranoside 6-*O*-sulfate were recorded. As is evident from table 2, sulfation at C-6 causes a downfield shift of H-6 and H-6' to δ 4.344 and 4.229, respectively. These chemical shift values are of the same order as those observed for Man-4' H-6 (δ 4.354) and H-6' (δ 4.218) in **A**. It should be noted that the H-6 and H-6' signals of Man-4' are essentially the same as those described for terminal GlcNAc, *O*-sulfated at C-6 (H-6, δ 4.337; H-6', δ 4.219) in GlcNAcβ(1-3)Galβ [13]. Summarizing the various data, the structure of **A** is proposed to be:

Table 1

^{1}H chemical shifts of structural-reporter groups of the constituent monosaccharides for the acidic high-voltage paper electrophoresis fraction **A**, together with the neutral HPLC fraction **c**, derived from *Panulirus interruptus* hemocyanin

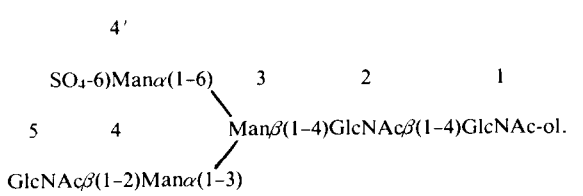
Reporter group	Residue[a]	Chemical shift [b,c] (ppm)	
		4′ 3-2-1-ol 5-4	S-4′ 3-2-1-ol 5-4
		Fraction **c**	Fraction **A**
NAc	GlcNAc-1-ol	2.055	2.055
	GlcNAc-2	2.077	2.077
	GlcNAc-5	2.055	2.055
H-1	GlcNAc-2	4.636	4.636
	Man-3	4.789	4.817
	Man-4	5.118	5.129
	Man-4′	4.918	4.911
	GlcNAc-5	4.551	4.551
H-2	GlcNAc-1-ol	4.239	4.221
	Man-3	4.255	4.235
	Man-4	4.186	4.187
	Man-4′	3.975	3.979
H-3	Man-4′	n.d.[d]	3.89
H-4	Man-4′	n.d.	3.698
H-5	Man-4′	n.d.	3.89
H-6	Man-4′	n.d.	4.354
H-6′	Man-4′	n.d.	4.218

[a] For numbering of monosaccharide residues and complete structures, see fig.1A

[b] Chemical shifts are given for neutral solutions at 27°C, in ppm downfield from sodium 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)

[c] S, sulfate

[d] n.d., not determined



Primary structural studies on sulfated *N*-linked carbohydrate chains have thus far been very limited. Some details have been reported for 4-*O*-sulfated Man in the carbohydrate chains of hen egg albumin [14] and 3/4-*O*-sulfated GalNAc in the carbohydrate chains of lutropin [15]. Man-6-sulfate has been discovered recently as a constituent of the *N*-linked carbohydrate chains of the lysosomal enzymes from *Dictyostelium discoideum* [16].

Table 2

^{1}H-NMR chemical shifts for the methyl α-D-glycopyranosides of mannose and mannose 6-*O*-sulfate

Protons	Chemical shift[a] (ppm)	
	Man	Man-6-S
H-1	4.761	4.763
H-2	3.929	3.938
H-3	3.751	3.626
H-4	3.640	3.700
H-5	3.604	3.824
H-6	3.898	4.344
H-6′	3.755	4.229
OCH_3	3.407	3.418

[a] Chemical shifts are given for neutral solutions at 27°C, in ppm downfield from sodium 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)

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