Eur. J. Biochem. *181*, 345–350 (1989)

A phosphodiester bridge between two arabinose residues as a structural element of an extracellular glycoprotein of *Volvox carteri*

Otto HOLST[1], Volker CHRISTOFFEL[1], Rüdiger FRÜND[2], Hermann MOLL[3] and Manfred SUMPER[1]

[1] Lehrstuhl Biochemie I der Universität, Regensburg
[2] Lehrstuhl Biophysik der Universität, Regensburg
[3] Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, Borstel

(Received November 1, 1988/January 18, 1989) – EJB 88 1278

The sulphated glycoprotein SSG 185 is the monomeric precursor of a highly aggregated structural element in the extracellular matrix of the multicellular green alga *Volvox carteri*. A phosphodiester of arabinose was isolated from a saccharide fragment of SSG 185. The structure of this phosphodiester was investigated by methylation analysis, ^{13}C-NMR, photometric methods and enzymatic assays and identified as D-Ara*f*-5-phospho-5-D-Ara*f*. The function of this phosphodiester bridge as a crosslink of different carbohydrate chains in SSG 185 is discussed.

It is now widely recognized that many developmental responses of cells are mediated by the extracellular matrix (ECM) with which those cells are in contact. A particularly favourable model system for analysing the role of the ECM in developmental processes is the multicellular flagellate *Volvox*. The structural organization is relatively simple and recent studies demonstrate that, as in higher plants and animals, the *Volvox* ECM is actively modified in developmentally specific ways [1–3].

The organization of the *Volvox* ECM has been analysed in detail at the light- and electron-microscopic level. Recently, the details of ECM architecture in the representative species of *Volvox* were summarized and a system of nomenclature proposed (for review, see [4]). Each *Volvox* organism is surrounded by a boundary zone which includes a crystalline lattice that is very similar to that of *Chlamydomonas reinhardii* being composed of a number of hydroxyproline-rich glycoproteins [5–7]. The ECM internal to the boundary zone was designated the cellular zone. The most distinct structural element of this zone is a highly regular pattern of fibrous layers that surround cells at a distance to form contiguous cellular compartments. This element exhibits a honeycomb-like organization and was designated cellular zone 3 [4].

A sulphated, hydroxyproline-rich glycoprotein (designated SSG 185) that was chemically characterized in some detail [3] could be identified by immunological techniques as the precursor of the cellular zone 3 structure of the ECM (Ertl, H., Wenzl, S. and Sumper, M., unpublished results). SSG 185 is a short-lived molecule, which is polymerized into highly aggregated material. Depolymerization *in vitro* is only achieved by proteolytic digestion, resulting in a 145-kDa glycopeptide, or by treatment with anhydrous HF. This fact indicates the existence of covalent crosslinks in the polymeric form of SSG 185. In this paper, we report the identification of the phosphodiester arabinose 5-phospho-5-arabinose as a structural element of the polymeric SSG 185 glycoprotein. A function of this element as a crosslink between different carbohydrate chains is suggested.

Correspondence to M. Sumper, Lehrstuhl Biochemie I der Universität, Universitätsstraße 31, D-8400 Regensburg, Federal Republic of Germany

Abbreviations. ECM, extracellular matrix; SSG 185, a sulphated, hydroxyproline-rich glycoprotein; GC/MS, gas chromatography/mass spectroscopy; Me_2SO, dimethylsulphoxide.

Enzymes. Acid phosphatase (EC 3.1.3.2); alkaline phosphatase (EC 3.1.3.1); galactose dehydrogenase (EC 1.1.1.48); snake venom phosphodiesterase I (EC 3.1.4.1); calf spleen phosphodiesterase (EC 3.1.16.1).

MATERIALS AND METHODS

Growth of Volvox carteri

Cultures of *Volvox carteri f. nagariensis*, strain HK 10 (female, Culture Collection of Algae, University of Texas, Austin, USA), were grown synchronously in *Volvox* medium [8] at 28 °C in an 8-h dark/16-h light (10000 lux) cycle [9].

General methods

Thin-layer chromatography (TLC) was performed either on silica gel plates (Merck, Darmstadt, FRG) using acetonitrile/water (85:15 or 70:30; by vol.) or on polyethyleneimine cellulose plates (Schleicher & Schüll, Dassel, FRG) with 1 M ammonium formiate, pH 3.4 as eluent. The silica plates were sprayed with orcinol (1 mg/ml) in 20% sulfuric acid [10] or with $KMnO_4$ [11]. Electrophoresis in 6% polyacrylamide gels was according to [12]. The gels were stained with Stains All [13].

Radio gas chromatography was performed with a Packard 423 gas chromatograph. The outlet end of the column was fitted with a 1:10 stream splitter. The radioactivity of 0.9 of the sample was measured as $[^{14}C]CO_2$ in a Packard model 894 gas proportional counter. One tenth of the sample served for mass detection of internal sugar standards. Neutral sugars were analyzed as their alditol acetates [14] on a 1 m × 2 mm glass column (3% SP-2340 on 100/120 Supelcoport, Supelco) with a linear temperature program from 180–240 °C/min).

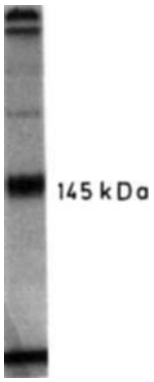

Fig. 1. *[^{32}P]Phosphate incorporation into the extracellular glycoprotein SSG 185. Volvox* spheroids at the developmental stage of gonidial cleavage were pulse labelled with [^{32}P]phosphate for 60 min. The 145-kDa fragment from SSG 185 was isolated according to [3] and applied to SDS/PAGE (6% acrylamide) and visualized by autoradiography

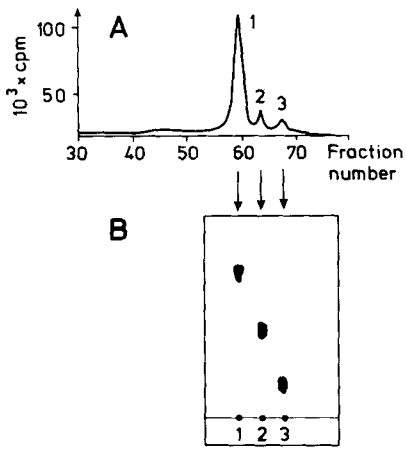

Fig. 2. *Chromatography profiles.* (A) Bio-Gel P 2 elution profile of hydrolysed ^{32}P-labelled 135-kDa fragment (0.5 M trifluoroacetic acid, 2 h, 100 °C). The column (70 × 0.7 cm) was equilibrated in 50 mM pyridine acetate, pH 5.2. Molecular size standards eluted in the following fractions (in parentheses): stachyose (56), raffinose (59), glucose 6-phosphate (64) and glucose (68). (B) Polyethyleneimine TLC (1 M ammonium formiate, pH 3.4) of the substances eluted in peaks 1, 2 and 3, respectively. 1, phosphodiester; 2, phosphomonoester; 3, inorganic phosphate

Gas chromatography was performed on a Varian 3700 gas chromatograph equipped with a Durabond 1701 capillary column (30 m, 1 ml/min helium as carrier gas) for the analysis of alditol acetates. The temperature was raised at a rate of 2 °C/min from 200 – 250 °C. Methylated sugars were analysed on a SE-54 column (25 m, hydrogen as carrier gas). The temperature program for the analysis of the reduced and permethylated phosphodiester was 160 °C for 3 min, then increased to 300 °C at a rate of 5 °C/min. Mono-*O*-acetyl-tetra-*O*-methyl-pentitol was chromatographed isothermally at 100 °C.

Electron-impact mass spectrometry at 70 eV and chemical ionization mass spectrometry (ammonia as reactant gas) were performed on a Hewlett-Packard 5985 GC/MS instrument equipped with the HP-1000 data system. The ion source temperature was 200 °C.

Radioactive labelling of SSG 185

300 ml *Volvox* suspension containing 10 – 15 spheroids/ml at the embryogenesis stage was poured over a 40-µm screen cloth and washed with glycerophosphate-free *Volvox* medium. The colonies were finally suspended in 1 ml. After the addition of 500 µCi [^{32}P]phosphate, the incubation was continued for 6 h. The colonies were then disrupted by ultrasonication and frozen.

Preparation of uniformly ^{14}C-labelled 135-kDa or 145-kDa fragment was performed according to [3].

Preparation of the 135-kDa fragment of SSG 185

Volvox spheroids from a 300-l culture were concentrated (600 ml packed spheroids), ultrasonically disrupted and frozen at −18 °C overnight. Crude extracellular matrix material was collected by centrifugation at 38000 × *g* for 16 h. The pellet was homogenized in 0.2 M NaCl/3% SDS and centrifuged at 10000 × *g* for 1 h. This treatment was repeated twice. The resulting sediment was delipidated with dichloromethane/methanol (1:1, by vol.) and collected by centrifugation at 25000 × *g* for 15 min. A total of five extractions were performed. The sediment was dried under a stream of nitrogen, powdered and treated with 1 M NaOH and 1 M $NaBH_4$ at 56 °C for 90 min (addition of 1 M $NaBH_4$ at this stage did not improve the yield of β-eliminated material and was therefore omitted in large-scale purifications). The resulting solution was adjusted to pH 8 and to 50 mM Tris/HCl, pH 8 and applied to a QAE Sephadex A 25 column (300 ml). After washing the column with three bed volumes of 1 M NaCl, 10 mM Tris/HCl, pH 8, the highly sulphated 135-kDa fragment was eluted with 4 M NaCl, 10 mM Tris/HCl, pH 8. After extensive dialysis, the final purification of the saccharide fragment was performed by preparative SDS/PAGE (6% polyacrylamide).

Preparation of the phosphodiester

Purified 135-kDa fragment (5 mg/ml) was mixed with ^{32}P-labelled 135-kDa fragment (10000 cpm/ml) and hydrolysed in 0.5 M trifluoroacetic acid at 100 °C for 2 h. The hydrolysate was applied to a Bio-Gel P 2 column (70 × 0.7 cm) equilibrated in 50 mM pyridine acetate, pH 5.2. Radioactive fractions were pooled and analysed on polyethyleneimine cellulose for the presence of the phosphodiester. Final purification of the phosphodiester was performed by preparative TLC on silica gel plates using acetonitrile/water, 85:15 (by vol.) as eluent.

Analytical methods

Complete hydrolysis of the phosphodiester was achieved in 0.5 M HCl at 100 °C for 48 h. Alternatively, the phosphodiester was hydrolysed in 2 M trifluoroacetic acid at 100 °C for 4 h, followed by a treatment with alkaline phosphatase in 50 mM $NaHCO_3$. The content of arabinose in the phosphodiester was determined by the phenol/sulfuric acid method [15] and by gas chromatography of alditol acetates with inositol as internal standard. Phosphate was determined according to [16].

Enzymatic assays

^{32}P-labelled phosphodiester was treated with phosphodiesterase either from snake venom (0.2 U in 50 mM Tris/

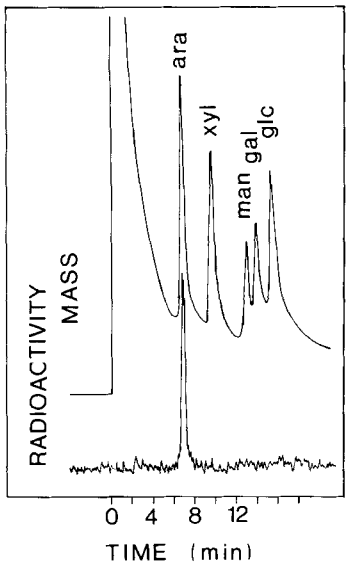

Fig. 3. *Chemical characterization of ^{14}C-labelled phosphodiester by radio gas chromatography.* The scan on top of the chromatogram records the mass signals of internal standard sugars. The radio scan analyses alditol acetates derived from the phosphodiester after acid hydrolysis and phosphatase treatment (Materials and Methods). A total of 9000 cpm of labelled material was injected

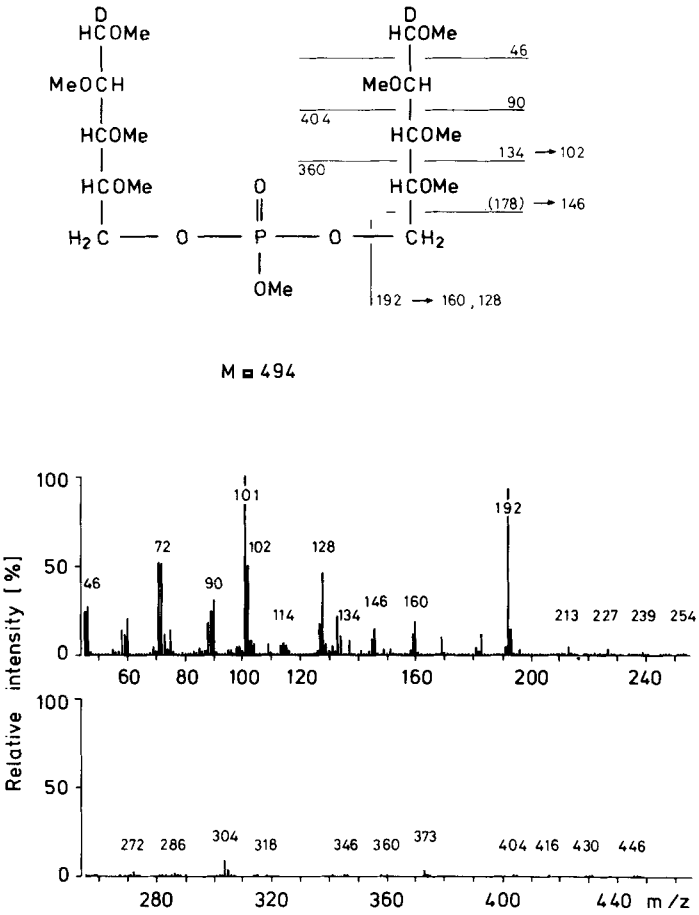

Fig. 4. *Electron-impact mass spectrum of the reduced and permethylated phosphodiester of arabinose derived from the 135-kDa fragment*

HCl, pH 7.5 at 37°C for 1 h) or from calf spleen (0.2 U in 50 mM Tris/HCl, pH 6.5 at 37°C for 1 h). The phosphodiester was also treated with acid phosphatase (0.2 U in 0.1 M ammonium acetate, pH 5.5 at 37°C for 30 min) and alkaline phosphatase (0.2 U in 0.01 M Tris/HCl, pH 8.8 at 37°C for 30 min). All enzymes were purchased from Boehringer, Mannheim, FRG.

L- and D-arabinose (20 μg each) and the hydrolysate of the phosphodiester (equivalent to 20 μg arabinose) were incubated at room temperature with galactose dehydrogenase (0.12 U) in 0.1 M Tris/HCl, pH 8.8, with a threefold excess of NAD^+. The absorbance of the test was read at 366 nm and production of NADH was measured for 20 min at appropriate intervalls.

Methylation analysis

Methylation analysis of the phosphodiester was performed according to [17]. Briefly, 200 μg reduced phosphodiester (NaB^2H_4 in water) were dissolved in methanol (0.1 ml) and treated with diazomethane (30 min, room temperature). After drying over P_2O_5, methylation with CH_3I was performed in absolute dimethylsulphoxide [18] in the presence of solid NaOH (10 mg, 15 min, room temperature). The methylated product was purified on a Sep-Pak C_{18} cartridge [19] and then analysed by GC/MS. After cleavage of the reduced and permethylated phosphodiester with $LiAlH_4$ in ether for 30 min at room temperature [20], the product was acetylated and again analysed by GC/MS.

^{13}C-NMR spectroscopy

^{13}C-NMR spectra were obtained at 75.475 MHz using a MSL 300 Bruker instrument with a 5-mm high-resolution, fixed-frequency probe head in the quadraturdetection mode. The field was stabilised by an internal deuterium lock. The distortionless enhancement by polarization transfer (DEPT) pulse sequence was used for sensitivity enhancement. The acquisition parameters were: spectral width, 25000 Hz; filter width, 30000 Hz; time domain words, 8 K; acquisition time, 0.164 s. The recycle delay was 1 s, the 90° ^1H pulse was 9.7 μs. The length for the magnetization transfer was set to 3 ms. For the sorting pulse at 135° ^1H pulse was used. The spectra were calibrated on methanol (49.6 ppm).

RESULTS

Newly synthesized monomeric SSG 185 aggregates within the ECM forming an insoluble polymer. This polymeric form remains insoluble even in hot SDS-containing solutions. Thus, low-speed centrifugation of a crude *Volvox* lysate in the presence of 3% SDS allows selective purification of polymeric SSG 185 material. Protease treatment (subtilisin or pronase) converts this insoluble material quantitatively into a soluble glycopeptide that exhibits, on SDS polyacrylamide gels, an apparent molecular mass of 145 kDa. Alkali treatment in the presence of $NaBH_4$ (β elimination of *O*-linked saccharides) converts the polymer into a saccharide fragment that migrates only slightly faster on SDS polyacrylamide gels (apparent molecular mass of 135 kDa). These procedures allow fast purification of defined fragments of the SSG 185 molecule.

As was shown previously by pulse-labelling experiments, SSG 185 is a highly sulphated glycoprotein. Similar pulse-labelling experiments with radioactive phosphate and sub-

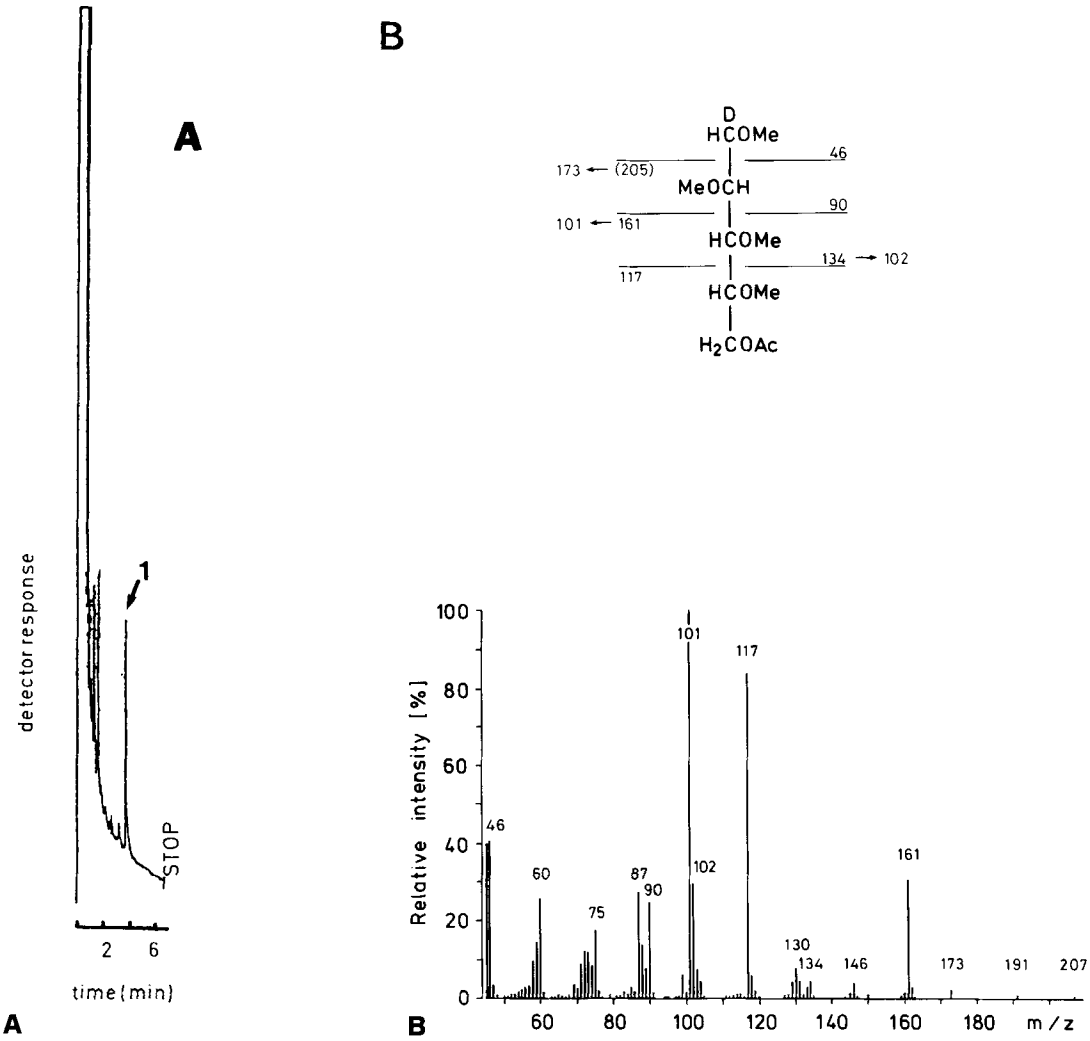

Fig. 5. *(A) Gas-liquid chromatography (25 m SE-54 column, 100 °C isothermally) of products obtained by cleavage of reduced and permethylated phosphodiester and subsequent acetylation.* (B) *Electron-impact mass spectrum of 5-O-acetyl-1,2,3,4-tetra-O-methyl-[1-^{2}H]pentitol from reduced and permethylated phosphodiester*

sequent isolation of the 145-kDa fragment [3] demonstrate additional incorporation of phosphate (Fig. 1). Even after alkali treatment, the ^{32}P label remains associated with the 135-kDa saccharide, indicating the presence of a sugar-linked phosphate.

For chemical characterization of the incorporated phosphate, a large-scale purification of the 135-kDa fragment of SSG 185 was performed as described in Materials and Methods. ^{32}P-labelled material was added as a marker. Homogeneous 135-kDa fragment was hydrolysed in 0.5 M trifluoroacetic acid at 100 °C for 2 h. Analysis of the hydrolysate on polyethyleneimine thin-layer plates resulted in the detection of three radioactive spots. One radioactive product comigrated with inorganic phosphate, the second migrated like a phosphomonoester and the third component, which was hardly retained by the ion-exchange layer, behaved like a phosphodiester. Preparative separation of these components was achieved on a Bio-Gel P 2 column (Fig. 2). The material of peak 1, eluting at the position of a trisaccharide was identified as the presumed phosphodiester (lane 1, Fig. 2B) and peak 2 material as the sugar phosphate (lane 2, Fig. 2B). Both substances were subjected to digestion with acid and alkaline phosphatase, as well as phosphodiesterase from snake venom and calf spleen, respectively. The substance in peak 2 (Fig. 2) was completely split by both the phosphatases. The substance in fraction 1 was neither cleaved by the phosphatases nor by the phosphodiesterases. The latter enzymes are specific for nucleic acids, therefore the observed resistance of the substance in fraction 1 does not exclude the presence of a phosphodiester linkage, as was demonstrated for other phosphodiesters of sugars, e.g. agrocinopine A [21].

Prolonged acid hydrolysis of the suggested phosphodiester (0.5 M trifluoroacetic acid, 100 °C, 30–120 min) resulted in increasing amounts of the phosphomonoester and inorganic phosphate. Therefore both these substances are likely to be degradation products of the suggested phosphodiester.

For the chemical characterization of the phosphodiester, the material of peak 1 of the Bio-Gel P2 column was applied to preparative TLC (silica-gel-60 plates). The eluted phosphodiester appeared as a homogeneous substance in different chromatographic systems (silica gel 60 plates developed either with acetonitrile/water, 85:15 by vol., or with 1-butanol/2-propanol/0.5 M boric acid, 30:50:20 by vol., staining with orcinol).

Table 1. *^{13}C-NMR resonances of* L-*arabinose,* D-*arabinose 5-phosphate and the phosphodiester from SSG 185*
Data given in parentheses were adapted from [23]

	Resonance of				
	C-1	C-2	C-3	C-4	C-5
	ppm				
α-Ara*f*	(101.9)	(82.3)	(76.5)	(83.8)	(62.0)
	101.9	82.3	76.5	83.8	61.9
β-Ara*f*	(96.0)	(77.1)	(75.1)	(82.4)	(62.0)
	95.9	77.1	75.2	82.3	61.9
α-Ara*f*-5-*P*	(102.2)	(82.2)	(76.7)	(83.1)	(65.1)
	101.9	82.0	76.4	82.9	64.5
β-Ara*f*-5-*P*	(96.3)	(77.0)	(75.1)	(81.1)	(66.2)
	96.0	76.9	75.0	81.3	65.7
α-*P*-diester	101.8	82.1	76.2	82.4	65.5
β-*P*-diester	96.1	76.9	74.9	80.7	67.1

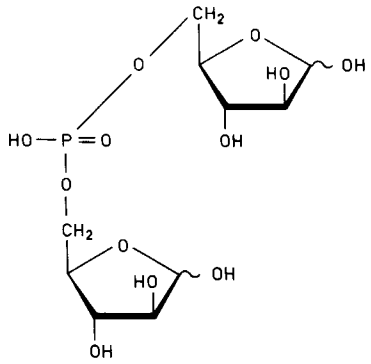

Fig. 6. *Structure of the phosphodiester of arabinose isolated from the glycoprotein SSG 185 of* Volvox carteri

Chemical characterization of the phosphodiester

The phosphodiester was completely split after 48 h in 0.5 M HCl at 100 °C or by a combination of acid hydrolysis and phosphatase treatment (Materials and Methods). Sugar analysis by thin-layer chromatography and by gas chromatography identified arabinose as the only sugar present in the hydrolysate. This is demonstrated by the experiment shown in Fig. 3. The phosphodiester was prepared from uniformly ^{14}C-labelled 135-kDa fragment (obtained by growing *Volvox* in the presence of $H^{14}CO_3^-$). After degradation by the combined acid/phosphatase treatment, sugars were analysed by radio gas chromatography. By spectrophotometric assays [15, 16] the molar ratio of arabinose and phosphate was found to be approximately 2:1 (1.06 nmol arabinose and 0.48 nmol phosphate/μl solution purified phosphodiester).

The strong acidic conditions required for complete cleavage of the phosphodiester made a linkage of phosphate to the anomeric carbon atom very unlikely. This was confirmed by reduction of the phosphodiester with $NaBH_4$ followed by acid hydrolysis. Only arabinitol could be detected in the hydrolysate. Therefore the anomeric centers of both the arabinoses are not substituted.

Galactose dehydrogenase is able to oxidize L-arabinose [22]. The arabinose present in the hydrolysate remained unaffected by this enzyme. Therefore, the arabinose molecules present in the phosphodiester should possess the D configuration.

Permethylation analysis

The reduced phosphodiester was treated with diazomethane, methylated with CH_3I/NaOH in Me_2SO and subjected to GC/MS. Fig. 4 shows the respective electron-impact mass spectrum and the fragmentation of the molecule (R_F = 3.23, relative to penta-*O*-acetyl-D-glucopyranose). The molecular mass of 494 Da was determined by chemical ionization mass spectrometry ($[M+1]^+ = 495$, $[M+18]^+ = 512$), confirming the chemical analysis of two arabinose residues and one phosphate/molecule. After cleavage of the reduced and permethylated phosphodiester with $LiAlH_4$ [20] and subsequent acetylation, a single product was identified on GC/MS (Fig. 5A). The corresponding mass spectrum (Fig. 5B) was found to be identical with that of 5-*O*-acetyl-tetra-*O*-methylpentitol. This result clearly shows that in the phosphodiester the ester linkage is established to the C-5 positions of both the arabinose residues.

^{13}C-NMR

^{13}C-NMR spectra were recorded from L-arabinose (20 mg), D-arabinose 5-phosphate (10 mg) and from the phosphodiester (3 mg). The data obtained are summarized in Table 1. The signals recorded from arabinose and arabinose 5-phosphate were identical to those published in [23]. All signals from the α- and β-configurated phosphodiester molecule could be identified. The C-1 signals were not shifted, showing again that both the arabinose residues possess unsubstituted anomeric carbon atoms. As for the arabinose 5-phosphate, only the carbon atoms, C-5 (3.6 ppm and 5.2 ppm downfield) and C-4 (1.4 ppm and 1.6 ppm upfield) were shifted in the phosphodiester compared to arabinose.

Taken together, the data presented above identified the phosphodiester D-Ara*f*-5-phospho-5-D-Ara*f* as a structural element in the extracellular glycoprotein SSG 185 from *Volvox carteri* (Fig. 6).

DISCUSSION

The extracellular glycoprotein SSG 185 is the monomeric precursor of a fibrous structural element that creates regular honeycomb-like compartments within the ECM of *Volvox carteri*. After its polymerization, the polymer is completely resistant towards chaotropic agents or boiling in the presence

of detergents. Therefore, formation of covalent crosslinks during the polymerization process appears highly likely. Chemical deglycosylation with anhydrous hydrogen fluoride at 0°C [24] of the polymeric material causes complete depolymerization, resulting in the formation of a single polypeptide chain with an apparent molecular mass of 60 kDa (unpublished results). This observation indicates that covalent crosslinks may be formed between the saccharide chains of the SSG 185 molecule rather than between the polypeptide chains of the monomeric units. In particular, this excludes the existence of diphenylether-linked bistyrosine in SSG 185, a crosslink discussed to be involved in cell-wall formation of higher plants [27].

The results presented in this paper demonstrate the existence of a phosphodiester bridge between the C-5 atoms of two arabinose residues. Obviously, this structural element could be a crosslink between different saccharide chains, where the phosphate links two saccharide chains either directly or as part of a (longer) saccharide bridge. However, the data available so far do not allow differentiation between intermolecular and intramolecular bridges. An estimate based on the yield of purified phosphodiester obtained from the 135-kDa fragment indicates the existence of more than ten phosphodiester bridges/molecule. Similar phosphodiester bridges are known in Gram-positive bacteria in the linkage of cell wall-polysaccharides to the peptidoglycan network [25, 26].

At present, no data are available concerning the biosynthetic pathway of this phosphodiester formation. Since aggregation of monomeric SSG 185 occurs within the extracellular matrix, phosphodiester formation of the suggested intermolecular bridges should take place extracellularly. If so, SSG 185 monomers have to be excreted in an activated state.

We are indepted to Susanne Stammler for expert technical assistance, to Prof. H. D. Lüdemann, R. Mengele (Universität Regensburg), Dr U. Zähringer and Dr H. Brade (Forschungsinstitut Borstel) for encouragement and help, and to Prof. J. Thiem (Universität Münster) for the kind gift of phosphodiester standards.

REFERENCES

1. Wenzl, S. & Sumper, M. (1982) *FEBS Lett. 143*, 311–315.
2. Gilles, R., Gilles, C. & Jaenicke, L. (1984) *Z. Naturforsch. 39C*, 584–592.
3. Wenzl, S., Thym, D. & Sumper, M. (1984) *EMBO J. 3*, 739–744.
4. Kirk, D. L., Birchim, R. & King, N. (1986) *J. Cell Sci. 80*, 207–231.
5. Catt, J. W., Hills, G. J. & Roberts, K. (1976) *Planta 131*, 165–171.
6. Roberts, K. (1974) *Philos. Trans. R. Soc. Lond. B, 268*, 129–146.
7. Roberts, K. (1979) *Planta 146*, 275–279.
8. Provasoli, L. & Pintner, I. J. (1959) in *The ecology of algae* (Tyron, C. A. & Hartman, R. T., eds), Special Publication No. 2, pp. 84–96, Pymatuning Laboratory of Field Biology, University of Pittsburgh.
9. Starr, R. C. & Jaenicke, L. (1974) *Proc. Natl Acad. Sci. USA 71*, 1050–1054.
10. Vaskovsky, V. E., Kostetsky, E. J., Svetaskev, V. J., Zhvkova, J. G. & Smirnova, G. P. (1970) *Comp. Biochem. Physiol. 34*, 163–177.
11. Stahl, E. (1962) *Dünnschichtchromatographie*, Springer-Verlag, Berlin.
12. Laemmli, U. K. (1970) *Nature 227*, 680–685.
13. Green, M. L., Pastewka, J. V. & Peacock, A. C. (1973) *Anal. Biochem. 56*, 43–51.
14. Laine, R. A., Esselman, W. J. & Sweeley, C. C. (1972) *Meth. Enzymol. 28*, 159–167.
15. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1955) *Anal. Chem. 28*, 350–356.
16. Ames, B. N. (1966) *Methods Enzymol. 8*, 115–121.
17. Holst, O., Thiem, J., Moll, H. & Brade, H. (1989) *Carbohydr. Res. 187*, in the press.
18. Ciucanu, J. & Kerek, F. (1984) *Carbohydr. Res. 131*, 209–217.
19. Waeghe, T. J., Darvill, A. G., McNeill, M. & Albersheim, P. (1983) *Carbohydr. Res. 123*, 281–304.
20. Zähringer, U. & Moll, H. (1987) *Jahresbericht Forschungsinstitut Borstel*, pp. 121–125.
21. Ryder, M. H., Tates, M. E. & Jones, G. P. (1984) *J. Biol. Chem. 259*, 9704–9710.
22. Bergmeyer, H. U. (1970) *Methoden der enzymatischen Analyse*, Vol. I, Verlag Chemie, Weinheim, p. 412.
23. Bock, K. & Pedersen, C. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 27–71.
24. Mort, A. J. & Lamport, D. T. A. (1977) *Anal. Biochem. 82*, 289–309.
25. Fujioka, M., Koda, S. & Morimoto, Y. (1985) *J. Gen. Microbiol. 131*, 1323–1329.
26. Voiland, A. & Michel, G. (1985) *Can. J. Microbiol. 31*, 1011–1018.
27. Fry, S. C. (1982) *Biochem. J. 204*, 449–455.