Iduronic acid: constituent of sulphated dolichyl phosphate oligosaccharides in halobacteria

Felix Wieland*, Johann Lechner and Manfred Sumper

Lehrstuhl für Biochemie I, Universität Regensburg, Universitätsstr. 31, D-8400 Regensburg, FRG

Received 3 October 1985; revised version received 24 October 1985

The occurrence of iduronic acid in the cell surface glycoprotein of halobacteria is described. This hexuronic acid is not only found in protein-linked oligosaccharides in halobacteria but does also exist in the corresponding dolichol-linked precursors. These findings were unexpected, as iduronic acid is a typical constituent of animal glycosaminoglycans, and its biosynthesis is so far known to occur by epimerization of a glucuronic acid residue within the completed carbohydrate chain linked to protein.

Iduronic acid *Dolichyl oligosaccharide* *Glycoprotein biosynthesis* *Halobacteria*

1. INTRODUCTION

Halobacteria, which belong to the kingdom of archaebacteria, possess a true glycoprotein [1] that is hexagonally arranged at the outer surface of their cell membranes [2]. This cell surface glycoprotein contains 2 different types of sulphated glycoconjugates [3]. Besides a repeating unit carbohydrate chain [4–6], oligosaccharides were detected, which consist mainly of Glc and GlcUA-sulphate, and are linked to the protein via asparaginylglucose [7]. Detailed biosynthetic studies have revealed that: (i) completely sulphated dolichyl monophosphate oligosaccharides serve as precursors of this type of glycoconjugates [8]; and (ii) transient methylation of a peripheral glucose residue of these intermediates is an obligatory step in the biosynthesis of sulphated glycoproteins in halobacteria [9]. Application of these oligosaccharide intermediates of a mild and effective method of carbohydrate hydrolysis and subsequent gas-liquid chromatography-mass spectroscopy (GLC-MS) revealed an as yet undetected hexuronic acid. Here we show that this hexuronic acid is iduronic acid, a finding that was unexpected for 2 reasons: (i) iduronic acid as a constituent of protein-linked carbohydrates has as yet exclusively been detected in eucaryotic organisms, and (ii) in all systems studied, iduronic acid is formed in glycoconjugates at the protein-linked level by epimerization of glucuronic acid residues [10], but has not been reported so far to occur in lipid oligosaccharide intermediates.

* To whom correspondence should be addressed at: Department of Biochemistry, Stanford School of Medicine, Stanford University Medical Center, Stanford, CA 94350, USA

2. EXPERIMENTAL

2.1. *Isolation of oligosaccharides*

Isolation of pronase-derived glycopeptides from the cell surface glycoprotein of *Halobacterium halobium* as well as isolation of halobacterial dolichol phosphate oligosaccharide sulphates were performed as described [7,8]. Oligosaccharides were isolated from dolichyl oligosaccharides after mild acid hydrolysis by partitioning between water and chloroform and subsequent chromatography on Bio-Gel P2 as in [8,9].

2.2. *Carbohydrate analysis*

Colorimetric assays for neutral hexoses were ac-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

cording to [11] with Glc as standard and for hexuronic acids according to [12] with glucuronic acid as standard. Preparation of monosaccharides for GLC as their alditol acetates was according to [13]. For mild and efficient hydrolysis of uronic acid-containing saccharides, samples were subjected to methanolysis in 0.5 M HCl/CH_3OH at 80°C for 24 h in a sealed tube under reduced pressure. By this method, which is less destructive than aqueous acid hydrolyses, neutral sugars give rise to their methylglycosides, and uronic acids to their corresponding methyluronic acid methyl esters. These compounds without further derivatization can be converted to their volatile pentafluoropropionyl derivatives according to [14], where trifluoroacetylation of methylglycosides is described. The advantage of pentafluoropropionylation over trifluoroacetylation is that virtually all peaks due to pentafluoropropionylation of Glc and GlcUA can be separated on GLC supports of medium polarity (one methylglycoside can give rise to up to 4 peaks according to the α- and β-anomers of the furanose as well as the pyranose form). For analysis by GLC-MS the samples after methanolysis were dried in a gentle stream of nitrogen, and after addition of 100 μl CH_2Cl_2 (Merck, p.A.) and of 100 μl pentafluoropropionic anhydride (Pierce) the tube was again sealed and incubated at 100°C for 30 min. After concentration by gently blowing nitrogen, aliquots were directly subjected to GLC. The samples must not be dried completely to avoid eventual loss of the highly volatile derivatives.

To optimize the yield of free iduronic acid, samples were hydrolyzed with sulphuric acid according to [15]. For conversion to their alditol acetates, the resultant monosaccharides were C_1-reduced with sodium [^{1}H]borohydride and carboxyl-reduced with sodium [^{2}H]borohydride as described [8].

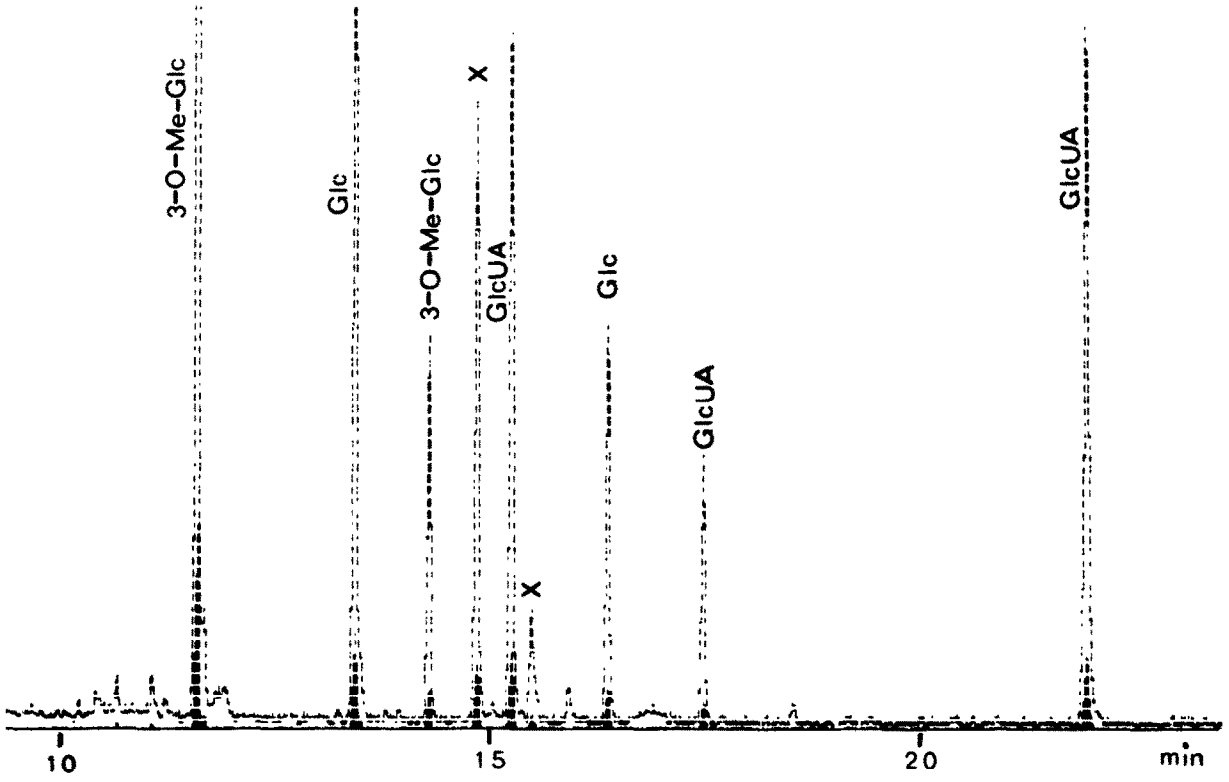

Fig.1. GLC-MS analysis of oligosaccharides B–J in fig.1 in [9]. The sample was methanolyzed and subsequently derivatized with pentafluoropropionic anhydride. The total ion profile (from m/e = 40–400 amu) is shown. Details are given in section 2.

2.3. *GLC-MS*

A Hewlett-Packard 5995 GLC-MS system equipped with a 30 m Durabond 1701 capillary column (ICT, Frankfurt) was used. With pentafluoropropionyl derivatives, the temperature immediately after injection was linearly raised by 2°C/min, starting at 120°C. Peralditol acetates were chromatographed from 200 to 250°C with a linear increase of 2°C/min, and with partially methylated peralditol acetates a program was run from 140 to 250°C, again with an increase of 2°C/min. Injector temperature was 220°C, and transfer line temperature 280°C, throughout.

3. RESULTS

Carbohydrate analyses after aqueous acid hydrolyses of Asn-Glc-linked oligosaccharides as

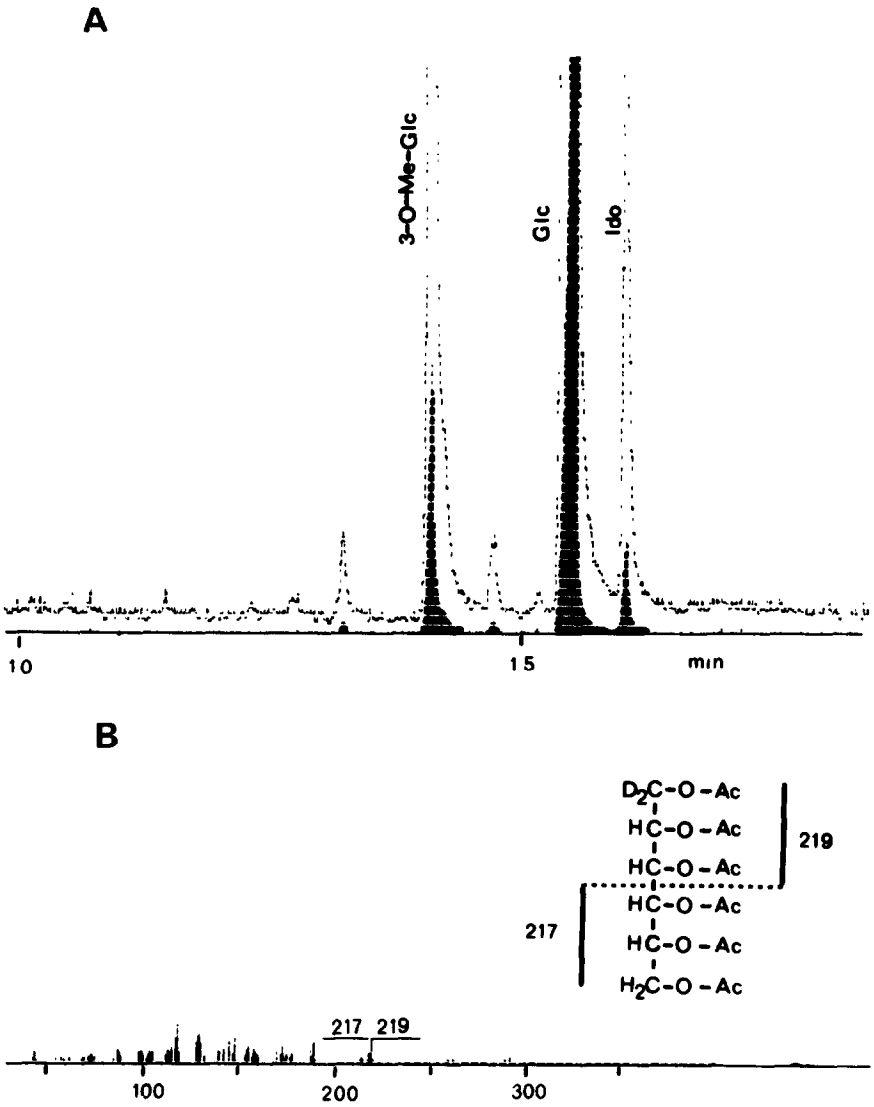

Fig.2. (A) GLC-MS analysis of the sample as in fig.1. The sample after methanolysis was hydrolyzed to yield the free monosaccharides, reduced C_1 with sodium [^{1}H]borohydride, carboxyl-reduced with sodium [^{2}H]borohydride and subjected to GLC-MS after conversion to the corresponding peralditol acetates. Total ion profile (from *m/e* = 40–400 amu) is shown. For details see text. (B) Mass spectrum of the material corresponding to the peak lettered Ido.

well as of their precursors isolated from dolichyl intermediates revealed the presence of Glc and GlcUA (and 3-*O*-methyl-Glc in some lipid intermediates). Using a different method of hydrolysis, the occurrence of an additional hexuronic acid could be established. After acid methanolysis of the oligosaccharides (e.g. material of peaks B–J of fig.1 in [9]) and subsequent analysis by GLC-MS of their pentafluoropropionyl derivatives (see section 2), a total ion profile was obtained as shown in fig.1. All of the peaks could be assigned to known constituents of the oligosaccharides, besides 2 that are lettered x in the figure. Their mass spectra showed fragments typical for hexuronic acids. Therefore, the methylglycosides after methanolysis were hydrolyzed and converted to the corresponding peralditol acetates, labeled with two ^2H atoms at carbon 6 and analyzed by GLC-MS. The corresponding total ion profile is shown in fig.2A. Besides 3-*O*-methyl-Glc and Glc (resulting from Glc plus GlcUA) an additional compound appeared, eluting from the column after Glc and yielding the mass spectrum of a C_6-[$^2H_2$]hexitol hexaacetate (see fig.2B). By comparison with commercially available authentic hexoses (Sigma), this compound was found to be C_6-[$^2H_2$]itol hexaacetate. Accordingly, the unknown hexuronic acid must be iduronic acid. For further analysis the reduced oligosaccharide B in fig.1 in [9] was subjected to permethylation. The permethylated saccharide containing uronic acid methyl esters was reduced with sodium [^2H]borohydride, and after hydrolysis, the resulting partially methylated alditols analyzed by GLC-MS as their acetate derivatives. A corresponding total ion profile is shown in fig.3. The following structural elements could be correlated with the 4 peaks identified: peak 1 corresponds to the reduced reducing-end Glc residue, peak 2 to a peripheral Glc (or 3-*O*-methyl-Glc) residue, and peak 4 to (1⟶4)-linked glucuronic acid residues. Peak 3 gave rise to a mass spectrum identical with that of peak 4. Therefore, this compound must be assigned to the iduronic acid residue, which accordingly occurs in (1⟶4)-linkage in the oligosaccharide. Analysis of several Asn-Glc-linked oligosaccharide peptides from the cell surface glycoprotein of halobacteria [7,8], as well as from their flagellae [16], revealed the presence of iduronic acid as well.

4. DISCUSSION

The hitherto unknown uronic acid of the sulphated oligosaccharides from the halobacterial cell surface glycoprotein is iduronic acid. This finding completes earlier data on the structure of these unusual glycoconjugates, which are linked to protein via the unit Asn-Glc [7–9]. The saccharides can be described as follows:

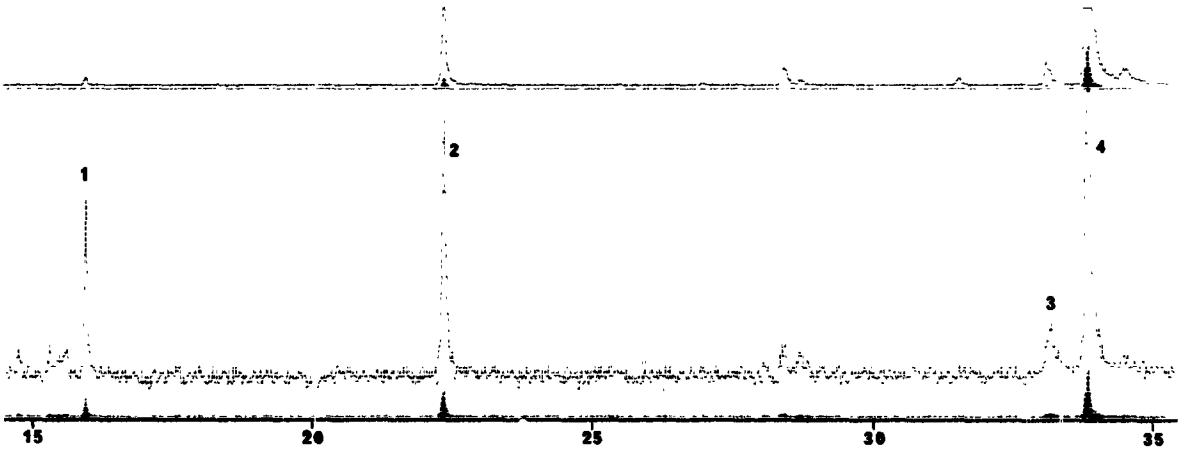

Fig.3. GLC-MS analysis of the material corresponding to peak B in fig.1 in [9] after permethylation. Total ion profile (from m/e = 4–400 amu) is shown. For details see section 2. (1) 4-*O*-Acetyl-1,2,3,5,6-penta-*O*-methylhexitol; (2) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol; (3 and 4) 1,4,5,6-[^2H]$_2$tetra-*O*-acetyl-2,3-di-*O*-methylhexitols.

HexUA(1⟶4)HexUA(1⟶4)HexUA(1⟶4)Glc, and
Glc(1⟶4)HexUA(1⟶4)HexUA(1⟶4)Glc, or
Glc(1⟶4)HexUA(1⟶4)HexUA(1⟶4)-
HexUA(1⟶4)Glc,

with one of the chain-standing HexUA residues in each of the saccharides being iduronic acid. Furthermore, this iduronic acid was also found to be present in the corresponding dolichol-linked precursors. Both results were unexpected, as iduronic is a main constituent of the animal glycosaminoglycans, and there is only one report of IdUA occurring in the kingdom of eubacteria, namely in a type-specific cellular polysaccharide of strain Hobb 10 of *Clostridium perfringens* [17]. In addition, the only biosynthetic pathway known so far leading to iduronic acid involves epimerization of chain-standing GlcUA residues, after completion of the carbohydrate at the protein-linked level [10], where sulphation also occurs. These striking differences in biosyntheses in halobacteria and eucaryotes could well be explained in terms of the following argumentation: as halobacteria lack organelles analogous to the Golgi apparatus (the site in mammalian cells of sulphation and epimerization of sulphated glycoproteins), these archaebacteria must have developed a different biosynthetic pathway leading to their sulphated glycoproteins. Our present working hypothesis is that the oligosaccharide is completed and sulphated being attached to the inside of the cell membrane via a dolichyl residues, thus able to serve as a compartmentalized substrate for a complex of the different (membrane-linked) biosynthetic enzymes using water-soluble precursor molecules for the construction of the sulphated oligosaccharide. After completion, the dolichol phosphate oligosaccharide sulphates are transported to the cell surface (most probably by a mechanism involving transient methylation of their peripheral Glc residue [9]), where transfer of the carbohydrate to the core protein occurs. Several lines of evidence indicate that in halobacteria this transfer actually does take place at the cell surface [4,9,18], which therefore could be regarded to be functionally analogous to the lumen of the rough endoplasmic reticulum in animal cells.

As to the iduronic acid, we do not yet know whether it is incorporated into the lipid-linked precursor from a nucleotide-activated induronic acid or is generated within the dolichyl phosphate oligosaccharides by epimerization of a glucuronic acid residue.

ACKNOWLEDGEMENTS

We thank Miss S. Stammler for expert technical assistance, and Mrs H. Trommer for typing the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 43, Regensburg).

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