Porin of *Rhodobacter capsulatus*: Biochemical and Functional Characterization

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Z. Naturforsch. **45c,** 576–582 (1990); received February 19, 1990

Rhodobacter capsulatus, Cell Wall, Outer Membrane, Porin, Oligomeric Structure

The major outer membrane protein of *Rhodobacter capsulatus* 37b4 (capsule-free) was isolated. Strong porin-activity was observed after reconstitution into artificial lipid bilayer membranes with a single channel conductance of 3.15 nS in 1 M KCl. The porin migrated as a broad, single band (M_r above 90,000) on sodium dodecyl sulfate polyacrylamide gel electrophoresis and dissociated into a single species of polypeptides (M_r 36,000) on treatment with EDTA (10 mM at 30 °C, 20 min) or by heating (100 °C, 5 min). Analytical ultracentrifugation studies demonstrated the native porin to be a trimer. The monomers chromatofocused as a single, sharp peak on fast performance liquid chromatography and only one band, corresponding to an isoelectric point of about 4.0, was obtained on isoelectric focusing. Gas-phase sequencing of the 23 N-terminal residues revealed Glu–Val–Lys–Leu–Ser–Gly–Asp–Ala–Arg–Met–Gly–Val–Met–Tyr–Asn–Gly–Asp–Asp–X–Asn–Phe–Ser–Ser.

Introduction

Porins, a family of channel-forming proteins allowing the uptake of small hydrophilic molecules, are main constituents of the outer membranes of Gram-negative bacteria [1–5]. Most porin monomers studied so far have molecular weights between 30,000 and 50,000. In the case of Enterobacteriaceae, they are trimeric, consisting of identical subunits [6–8].

Among the phototrophic bacteria, polypeptide patterns of outer membranes are known only for a few Rhodospirillaceae species [9, 10]. Porins have been isolated from *Rhodobacter sphaeroides* ATCC 17023 [11] and the closely related *Rhodobacter capsulatus* strain St. Louis [12, 13]. Interestingly, mild conditions such as an EDTA-treatment caused the oligomers of these two porins to dissociate into their constituent polypeptide chains [11, 12]. In another study, native porin of *R. sphaeroides* ATCC 17023 was reported to exist as heterooligomers, consisting of M_r 47,000 subunit and additional polypeptides of smaller mass [9]. The N-terminal Ala was found to be blocked due to bound fatty acids [14, 15]. Recently, the porin of *Paracoccus denitrificans,* closely related to *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* [16] has been isolated [17]. Chemical cross-linking studies suggested a dimeric structure for the homooligomers.

In this study we present data on the biochemical and functional properties of the major outer membrane protein of *R. capsulatus* 37b4, a non-capsulated strain with known lipopolysaccharide and rigid layer compositions [18, 19]. A precursor of this porin was recently found [20], and crystallization of this porin was successfully achieved [21, 22].

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel-electrophoresis; EDTA, ethylene-diaminetetraacetate; Octyl-POE, octyl pentaoxyethylene; PMSF, phenylmethylsulfonyl fluoride.

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0341–0382/90/0600–0576 $ 01.30/0

Materials and Methods

Source and cultivation of bacteria, isolation of porin

Rhodobacter capsulatus 37b4 was taken from the strain collection of the Institut für Biologie, Mikrobiologie, der Universität Freiburg i. Br., F.R.G. Mass cultures were grown photo- or chemoheterotrophically at 32 °C in R8ÄH medium [23] containing 0.3% yeast extract.

Saline treatment (0.9% NaCl at 37 °C for 2 h) of whole cells was performed as described elsewhere [11]. For isolation of porin from cell envelopes freshly harvested cells were disrupted with glass-beads (0.17–0.18 mm in diameter) (cells to glass-beads ratio: 1:2, w/w) in a cooled (4 °C) Vibrogen shaker (type Vi 2, E. Bühler, Tübingen, F.R.G.) for 15 min at full speed. Phenylmethylsulfonyl fluoride (PMSF, 1 mM final concentration) was added to inhibit serine proteases. The cell homogenate was passed through a glass-filter (type 6-1, Schott, Mainz, F.R.G.). Whole cells were separated by centrifugation (2,000 × *g*, 4 °C, 10 min). From the supernatant, cell envelopes were obtained by sedimentation at 63,000 × *g* and 4 °C for 30 min and were washed three times with Tris/HCl buffer (20 mM, pH 8.0). Porin was obtained from the cell envelopes by differential temperature extraction in the presence of sodium dodecyl sulfate (SDS). The cell envelopes were first extracted in buffer containing 2% SDS, 10% glycerol, 20 mM Tris/HCl (pH 8.0) at 50 °C for 30 min. The pellet of the following centrifugation (113,000 × *g*, 20 °C, 1 h) was resuspended in the same buffer including 0.5 M NaCl at 37 °C for 30 min. After centrifugation (113,000 × *g*, 20 °C, 1 h) the supernatant was further purified by gel filtration on Sephadex G-200 as given in detail in reference [11].

Isolation of cell walls

Details of isolation of cell wall fractions from cell homogenates by differential and sucrose gradient centrifugation are given in [10]. Since the cell envelope fractions of the phototrophically grown cells were usually contaminated by thylakoids, they were extracted by Triton X-100 (2%, wt/vol, at 23 °C, 20 min) according to [24].

Isoelectric focusing, chromatofocusing

Experimental conditions of isoelectric focusing were as described by O'Farrell [25]. Chromatofocusing was performed by fast performance liquid chromatography (FPLC) using a Mono P HR 25/5 column (Pharmacia, Uppsala, Sweden).

Measurement of porin activity

Experimental details of the measurement of porin-activity by the black lipid film method are described elsewhere [1, 2].

Fatty acid and amino acid analysis

Phospholipids were determined by chloroform/methanol-extraction 2:1 (v/v) [26] and by assaying total phosphorus [27]. The analyses for fatty acids and sugars were carried out as described earlier [19]. SDS-polyacrylamide gel electrophoreses (PAGE) was performed according to ref. [28], using a 15% (w/v) polyacrylamide slab gel. The mass standards used were: phosphorylase b (94 kDa), bovine serum albumine (68 kDa), ovalbumine (43 kDa), carbonic anhydrase (30 kDa), soy bean trypsin-inhibitor (20 kDa) and α-lactalbumine (14.4 kDa). The amino acid composition of the porin, after hydrolysis in 4 N HCl (100 °C, 18 h), was determined with a Biotronic LC 6001 automatic amino acid analyzer.

Sequence analysis

Automated Edman degradation was performed in a pulsed-liquid protein sequencer 477 A equipped with an on-line microbore PTH-amino acid analyzer 120 A (Applied Biosystems) [29]. All reagents and solvents were from ABI. A TFA-activated glass fiber filter was coated with 3 mg of Bio-Brene Plus prior to precycling. Lyophilized porin (6.5 µg, corresponding to 200 pmol) was dissolved in 30 µl of 0.1 M NaOH or neat TFA, respectively, and applied to the filter disk. Sequencing was carried out using the standard programs, which include sample treatment with 100% TFA at 48 °C in each cycle. The sequence analysis was carried out four times, each time yielding identical results.

Analytical ultracentrifugation, circular dichroism spectroscopy

Sedimentation studies were performed with a Beckmann Model E analytical ultracentrifuge with a photoelectric scanning absorption system [30]. Sedimentation velocity experiments were per-

formed at 21 °C and 52,000 rpm rotor speed using an AN-D rotor, sedimentation equilibrium studies at 20 °C and 12,000 rpm using an AN-G rotor. All sedimentation studies were made with porin in 1% Octyl-POE (slightly polydisperse octyl pentaoxyethylene C_8E_n, $n = 2-9$, with a sharp peak at $n = 5$, its properties are indistinguishable from C_8E_5; Bachem, Bubendorf, Switzerland) in 50 mM NaP_i, 100 mM NaCl, pH 7.0.

Circular dichroism spectra were measured using a Cary-61 spectropolarimeter. Samples of the porin were dissolved in a buffer containing 50 mM NaP_i, 0.1 M NaCl, 0.2 mM EDTA, 0.2 mM dithiothreitol, 1% Octyl-POE (pH 7.5).

Results

Extraction of porin by salt or detergent

Treatment of whole cells of *Rhodobacter capsulatus* 37b4 with saline resulted in the release of a protein-phospholipid-LPS-complex ("NaCl-porin") in a yield of about 120 mg/100 g cell wet weight. The protein, neutral sugar and phosphorus content of the complex was 45%, 16%, and 1.7%, respectively. The fatty acid pattern (total: 5.7% of dry weight) comprised mainly $C_{18:1}$, $C_{18:0}$ and $C_{16:0}$. Lipopolysaccharide constituents (acofriose, rhamnose, glucosamine, and 3-OH-10:0) were found. The polypeptide pattern was dominated by a single major protein that migrated with a mobility corresponding to a mass of about 90 kDa on SDS-PAGE. Between 60 °C and 70 °C it dissociated into its subunits of 36 kDa. Comparable observations were made with the polypeptide pattern of the outer membrane. Since the NaCl-porin fraction contained proteins other than porin, the latter one was also obtained by SDS-extraction of cell envelope fractions [31, 32] and purified by gel filtration on a Sephadex-G 200 column. The protein ("SDS-porin") retained its heat-modifiability during the isolation procedure (Fig. 1). Like the NaCl-porin, the oligomer migrated as a broad, single band corresponding to a M_r of 94,000, and boiling in sample buffer or treatment with 10 mM EDTA at 30 °C (20 min) resulted in the dissociation into subunits of 36 kDa. In some cases, however, a small portion of porin dissociated also at room temperature into monomers during isolation. The porin monomers eluted as a single sharp peak on chromatofocusing on a Mono P HR 25/5 FPLC-column. Only one band, corresponding to an isoelectric point of 4.0, was observed on isoelectric focusing of the monomer.

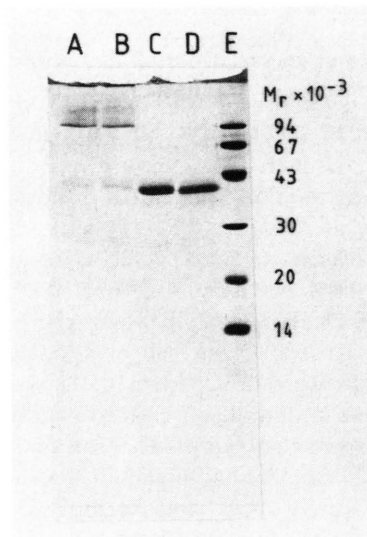

Fig. 1. SDS-PAGE of isolated porin from *Rhodobacter capsulatus* 37b4, obtained from cell envelopes by SDS-extraction (SDS-porin). The porin was solubilized in sample buffer at 30 °C (A), 50 °C (B), or 70 °C (C) for 20 min, each, or 100 °C for 5 min (D). Standard proteins, (E); acrylamide concentration, 15%; protein applied to the gel, 20 µg/sample.

The SDS-porin was free from lipopolysaccharide, as indicated by the absence of detectable amounts of neutral sugars, 2-keto-3-deoxyoctonate, and glucosamine. Similarly, (0.1%) residual phosphate and (2%) fatty acids (mainly $C_{18:1}$ and $C_{16:0}$) were removed below detection limit

Table I. Average single-channel conductance of the porin from *Rhodobacter capsulatus* 37b4 porin in different salt solutions.

Salt	*c* [M]	[nS]
KCl	1.0	3.15
LiCl	1.0	1.3
$CH_3COO^-K^+$	1.0	2.0
$CaCl_2$	0.5	1.6

The solution contained 10 ng/ml porin and less than 0.1 µg/ml SDS or Triton X-100. The pH of the aqueous salt solutions was around 6. The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. $T = 25$ °C; $V_m = 20$ mV. The pore size was determined by recording at least 100 conductance steps and averaging over the distribution of values.

Table II. Amino acid composition (nmol/mg dry weight) of the porin of *Rhodobacter capsulatus* 37b4.

Amino acid[1]	nmol/mg	mol %/mg
Asx	782	14.1
Thr	417	7.5
Ser	403	7.2
Glx	409	7.4
Gly	675	12.1
Ala	656	11.8
Val	468	8.4
Met	57	1.0
Ile	161	2.9
Leu	483	8.7
Tyr	315	5.7
Phe	315	5.7
His	48	0.9
Lys	216	3.9
Arg	152	2.7

[1] Cys, Trp, and Pro not determined.

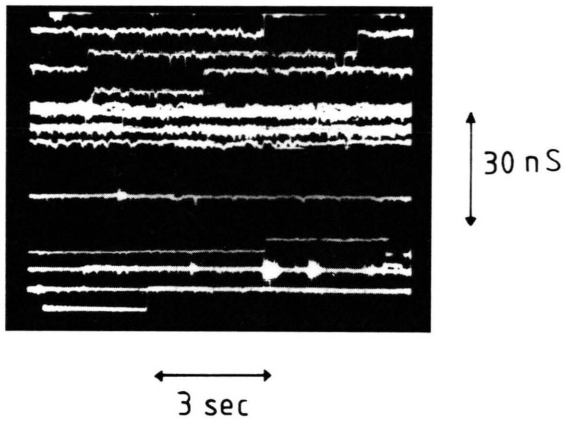

Fig. 2. Stepwise increase of the membrane current after the addition of porin from *Rhodobacter capsulatus* 37b4. The aqueous phase contained 1 M KCl and 10 ng porin/ml. The membrane was formed from diphytanoyl-phosphatidyl choline/*n*-decane. The applied voltage was 20 mV, $T = 25$ °C.

by a following chloroform/methanol extraction. The amino acid composition of the SDS-porin is given in Table II.

Porin-activity

The addition of small amounts (1–10 ng) of SDS- or NaCl-porin to lipid bilayer membranes of small surface (1 mm^2) allowed the resolution of stepwise conductance increases. A single record observed with 10 ng/ml of NaCl-porin using a diphytanoyl phosphatidyl choline membrane in the presence of 1 M KCl is shown in Fig. 2. The values of the single channel conductance of the pores were distributed around a mean, the conductance fluctuations were exclusively directed upwards. The average single channel conductance was 3.15 nS in 1 M KCl. An effective channel diameter of 1.5 nm was estimated for the oligomeric pore from the average value of the single channel conductance in 1 M KCl (according to the equation cited in [2]), assuming that the porin forms large water-filled transmembrane channels. The results obtained with the SDS-porin were comparable to those of the NaCl-porin. The porin was also permeable to a variety of other ions in the single channel experiments (Table I). Zero-current membrane measurements were performed in the presence of salt gradients using ten-fold gradients of KCl. Since the potential was found to be positive on the more diluted side of the membrane, the pore seems to be selective for cations over anions. Heating (100 °C, 5 min) of the porin oligomer in 0.2% SDS, 20 mM Tris-HCl buffer, pH 8.0, as well as EDTA-treatment (10 mM at 30 °C, 20 min) resulted in the loss of the porin activity in lipid bilayer membranes.

Sensitivity to proteases; N-terminal sequence analysis

The porin was resistant to proteases such a trypsin, when embedded in cell envelopes or cell walls (Fig. 3). It was resistant to trypsin even when cell envelopes pretreated with 2% SDS at 50 °C were used. Solubilized SDS-porin, however, was totally degraded into small fragments on treatment with trypsin for 30 min (not shown). Digestion of cell envelopes or cell walls with pronase E resulted in a partial degradation of the porin.

Amino acid sequence analysis revealed as amino terminal sequence: Glu–Val–Lys–Leu–Ser–Gly–Asp–Ala–Arg–Met–Gly–Val–Met–Tyr–Asn–Gly–Asp–Asp–X–Asn–Phe–Ser–Ser. The X in position 19 stands for an unidentified amino acid (cysteine was not detectable with the method used, this means that X could not be defined more precisely). An amide bond between a fatty acid and the N-terminus of a protein would be expected to be stable against sequencing conditions (100% TFA at 48 °C for 5 min in each cycle).

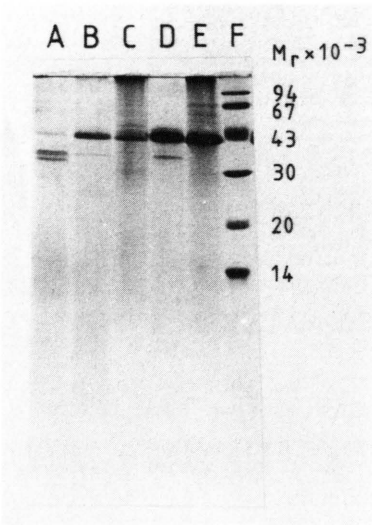

Fig. 3. Effect of trypsin and pronase E on the SDS-PAGE gel pattern of cell walls (Triton-extracted, 2% wt/vol, 23 °C, 20 min) of *Rhodobacter capsulatus* 37b4. Cell walls were treated with pronase E for 24 h (A), or trypsin for 24 h (B), or 2% SDS at 50 °C prior to the trypsin-treatment (D), or 2% SDS at 50 °C. Controls: cell walls, untreated (C) or extracted with 2% SDS at 50 °C (E), both without the following enzyme-treatment. Protein standards, (F); acrylamide concentration, 15%; protein applied to the gel, 20 µg/sample.

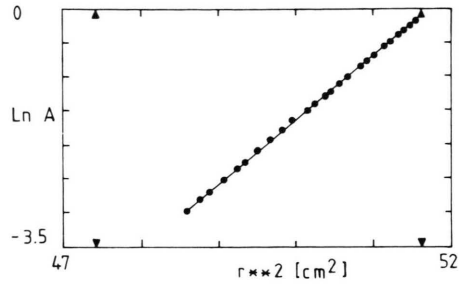

Fig. 4. Determination of the molecular weight of the porin oligomer by analytical ultracentrifugation to equilibrium in presence of 1% Octyl-POE. The molecular weight as derived from the slope shown is 108,000. Absorbance was scanned at 280 nm. Note that the regression line is linear to the bottom of the cell indicated by triangles, as is the meniscus.

Therefore, no evidence for a blocked N-terminus was found.

Secondary structure, oligomeric structure

Circular dichroism spectra between 205 nm and 280 nm were taken for the oligomeric porin in neutral water containing 1% Octyl-POE. The only significant absorption occurred at 219 nm, which is indicative for the presence of β-sheet conformation. The absence of nadir at 207 and 222 nm indicated that no detectable fraction existed in α-helix conformation.

Oligomeric porin from *R. capsulatus* 37b4 solubilized in 1% Octyl-POE was subjected to sedimentation velocity analysis. One major component sedimenting as a sharp boundary with a sedimentation coefficient of 4.4 ± 0.2 S was obtained. The molecular mass of this homogeneous complex was determined by sedimentation equilibrium centrifugation (Fig. 4). The average molecular mass of the main component was calculated from these data as 108,000 Da. For the monomeric porin a respective value of 34,000 to 35,000 Da was obtained. The oligomer of the *R. capsulatus* 37b4 porin, therefore, consists of three subunits. It should be noted that in preliminary experiments, considerable quantities of higher aggregates were seen. They could be correlated to reaggregation of denaturated trimers. Avoiding lyophilization yielded monodisperse preparations.

Discussion

Homogeneity of the porin from *Rhodobacter capsulatus* 37b4 was revealed by several criteria. It migrated as a large protein species on SDS-PAGE of unboiled samples and dissociated into a single band upon heat treatment. Only a single sharp peak was formed on chromatofocusing by FPLC. The respective pH corresponded to that observed on isoelectric focusing with no additional band observed.

The mass of 108 kDa, determined by equilibrium sedimentation centrifugation of the oligomer, demonstrates that the porin consists of three subunits with a M_r of 36 kd. They are identical, as shown not only by their migration on SDS-PAGE but also by the amino acid sequence determined over the N-terminal 23 residues (in part determined also by M. Tadros, unpublished data). Trimeric structure of the *Rhodobacter capsulatus* 37b4 porin was recently confirmed by X-ray analysis [21], where the trigonal space group supports

the notion of trimers. They were similar to those obtained with two-dimensional crystalline sheets of the matrix porin of *Escherichia coli* [33]. Thus, the oligomeric structure of the trimeric porin of *Rhodobacter capsulatus* 37 b 4 is different from that of the closely related *Paracoccus denitrificans,* where the presence of dimers has been claimed as judged from chemical cross-linking studies [17].

In the presence of EDTA, or likely also by lyophilization, the trimers appear to dissociate into the monomers. This could clearly be seen in early analytical ultracentrifugation experiments, when a sizable fraction of the porin existed as large, ill-defined aggregates [J. P. Rosenbusch, unpublished results]. Controlling this situation has led to the predominant structure to occur in trimeric form, as shown by the straight line in the dependence of absorption and the distance from the center of rotation on the ultracentrifugation to equilibrium. The EDTA-sensitivity was exhibited also with the porins from *Rhodobacter sphaeroides, Rhodobacter capsulatus,* strains St. Louis and ATCC 11166 (type strain) [34], and from the closely related *Paracoccus denitrificans* [17]. Divalent cations seem to play a major role in stabilizing the oligomeric structure of these porins. With the porins from *Rhodobacter sphaeroides, Pseudomonas aeruginosa* and *Anabaena variabilis* porin activity has been observed with the monomeric state [11, 35, 36], although in case of *Rhodobacter sphaeroides* the possibility of reaggregation of porin monomers to form oligomers on insertion into the artificial lipid bilayers was not excluded. In the lipid bilayer experiments, the monomers obtained by the EDTA-treatment from the oligomers of *Rhodobacter capsulatus* 37 b 4 showed no significant porin-activity. This might indicate a stable oligomeric structure to be necessary for porin-function. On X-ray analysis, however, a channel is actually seen to span the monomer [22], and a recent study on PhoE porin from *Escherichia coli* came also to this conclusion [37].

The oligomeric porin from *Rhodobacter capsulatus* 37 b 4 has properties known from the *Escherichia coli* porin, such as a major portion of β-sheet structure with no evidence for α-helical regions, protease resistance (even after the Triton-extraction) as long as it remains embedded in the membrane, relative high hydrophilicity as judged from amino acid composition, and weak selectivity for cations. The effective radius of the oligomeric pore, determined in lipid bilayer experiments (0.75 nm) is comparable to that found in the liposome swelling assay with *Rhodobacter capsulatus* St. Louis (0.8 nm) [13] and in bilayer experiments with the type strain of *Rhodobacter capsulatus* (0.74 nm) [34]. We found no evidence for a blocked N-terminus in the *Rhodobacter capsulatus* 37 b 4 porin, as it has been claimed for the porin of *Rhodobacter sphaeroides* [14, 15]. The purified, chloroform-methanol extracted porin contained only traces of 18 : 0 and 18 : 1 (probably originating from cell wall lipoprotein), and free Glu was found as the N-terminal amino acid (see also ref. [38]). The N-terminal sequence (the total sequence is in progress) was identical with that obtained with the porin from the type strain (ATCC 11166) of *Rhodobacter capsulatus* [34], although this porin migrated with M_r 43 kd on SDS-PAGE.

Acknowledgements

The authors thank G. Ellet-Lögler for the preparation of the antisera and U. J. Jürgens for amino acid analyses. The stimulating discussions with R. Dierstein are gratefully acknowledged. The work was supported by the Deutsche Forschungsgemeinschaft and by the Swiss National Science Foundation.

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