The Lipoprotein and Peptidoglycan of *Rhodobacter sphaeroides*

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The lipoprotein-peptidoglycan complex (rigid layer) from *Rhodobacter sphaeroides* was isolated. Treatment of the complex with N,O-diacetyl-muramidase cleaved off from the rigid layer the lipoprotein moiety with covalently bound peptidoglycan-fragments. The lipoprotein with the bound fragments showed a broad single band (M_r about 19,000) on SDS-polyacrylamide gels and had an isoelectric point of about 5.6. There was no serological cross-reaction with the rigid layer of *Escherichia coli* K12. The lipid moiety of lipoprotein with residual amino acids (mainly Gly, Ser, Glu, Asp) was obtained by Pronase E treatment of the rigid layer and chloroform-methanol extraction. It was free from phosphate and contained amide- and esterbound 18:1 and esterbound 16:0 and 18:0.

The isolated peptidoglycan, after enzymatical cleavage from the lipoprotein, had a chemical composition indicating A1γ-type structure. Comparable studies, performed with *Rhodobacter capsulatus* 37b4 resulted in essentially similar results for rigid layer, lipoprotein and peptidoglycan compositions.

Introduction

The outer membrane of Gram-negative bacteria generally contains a lipoprotein covalently bound to the peptidoglycan and thought to contribute in stabilizing the structure of the cell wall [1–4]. In many cases the lipoprotein exists also in a free form [5]. Well characterized is the lipoprotein of *Escherichia coli*. The protein moiety is attached to the peptidoglycan *via* its C-terminal Lys, replacing D-Ala on the A_2pm-residue. The fatty acids are bound as esters to the glyceryl Cys at the N-terminal end, or amidically to the N-terminal *a*-amino group.

Little is known about peptidoglycan-bound (lipo-)proteins in phototrophic bacteria. Recently, protein covalently bound to the peptidoglycan and presumably being a lipoprotein has been described for *Rhodobacter capsulatus* strains [6]. In addition, a large capsule polysaccharide is strongly attached to this lipoprotein-peptidoglycan complex. We report here on the peptidoglycan-lipoprotein complex of *Rhodobacter sphaeroides,* which is closely related to *Rhodobacter capsulatus* in classical and molecular taxonomy [7–9]. In addition, data on the rigid layer of the noncapsulated *Rhodobacter capsulatus* 37b4 are presented.

Abbreviations: A_2pm, diamino pimelic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel-electrophoresis.

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Materials and Methods

Bacterial strain and cultivation

Rhodobacter sphaeroides ATCC 17023 was obtained from the American Type Culture Collection, Rockville, M.D., U.S.A., *Rhodobacter capsulatus* 37b4 from the strain collection of the Institut für Biologie II, Mikrobiologie, der Universität Freiburg i. Br., F.R.G. Both strains were cultivated chemotrophically [10].

Preparation of cell-envelopes and rigid layers

The method used for isolation of cell envelopes from cell homogenates by differential centrifugation is given in reference [10]. The rigid layer was obtained by a three fold extraction of cell-envelopes in boiling SDS (4% in water, 100 °C, 15 min) according to reference [11]. After centrifugation (140,000 × *g*, 20 °C, 1 h), the sediment was washed with distilled water until it was free of SDS.

The rigid layer was digested with N,O-diacetyl-muramidase from *Chalaropsis* sp. [12] in 20 mM ammoniumacetate buffer, pH 4.8 at 37 °C for 18 h. After centrifugation (140,000 × *g*, 4 °C, 1 h) the supernatant was dialyzed against distilled water and lyophilized.

Pronase E-digestion and extraction of lipids

For removal of the nonpeptidoglycan amino acids, the rigid layer was digested with Pronase E (from *Streptomyces griseus*, Serva, Heidelberg, F.R.G., 6 U/mg protein). After centrifugation (140,000 × *g*, 4 °C, 1 h) the insoluble peptidoglycan was again extracted with SDS (4%, 100 °C, 15 min) and the detergent removed (as above).

Chloroform-methanol extraction (2:1; v/v, 25 °C, 2 min) for removal of lipids was according to reference [13] with a 5,000 × *g*, 4 °C, 10 min centrifugation for phase separation.

SDS-PAGE, isoelectric focusing, thin layer electrophoresis, agar-gel-precipitation

SDS-PAGE was performed according to reference [14]. Gels were stained for proteins with Coomassie blue or for polysaccharides with Schiff's reagent. For isoelectric focusing see reference [15]. Thin layer electrophoresis for peptide separation was performed in pyridine/acetic acid/water (1:2:250; v/v/v), pH 4.4, at 400 V and 4 °C for 150 min. Peptides were detected by staining with Fluorescamine (0.05% in acetone) for preparative purposes.

Antisera against lipoprotein of *Escherichia coli* Kl2 were kindly obtained from W. Bessler, Institut für Immunologie der Universität Freiburg i.Br., F.R.G. Ouchterlony agar-gel precipitation was performed as described previously [16].

Analytical chemical methods

Amino acids and amino sugars were determined after hydrolysis with 4 M HCl (105 °C, 18 h) on an automatic amino acid analyzer, model LC 6001 (Biotronik). Determination of neutral sugars was as described elsewhere [10]. Uronic acids, released by 0.5 M H_2SO_4, 100 °C, 4 h, were detected by high-voltage paper electrophoresis. Fatty acids were determined gas-liquid chromatographically as methyl-esters using an EGSS-X packed fused silica capillary column [17]. Hydroxylaminolysis of fatty acids and determination of phosphate were performed according to references [18] and [19], respectively.

Results

Rigid layer

The rigid layer of *Rhodobacter sphaeroides* ATCC 17023 was obtained as SDS insoluble fraction from cell-envelopes in a yield of about 25% of cell wet weight. It consisted of about 38% protein, 46% peptidoglycan and 2.7% fatty acids with 18:1 dominating over 18:0 and 16:0 (Table I). Chloroform-methanol extraction of the rigid layer did not remove the fatty acids from the rigid layer. Hydroxylaminolysis performed with the rigid layer revealed about 1/3 of 18:1 to be amide bound, while the small amounts of 16:0 and 18:0 were mainly ester-bound. The rigid layer fraction was free from phosphate indicating the absence of phospholipids, and neutral sugars (except glucose) or uronic acids were not found.

The rigid layer of both *Rhodobacter sphaeroides* ATCC 17023 and *Rhodobacter capsulatus* 37b4 (see below) did not react with antisera raised against lipoprotein of *Escherichia coli* Kl2 in the Ouchterlony test, while the control experiment with the rigid layer of *Escherichia coli* was positive. There was also

Table I. Amino acids, amino sugars, and fatty acids (nmol per mg fraction dry weight) in the rigid layer, in the isolated peptidoglycan, and in the lipid moiety with bound amino acid residues of the lipoprotein of *Rhodobacter sphaeroides* ATCC 17023. Values in parentheses give molar ratios.

Component	Rigid layer	Peptidoglycan	Lipid moiety[a]
Asp	251 (0.35)	–[b]	(1.00)
Thr	233 (0.33)	–	–
Ser	105 (0.15)	–	(1.88)
MurNAc	578 (0.82)	741 (0.69)	–
Glu	800 (1.13)	1,426 (1.33)	(0.85)
Gly	306 (0.43)	155 (0.14)	(2.93)
Ala	654 (0.92)	1,155 (1.08)	(0.54)
Cys	ND[c]	ND	ND
Val	319 (0.45)	–	(0.67)
Met	ND	ND	ND
A_2pm	709 (1.00)	1,072 (1.00)	–
Ile	49 (0.07)	–	(1.02)
Leu	108 (0.15)	–	–
Tyr	168 (0.24)	–	–
Phe	31 (0.04)	–	–
GlcNAc	859 (1.21)	873 (0.81)	–
His	44 (0.06)	–	–
Orn	ND	ND	ND
Lys	237 (0.33)	91 (0.08)	(0.94)
Arg	46 (0.06)	–	(0.71)
16:0	7 (0.01)	–	+[d]
18:0	19 (0.03)	–	+
18:1	71 (0.01)	–	+

[a] Molar ratios determined only.
[b] –, Absent.
[c] ND, not determined.
[d] +, Present but not quantified.

no crossreaction with porins from *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*.

Digestion of the rigid layer with N,O diacetyl-muramidase

Treatment of the rigid layer with N,O-diacetyl-muramidase rendered soluble the lipoprotein moiety. The enzyme-digested rigid layer showed on SDS-gels one single broad band (M_r about 19,000, Fig. 1) beside the band of the enzyme, while the untreated complex did not show any band on the gel. The lipoprotein also stained with Schiff's reagent (Fig. 1) due to the peptidoglycan residues covalently bound to the lipoprotein. The different size of these residues is probably the reason for the broad band of the lipoprotein on SDS-gels. The isoelectric point was found to be about 5.6 (Fig. 2).

Peptidoglycan and lipid moiety of lipoprotein

To remove the lipoprotein moiety from the peptidoglycan the rigid layer was digested with Pronase E. The isolated peptidoglycan had an amino acid composition (in molar ratios) of A_2pm:MurN:Glu:Ala:GlcN = 1.0:0.7:1.3:1.1:0.8 (Table I). In addition, two amino acids, Gly and Lys were constantly found in the fraction in about 10% amounts relative to the A_2pm content.

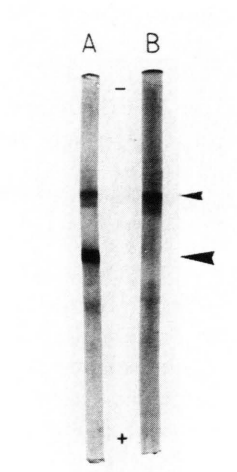

Fig. 2. Isoelectric focusing of the lipoprotein (with bound peptidoglycan residues) solubilized by muramidase-treatment of the rigid layer of *Rhodobacter sphaeroides* ATCC 17023. Track A, muramidase-treated rigid layer; track B, N,O-diacetyl-muramidase. Staining: Coomassie-blue. Large arrow: rigid layer protein; small arrow: N,O-diacetyl-muramidase.

The supernatant of the Pronase E digested rigid layer was investigated for lipid containing peptides. It was extracted with chloroform-methanol and the chloroform-phase obtained was separated for peptides by thin layer electrophoresis (Fig. 3). One pep-

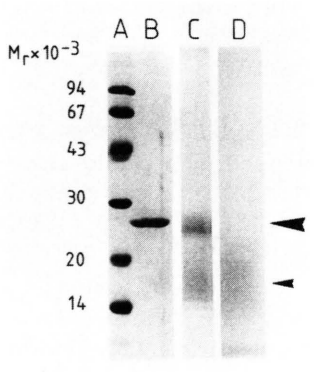

Fig. 1. SDS-PAGE of the N,O-diacetyl-muramidase-digested rigid layer of *Rhodobacter sphaeroides* ATCC 17023. Track A, molecular weight standard proteins; track B, N,O-diacetyl-muramidase; tracks C and D, muramidase-treated rigid layer. Staining was with Coomassie blue (track C) or with Schiff's reagent (track D). Samples were heated in sample buffer at 100 °C for 5 min before application to the gel. Large arrow: N,O-diacetyl-muramidase; small arrow: rigid layer protein.

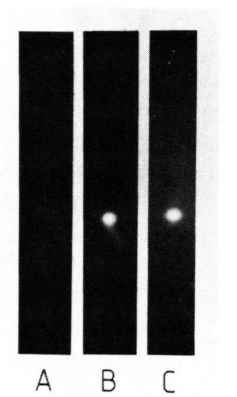

Fig. 3. Thin-layer electrophoresis of rigid layer peptides after muramidase- and Pronase E-digestion and chloroform-methanol extraction. The chloroform phase was separated in pyridine–acetic acid–water (1:2:250, v/v/v), pH 4.4, at 400 V for 150 min. Fragments were detected by Fluorescamine. Track A, chloroform-methanol extracted Pronase E (control); track B, peptides obtained with *Rhodobacter sphaeroides* ATCC 17023; track C, peptides obtained with *Rhodobacter capsulatus* 37b4.

tide could be detected by staining with Fluram. The fragment was isolated and analyzed for fatty acids and amino acids. It contained 18:0, 18:1 and 16:0, which is the same fatty acid pattern like that of the rigid layer (Table I). Gly, Ser, Glu and Asp were the dominating amino acids found.

Rigid layer, lipoprotein, and peptidoglycan of Rhodobacter capsulatus 37b4

The rigid layer of the noncapsulated *Rhodobacter capsulatus* strain 37b4 as well as the respective muramidase-digested complex and the isolated peptidoglycan had comparable chemical compositions as found with *Rhodobacter sphaeroides* (Table II). Again, the rigid layer was free from neutral sugars other than glucose and from uronic acids and showed the same characteristic fatty acid spectrum (1.8% total fatty acid content of rigid layer dry weight) as observed with the rigid layer of *Rhodobacter sphaeroides*. The supernatant of the Pronase E digested rigid layer was investigated thin-layer chromatographically for lipid-containing peptides in the chloroform-phase after chloroform-methanol extraction (Fig. 3). Again, the amino acid and fatty acid composition of the only peptide detectable with Fluram was qualitatively and quantitatively similar to that observed with *Rhodobacter sphaeroides* (Table II).

Table II. Amino acids, amino sugars, and fatty acids (nmol per mg fraction dry weight) in the rigid layer, in the isolated peptidoglycan, and in the lipid moiety with bound amino acid residues of the lipoprotein of *Rhodobacter capsulatus* 37b4. Values in parentheses give molar ratios.

Component	Rigid layer	Peptidoglycan	Lipid moiety[a]
Asp	96 (0.26)	–[b]	(1.00)
Thr	130 (0.35)	–	–
Ser	202 (0.54)	–	(1.88)
MurNAc	351 (0.94)	724 (0.86)	–
Glu	605 (1.63)	1,216 (1.44)	(0.85)
Pro	ND	ND	ND
Gly	201 (0.54)	250 (0.03)	(2.93)
Ala	845 (2.27)	1,227 (1.45)	(0.54)
Cys	ND[c]	ND	ND
Val	226 (0.61)	–	(0.67)
Met	ND	ND	ND
A_2pm	372 (1.00)	844 (1.00)	–
Ile	87 (0.23)	–	(1.02)
Leu	127 (0.34)	–	–
Tyr	71 (0.19)	–	–
Phe	29 (0.08)	–	–
GlcNAc	371 (1.00)	1,098 (1.30)	–
Trp	ND	ND	ND
His	33 (0.09)	–	–
Orn	ND	ND	ND
Lys	39 (0.10)	51 (0.06)	(0.94)
Arg	12 (0.03)	–	(0.71)
16:0	7 (0.02)	–	+[d]
18:0	11 (0.03)	–	+
18:1	47 (0.13)	–	+

[a] Molar ratios determined only.
[b] –, Absent.
[c] ND, not determined.
[d] +, Present but not quantified.

Discussion

The question of whether or not having a lipoprotein in the rigid layer of *Rhodobacter sphaeroides* ATCC 17023 may be answered positively. First, a fatty acid pattern with 18:1, 18:0 and 16:0 was not removable from the rigid layer by chloroform-methanol extraction. Phospholipid contamination can be excluded due to the absence of phosphorus. Second, hydroxylaminolysis revealed that about 30% of 18:1 is amide bound, in accordance with the data obtained with lipoprotein of *Escherichia coli,* where more than half of the main lipoprotein fatty acid (18:1) is amide-linked [20]. A strong argument is also the result of thin layer electrophoresis, where one single peptide with a lipid moiety was detected. Fatty acid compositions of lipoproteins from *Rhodobacter sphaeroides* (essentially identical to that of *Rhodobacter capsulatus*) and *Escherichia coli* [20] are different, but in both cases their ester-linked fatty acids are similar to those of the respective phospholipids [20–21].

After cleavage of the lipoprotein with Pronase E, some Lys and Gly still remained in the peptidoglycan fraction. Lys represents the link between lipoprotein and peptidoglycan in *Escherichia coli* [2], *Proteus mirabilis* [22] and *Pseudomonas aeruginosa* [23]. Thus, Lys may represent the C-terminus in the lipoprotein of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* as well, although experimental proof still has to be given as is still unclear the structural role of the Gly found. The lower D-Ala content, relative to A_2pm, in the *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* peptidoglycans will be explained by either partial lack of the terminal D-Ala, as is observed also with the *Escherichia coli* peptidoglycan [2] or by partial replacement of the C-terminal amino acid of the lipoprotein.

In another study [24], two outer membrane proteins of *Rhodobacter sphaeroides* ATCC 17023 with M_r of 8,000 and 10,000 on SDS-PAGE were suggested to be equivalent to the lipoprotein in *Escherichia coli*. It was suggested there that the 8,000 band may be the analog to the free form and the 10,000 band the analog to the bound form. No evidence for such a protein in the rigid layer of *Rhodobacter sphaeroides* ATCC 17023 was obtained in our study.

In accordance with absence of serological cross-reaction the amino acid compositions of the lipoproteins from *Rhodobacter sphaeroides, Rhodobacter capsulatus, Escherichia coli* [25] and *Proteus mirabilis* [22, 26] are different.

With the capsulated *Rhodobacter capsulatus* St. Louis firmly attached capsule material is found in large amounts in the rigid layer fraction [6]. In accordance with the fact of *Rhodobacter sphaeroides* ATCC 17023 to be noncapsulated, neutral sugars and/or uronic acids indicating a capsule were not found in the rigid layer of this strain or of the noncapsulated *Rhodobacter capsulatus* 37b4. When the rigid layer of *Rhodobacter capsulatus* St. Louis is digested with Pronase E, the capsule polysaccharide gets solubilized together with a characteristic amino acid pattern (Gly, Glu, Ser, and Ala dominating [6]). Gly, Glu, Ala, and Ser were also dominating in the lipopeptide moieties obtained by Pronase E-treatment and chloroform-methanol extraction with *Rhodobacter capsulatus* 37b4 and *Rhodobacter sphaeroides* ATCC 17023, indicating possible similarities in this structural region of the lipoprotein.

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