JOURNAL OF BACTERIOLOGY, July 1984, p. 199–205
0021-9193/84/070199-07$02.00/0

Vol. 159, No. 1

Porin from *Rhodopseudomonas sphaeroides*

JÜRGEN WECKESSER,†* LEORA S. ZALMAN,‡ AND HIROSHI NIKAIDO

Department of Microbiology and Immunology, University of California, Berkeley, California 94720

Received 5 March 1984/Accepted 25 April 1984

A protein homooligomer was purified from both the cell envelope fractions and the saline extracts of *Rhodopseudomonas sphaeroides* cells. This oligomer exhibited strong porin activity when reconstituted into proteoliposomes with egg phosphatidylcholine. In the saline extracts of both chemotrophically and phototrophically grown cells, the porin oligomer was the most predominant polypeptide, which produced pores whose behavior toward various sugars could be approximated by hollow cylinders of 0.62 nm in radius. The oligomer was dissociated, in the presence of EDTA, into monomers that migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as though their molecular weight was about 47,000. The monomer was active in the reconstitution assay and produced pores with sizes comparable to those produced by the oligomer. Circular dichroism spectra indicated the predominance of β-sheet structure in both the oligomeric and EDTA-dissociated monomeric forms. Drastic conditions, for example, precipitation with 10% trichloroacetic acid or heating for a few hours at 100°C in sodium dodecyl sulfate, were necessary to denature the protein into a form with a reduced content of β-sheet structure.

Small hydrophilic molecules, such as most of the nutrients, penetrate the cell wall of gram-negative bacteria through pores in the outer membrane (15). The pores are formed by the porin proteins with characteristic properties. Many of them form homooligomeric complexes in the outer membrane, and stable trimers composed of identical subunits were isolated from *Escherichia coli* in the presence of sodium dodecyl sulfate (SDS) (14). Each of the subunits of *E. coli* porin seems to have a water-filled pore of ca. 0.6 nm in radius (16), but the subunits become denatured and lose their channel-forming activity upon dissociation of the trimeric structure (14). In *Pseudomonas aeruginosa*, the pore size is larger (about 1 nm in radius) (8, 9), and monomeric porin molecules that were active in reconstitution assays could be isolated in the presence of SDS (22).

Porins of phototrophic bacteria have not yet been identified, and polypeptide patterns of the outer membrane are known only for a few species (3, 6a, 10). The outer membrane of *Rhodopseudomonas sphaeroides* 2.4.1 was reported to contain, as the major protein complexes, three heterooligomers, each containing a shared 47-kilodalton (kdal) protein and one of the additional polypeptides of 29, 26.5, and 21.5 kdal, respectively, on the basis of the two-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) in which the protein oligomers were dissociated by heating at 75°C before the electrophoresis in the second dimension (3, 6). Deal and Kaplan (4) suggested that these heterooligomeric structures perform the porin functions in *R. sphaeroides*.

In the present work, we isolated a homooligomeric protein complex that travels at a position corresponding to 68 kdal on SDS-PAGE from *R. sphaeroides* 2.4.1 and showed that this oligomer dissociated into subunits of apparently 47 kdal upon EDTA treatment or heating. In contrast to the conclusions reached by another laboratory (3, 4, 6), we found that the major portion of the 47-kdal polypeptide existed as a homooligomer both in the saline extract of the whole cells and in the cell envelope fraction. The oligomer, as well as the monomer prepared by EDTA treatment, had a strong pore-forming activity in a reconstitution assay.

MATERIALS AND METHODS

Source and cultivation of bacteria. *R. sphaeroides* 2.4.1 (ATCC 17023) was obtained from the American Type Culture Collection. Mass cultures were grown in R8AH medium (19) with 0.3% yeast extract aerobically in the dark at 37°C; one liter of medium was put into a 6-liter Erlenmeyer flask, and the culture was aerated by shaking at 200 rpm on a New Brunswick model G52 platform shaker. Cultures were also grown phototrophically in the same medium but anaerobically in the light (ca. 2,000 lx; 37°C).

Another strain of *R. sphaeroides* (here called strain UC), from the culture collection of this department and originally obtained from C. B. van Niel, was used in the preliminary experiment. This strain was grown aerobically in the dark in a medium containing 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, and 10 g of Difco yeast extract per liter, adjusted to pH 7.0, at 30°C.

Isolation of cell envelope fraction and the outer membrane. Freshly harvested late-exponential-phase cells (3.3 g [wet weight] from 1-liter culture) were disrupted by French pressure cell treatment, and the outer membrane was isolated as described by Hancock and Nikaido (9), except that lysozyme was not added. The preparation before the step sucrose gradient centrifugation was used as the cell envelope. Most of the outer membrane was recovered at the interphase between 58 and 64% (wt/vol) sucrose, and this fraction was used in the purification of the porin.

Preparation of saline extracts. A fully grown chemotrophic culture of *R. sphaeroides* was diluted 10-fold with fresh medium, and the shaking at 37°C was continued for 24 h. Since the doubling time of this strain under these conditions was about 2 h, this produced a culture well into the stationary phase. The cells freshly harvested from such a culture (10 g [wet weight]) were suspended in 50 ml of 0.9% NaCl containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) (added to prevent the possible autolytic

* Corresponding author.
† Present address: Institut für Biologie II, Lehrstuhl für Mikrobiologie der Universität, D-7800 Freiburg i. Br., Federal Republic of Germany.
‡ Present address: Department of Microbiology, University of California, Los Angeles, CA 90024.

degradation of proteins), and the suspension was incubated at 37°C with shaking for 2 h (20). The cells were then removed by centrifuging at 31,000 × *g* for 1 h, and the supernatant was dialyzed against distilled water at 4°C for 2 days with frequent changes of water and was finally lyophilized.

Purification of porin by gel filtration. The cell envelope, the outer membrane, or the saline extract was solubilized in a solution containing 0.3% lithium dodecyl sulfate (LDS; Sigma Chemical Co.), 0.3 M LiCl, 5 mM Tris-chloride buffer (pH 7.2), and 0.3 mM NaN_3. In some cases the solution also contained 5 mM Na_2 EDTA. The sample was applied to a column (1.5 by 90 cm) of Sephacryl S-200 (Superfine; Pharmacia Fine Chemicals), equilibrated with the same solution containing LDS and LiCl (and in some cases also EDTA), and the column was eluted with the same salt solution. The gel filtration step was carried out either at room temperature or at 4°C.

Liposome swelling assay. The pore-forming activity of various fractions was determined by preparing proteoliposomes by adding acetone-extracted egg phosphatidylcholine (2.4 μmol) and dicetylphosphate (0.1 μmol), as described previously (12, 16). The dried phospholipid-protein mixture was resuspended in 0.6 ml of 15% (wt/vol) Dextran T-40 (Pharmacia Fine Chemicals) in 5 mM Tris-chloride buffer (pH 7.4). The proteoliposomes (0.02 ml) were then diluted into 0.6 ml of isotonic solutions of various sugars, and permeability was determined from the initial rates of change of optical density of liposomes at 500 nm. The isotonic concentration for sugars was determined by diluting liposomes into stachyose solutions of different concentrations. The results were normalized to the rate of swelling of proteoliposomes in L-arabinose.

Circular dichroism spectra. A Jasco J-500 C spectropolarimeter was used, and data from at least eight consecutive measurements were averaged by the Jasco data processor.

Analytical methods. Phospholipids were determined by assaying total phosphorus (2). The Lowry method (11) was routinely used for protein determination with bovine serum albumin as the standard. The concentration of samples used for the circular dichroism experiment, however, was calculated from the optical density at 280 nm and the reported amino acid composition of the 47-kdal protein (5). The analysis for sugars and fatty acids was carried out as described earlier (19). SDS-PAGE was performed as described by Lugtenberg et al. (13). The samples were solubilized at room temperature in the sample buffer containing 62.5 mM Tris-chloride (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. In many cases the solution was applied to the gel without further heating, and in others the sample was heated in a boiling-water bath before application. The mass standards used were: phosphorylase *b* (94 kdal), bovine serum albumin (68 kdal), ovalbumin (43 kdal), carbonic anhydrase (30 kdal), soy bean trypsin inhibitor (21 kdal), and lysozyme (14.3 kdal).

RESULTS

Preliminary studies. In the initial series of experiments (L. S. Zalman, Ph.D. thesis, University of California, Berkeley, 1982), porin of *R. sphaeroides* UC was obtained from cell envelope fractions. When chemotrophically grown cultures attained the Klett reading of 100 (red filter), the cells were harvested by centrifugation and washed, and the cell envelope fraction was isolated as described above. This fraction contained major protein(s) which migrated on SDS-PAGE with an apparent molecular weight of 47,000 when the sample was heated at 100°C in the sample buffer (see above) before application. However, when the sample was solubilized at room temperature, the major band at the apparent molecular weight of 47,000 was replaced with a heavy band at an apparent molecular weight of about 68,000 (data not shown).

The envelope fraction was first extracted with 1% SDS containing 10 mM Tris-chloride (pH 7.4) for 1 h at 37°C, and the insoluble pellet was further extracted with 1% SDS containing 1 M NaCl, 10 mM EDTA, and 10 mM Tris-chloride buffer (pH 7.4), again for 1 h at 37°C. This second extraction produced a supernatant enriched very strongly in the 47-kdal major protein, which could be further purified by gel filtration as described above, using an elution buffer containing 10 mM EDTA. This produced a fraction which was active in the liposome reconstitution assay (see above) and contained only a few, minor contaminating proteins. These results identified the 47-kdal major protein as the porin, or at least one of the porins, of *R. sphaeroides*. However, the quaternary structure of the purified protein was different from that in the envelope fraction because the purified protein did not migrate as a 68-kdal complex on SDS-PAGE even when the sample was not heated in the sample buffer. Because of this observation, we tried to find a milder method of purification that preserved the quaternary structure of the protein.

Purification of porin from the saline extract. One of us has previously found that extraction of the whole cells of *Rhodopseudomonas capsulata* with 0.9% NaCl for 2 h at 37°C resulted in the release of a lipopolysaccharide-phospholipid-protein complex (20) that was greatly enriched in porin (7). Application of this method to *R. sphaeroides* 2.4.1 resulted in the successful isolation of a comparable complex in a yield of about 250 mg/100 g (wet weight) cells. The protein content of the complex was between 15 and 25% in different preparations, the neutral sugar content (assayed as glucose) was about 15%, and that of the phosphorus was 1.2% of the dry weight. The fatty acid content was about 10%, and the predominant species were those that are typically present in phospholipids of these organisms, i.e., $C_{18:1}$, C_{18}, and C_{16}. Fatty acids that are typical of the lipopolysaccharide (17a), such as $C_{10}OH$, $C_{14}OH$, and 3-oxo-C_{14}, did not each exceed 0.6% of the dry weight. When the material was analyzed by SDS-PAGE after solubilization at room temperature in the sample buffer, the polypeptide pattern was seen to be dominated by a band of an apparent molecular weight of 68,000, which appeared to dissociate into monomers of apparent molecular weight 47,000 upon treatment at 100°C in the sample buffer (Fig. 1). When the material solubilized at room temperature was purified by gel filtration on Sephacryl S-200 in the absence of EDTA, the proteins appeared to elute as a broad peak (Fig. 2A), although some nonprotein material absorbing at 280 nm was also eluted, especially in later fractions. The SDS-PAGE analysis of the eluates showed that the protein still behaved like a 68-kdal protein, i.e., it still retained its oligomeric structure (Fig. 2B).

Porin activity of the oligomeric protein. When the saline extract containing 0.1 μg of protein was reconstituted with 2.4 μmol of phosphatidylcholine and 0.1 μmol of dicetylphosphate (see above), the liposomes gave the initial swelling rate of 0.28 optical density (at 500 nm) (OD_{500}) unit per min in an isotonic solution of L-arabinose. Rates as high as 0.5 OD_{500} unit per min were obtained when 0.1 μg of the *R. sphaeroides* porin purified by gel filtration (see above) was used. Since purified *E. coli* OmpF porin (0.1 μg) yielded

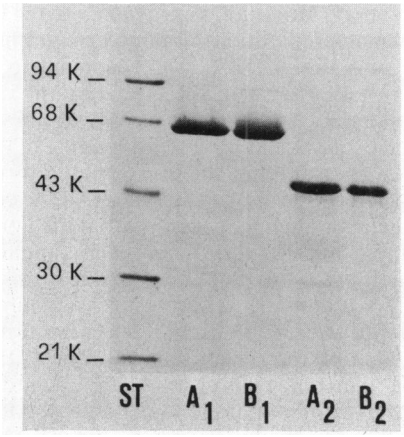

FIG. 1. Protein patterns of saline extracts from *R. sphaeroides* 2.4.1. The cells were grown either chemotrophically (A_1 and A_2) or phototrophically (B_1 and B_2) and were extracted with 0.9% NaCl as described in the text. Samples, each containing about 10 μg of protein, were analyzed by SDS-PAGE, after solubilization in the sample buffer either at room temperature (A_1 and B_1) or at 100°C for 15 min (A_2 and B_2). Proteins used as molecular weight standards are given in the text. K, Kilodaltons.

a rate of 0.15 to 0.2 OD_{500} unit per min under the same conditions, it appears that *R. sphaeroides* porin produces somewhat higher permeability than does the *E. coli* OmpF porin of the same weight.

The size of the channel produced by the *R. sphaeroides* 2.4.1 porin was estimated by measuring the effect of the solute size on permeability, using sugars ranging from 150 to 504 in molecular weight (Fig. 3). When these results were fitted with the equation proposed by Renkin (17), a value of about 0.62 nm in radius was shown to produce the best fit. The same value was obtained regardless of whether the porin used was the crude saline extract or a preparation purified by gel filtration on Sephacryl S-200.

Formation of monomers and their activity. When the saline extracts containing the oligomeric porin were fractionated on a Sephacryl S-200 column in a solution containing 0.3% LDS, 0.3 M LiCl, 5 mM Tris-chloride buffer (pH 7.2), and 3 mM NaN_3, as above but with the addition of 5 mM EDTA, the eluted porin migrated completely as 47-kdal monomers when analyzed by SDS-PAGE without heating of the sample (Fig. 2). These results suggest that the oligomer has dissociated into monomeric units either during or before the gel filtration step. The later position of elution of the protein from the gel filtration column in the presence of EDTA (Fig. 2A) also supported this notion. We therefore assume that the porin molecules travelling as 47-kdal proteins on SDS-PAGE when applied without heating are indeed fully dissociated monomers, although we have not excluded rigorously the possibility that the protein existed as weakly associated oligomers when it emerged from the Sephacryl column. The dissociation into monomers (in this provisional sense) occurred even without the gel filtration step, when high concentrations of EDTA (such as 25 mM) were used for the solubilization of the sample (Fig. 2B, lane C). These results explain the initial difficulty (see above) in obtaining the *R. sphaeroides* UC porin in its oligomeric form, since both the extraction of porin and the purification by gel filtration were done in the presence of EDTA.

Monomer fractions, obtained by the gel filtration procedure described above, contained less than 1% (by weight) phospholipid fatty acids and only traces of $C_{10}OH$ as the only lipopolysaccharide-specific fatty acid detected: this indicates a very low degree of contamination by phospholipids and lipopolysaccharides. However, we did not analyze solvent-extracted samples, and thus we could not test for the presence of covalently bound fatty acid molecule, recently reported to be present on this protein (5). When the mono-

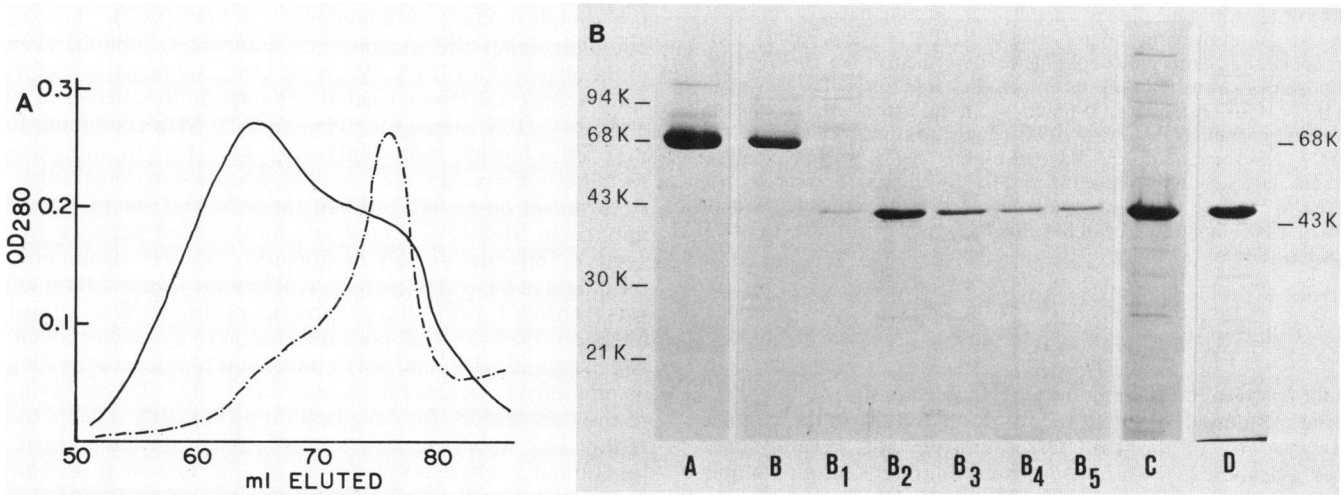

FIG. 2. (A) Gel filtration of saline extacts of *R. sphaeroides* 2.4.1. Saline extract (about 25 mg [dry weight]; containing about 5 mg of protein) was fractionated at room temperature on a column of Sephacryl S-200 as described in the text. The elution buffer contained no EDTA (———) or 5 mM Na_2 EDTA (– · – ·), and 1.0-ml fractions were collected. In both cases the peaks containing predominantly proteins were followed by large UV-absorbing peaks that contained little protein, eluted mainly at 110 to 140 ml. Blue dextran 2000 was eluted at about 55 ml. (B) SDS-PAGE patterns of column fractions. Lanes: A, fraction 65 from the run without EDTA in A; B, saline extract before application to the column; B_1 through B_5, fractions 66, 74, 80, 86, and 92 from the run with EDTA in A; C, saline extract dissolved in the column buffer containing 25 mM EDTA; D, pooled fractions from the run with EDTA, used in the proteoliposome swelling experiments of Fig. 3 and 4. All samples were applied to PAGE after solubilization at room temperature, and the gel was stained with Coomassie brilliant blue.

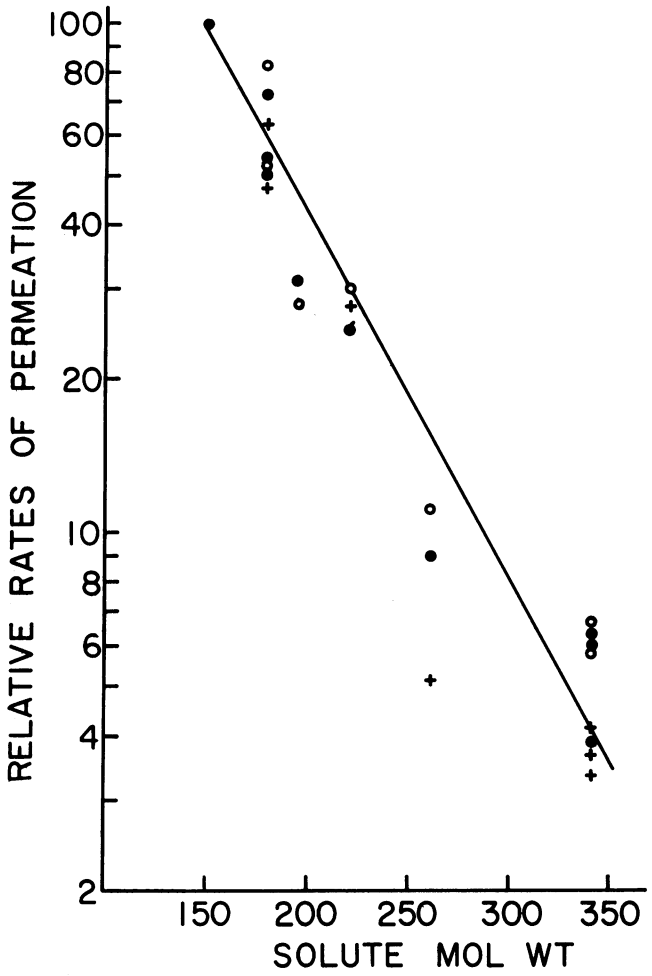

FIG. 3. Swelling rates of proteoliposomes containing *R. sphaeroides* 2.4.1 porin in isotonic solutions of various sugars. Saline extracts (0.1 to 0.2 μg of protein) from chemotrophically grown cells (●) or phototrophically grown cells (○), or monomeric porin isolated by gel filtration in the presence of EDTA (+) (see Fig. 2), were reconstituted with phospholipids as described in the text. Proteoliposomes were diluted into isotonic solutions of L-arabinose (M_r = 150), D-glucose, D-mannose, D-galactose (M_r = 180), α-methyl-D-mannoside (M_r = 194), *N*-acetyl-D-glucosamine (M_r = 221), 2,3-diacetamido-2,3-dideoxy-D-glucose (M_r = 262), and sucrose, lactose, and maltose (M_r = 342). Swelling rates were normalized to the rate with L-arabinose. Note that the monomer fraction used was completely free of the oligomeric porin, as shown in lane D of Fig. 2B.

mer fraction, containing only negligible amounts of oligomer (Fig. 2B, lane D), was tested for porin activity in the swelling assay, rates of about 0.1 OD_{500} unit per min were obtained for L-arabinose with liposomes containing 0.1 μg of monomer per 2.4 μmol of phosphatidylcholine–0.1 μmol of dicetylphosphate. Thus the activity of the monomeric porin was at least in the same order of magnitude as that of the oligomer. The liposomes reconstituted from the monomers exhibited a pore size similar to that observed in liposomes reconstituted from the oligomers, i.e., 0.62-nm radius (Fig. 3).

The question of the possible reaggregation of the monomers into oligomeric structure was investigated in two ways. When a monomer preparation, obtained by gel filtration in the presence of 5 mM EDTA, was reapplied to the same column but without EDTA in the equilibration and eluting buffers, the material eluted produced only the 47-kdal band when analyzed by SDS-PAGE without heating of the sample (data not shown). It seems therefore that reformation of oligomeric structure did not occur under these conditions. However, reaggregation might occur within the lipid bilayers of the liposomes, and this could explain the apparent channel-forming activity of the monomers. To test this possibility, liposomes were made with various amounts of the monomeric porin. If the formation of pores occurred only in oligomers created by the reaggregation process, the permeability of the liposomes may show an upward concave dependence on the amount of monomer added. Although we started with an amount as low as 0.018 μg per reconstitution mixture (Fig. 4), a linear dependence was obtained, a result consistent with the notion that monomers may form channels. However, we stress that this is not a decisive piece of evidence because the reaggregation could take place with a rapid enough kinetics so that all porins become oligomeric by the time of the swelling assay.

Secondary structure in the porin preparations. Porin oligomer and monomer preparations in 0.25% SDS containing 10 mM phosphate buffer (pH 7.0), purified by gel filtration through Sephacryl S-200, were studied by measuring the circular dichroism spectra between 200 and 240 nm. Only one negative peak at 219 nm was seen with both preparations (Fig. 5), a result indicating the presence of large amounts of β-sheet structure (1). As with *E. coli* porin, there was no indication of the presence of significant amounts of alpha helices, i.e., there was no negative peak or shoulder at 207 or 222 nm (1). The spectra of monomer and oligomer were very similar, suggesting that monomers retain essentially unaltered overall confirmation upon dissociation.

Thermal denaturation of the porin. We studied the effect of heating, both on the physical structure and on the biological activity of the porin. As indicated by our initial observation (see above) that the porin migrated, after heating at 100°C in the sample buffer, as monomers on SDS-PAGE, heating could dissociate the oligomer into monomeric subunits even in the absence of EDTA. When the crude saline extract of *R. sphaeroides* 2.4.1 was treated at temperatures between 20 and 100°C (10°C steps) for 30 min in 0.2% SDS containing 10 mM Tris-chloride buffer (pH 7.0), 50% loss of the pore-forming activity, assayed by the liposome reconstitution, occurred at 80 to 90°C. When these heated samples were applied to SDS-PAGE analysis, most of the material was seen to migrate as the monomeric subunits again after treatment at 80 to 90°C or higher. When the same experiment was repeated with the oligomeric porin purified by gel filtration, 50% loss of pore-forming activity occurred between 60 and 70°C, and 50% conversion to a form migrating as monomers occurred between 70 and 80°C. This lowering of the temperature for denaturation presumably reflects the removal of stabilizing components. When the monomeric, functionally active porin was used, the loss of functional activity occurred again at 60 to 70°C; in this case the protein migrated on SDS-PAGE always as a 47-kdal polypeptide both before and after heating.

Study of the heated samples with the circular dichroism technique, however, revealed no gross changes in the secondary structure of the protein after it had been subjected to conditions leading to its complete inactivation. In fact, even heating for 30 min at 100°C in 0.25% SDS containing 10 mM

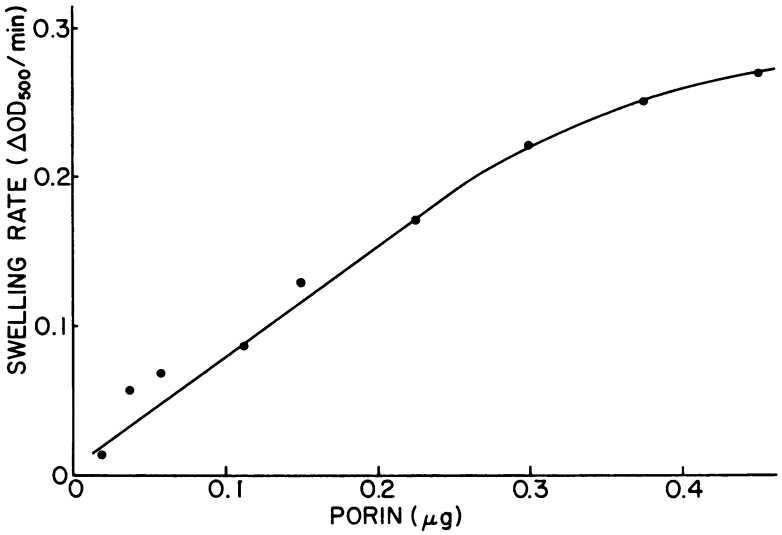

FIG. 4. Swelling rates of proteoliposomes reconstituted with various amounts of monomeric porin. The monomeric porin purified by gel filtration in the presence of EDTA (Fig. 2) and shown to be free of oligomers (Fig. 2b, lane D) was reconstituted with a phosphatidylcholine-dicetylphosphate mixture as described in the text. The permeability of the porin channel was measured by the swelling rate upon dilution into isotonic L-arabinose solution.

phosphate buffer (pH 7.0) did not visibly alter the circular dichroism spectrum. Thus the overall structure of this protein is unusually stable, and gross denaturation of the protein, as indicated by the decrease the in β-sheet structure and increase in the negative ellipticity near 200 nm, indicative of the formation of random coils, did not occur until the porin was subjected to very harsh treatments, such as partition in a hot phenol-water mixture (21), treatment with a chloroform-methanol (1:1, vol/vol) mixture at room temperature, precipitation with trichloroacetic acid followed by heating, or heating for 150 min in 0.25% SDS containing 10 mM phosphate buffer (pH 7.0) and 10% (wt/vol) 2-mercaptoethanol (one representative example is shown in Fig. 5). This gross alteration of the secondary structure apparently caused a slight decrease in the rate of migration of the protein on SDS-PAGE (data not shown).

Porin isolated from cell envelope fraction. Most of the studies described above were carried out by using preparations derived from the saline extract fraction. It was therefore important to establish that the functional and structural properties of the porin observed were not influenced by the process of release of the saline extract fraction from the outer membrane. For this purpose, we examined the pore-forming activity of the cell envelope fractions and isolated, from the outer membrane, a fraction strongly enriched in porin.

When fragments of the cell envelope fraction (0.4 μg of protein) were reconstituted with 2.4 μmol of phosphatidylcholine and 0.1 μmol of dicetylphosphate, the rate of swelling in L-arabinose solution was 0.13 OD_{500} unit per min; this was in the expected range, when we consider that porin represented only a portion of the total protein in this preparation. The pore size, determined from the solute size dependence of permeability, was similar to that estimated by using porins derived from saline extracts (data not shown).

Fractionation of the cells disrupted with the French press treatment by sucrose density gradient centrifugation resulted in the isolation of an outer membrane fraction (see above).

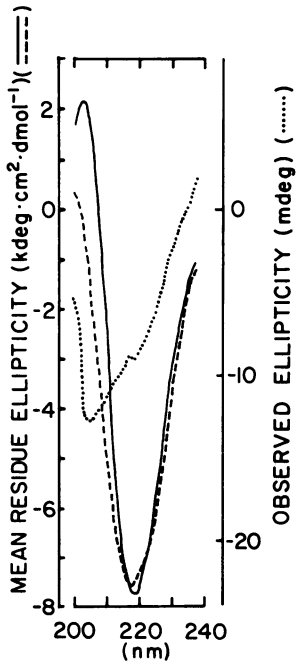

FIG. 5. Circular dichroism spectra of *R. sphaeroides* 2.4.1 porin. Spectra were measured in 10 mM sodium phosphate buffer (pH 7.0) containing 0.25% SDS. Symbols: ———, oligomeric porin purified by gel filtration in the absence of EDTA; – – –, monomeric porin obtained by gel filtration in the presence of EDTA; · · ·, porin precipitated by 10% trichloroacetic acid and dissolved by heating at 100°C in 10 mM phosphate buffer (pH 7.0) containing 0.25% SDS and 10% 2-mercaptoethanol for 30 min, followed by extensive dialysis against the same buffer without 2-mercaptoethanol. (Various other drastic treatments mentioned in the text also produced spectra similar to this. Because of the uncertainty in the recovery of the last sample, its spectrum is shown as the observed ellipticity. However, if the recovery was complete, the mean residue ellipticity at 205 nm was about −10,000 degrees (deg) per cm^2 dmol^{-1}.

SDS-PAGE analysis of this preparation showed only one major protein band at the position of 68 kdal when the extraction was carried out at room temperature (Fig. 6, lane A_1), without any signs of the presence of 72- and 75-kdal bands reported by another laboratory (3). When the solubilization was done at 100°C, the band disappeared to give rise to a single heavy band at 47 kdal (Fig. 6, lane A_2). The relative lightness of the 68-kdal band in lane A_1 is a phenomenon seen very often with porins and is due to the failure of oligomeric porins to become solubilized at room temperature, probably due to their interaction with the underlying peptidoglycan layer (15).

The outer membrane fraction was extracted with 0.3 M LiCl containing 3% LDS at room temperature for 5 min. The extract, containing the porin in the oligomeric form as judged by SDS-PAGE, was fractionated on a column of Sephacryl S-200, equilibrated, and eluted with 0.3 M LiCl containing 0.2% LDS and 5 mM Tris-chloride buffer (pH 7.2) at 4°C. The porin was eluted as a single peak and migrated as an oligomer on SDS-PAGE (Fig. 6, lane B_1). There was no indication of the heterogeneity of this oligomeric protein complex, in contrast to the reports from another laboratory (3, 4, 6) and dissociation of this complex by heating did not produce proteins of 20 to 30 kdal (Fig. 6, lane B_2).

Comparison between chemo- and phototrophically grown cells. The porin described so far was obtained from chemotrophically grown cells. When phototrophically grown cells were incubated in 0.9% NaCl, a similar extract, containing predominantly the 68-kdal porin oligomer, was obtained. The porin dissociated into the 47-kdal subunits upon heating at 100°C. There was also no discernible difference in the absolute magnitude of permeabiliy of the liposomes or in the apparent pore size (Fig. 3) between the saline extracts obtained from phototrophically and chemotrophically grown cells.

DISCUSSION

In this study we showed that the major protein component of the saline extract of *R. sphaeroides* had a strong porin activity. This protein is by far the major protein in the outer membrane of this organism. Since the pore-forming activity of the cell envelope in reconstitution assays can be explained more or less quantitatively by the level of the 47-kdal protein present, this protein can be regarded as the major porin of this organism. Interestingly, the protein shares many properties characteristic of porins from other bacteria (15). Thus it exists in an oligomeric form even in the presence of SDS but can be dissociated into subunits upon heating in SDS; it also seems to be associated with the underlying peptidoglycan layer through a noncovalent interaction sensitive to ionic strength, as its efficient extraction with SDS requires the presence of high concentrations of NaCl or LiCl.

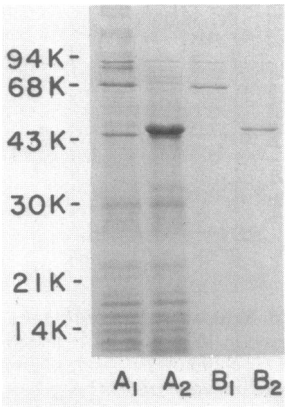

FIG. 6. Porin purified from the outer membrane of *R. sphaeroides* 2.4.1. The outer membrane was isolated, and the porin fraction was purified by gel filtration at 4°C in the absence of EDTA, as described in the text. For SDS-PAGE analysis, 15 μg of the outer membrane fraction (lanes A_1 and A_2) and 2 μg of the peak fraction from the column (lanes B_1 and B_2) were applied, and the gel was stained with Coomassie brilliant blue. Solubilization in sample buffer was done at room temperature (lanes A_1 and B_1) or at 100°C for 10 min (lanes A_2 and B_2).

As found with *R. capsulata* (7), we found that saline extraction of whole cells yielded preparations greatly enriched for porin, relative to other cell envelope proteins (cf. Fig. 1 and 6). Such preparations presumably exist in the form of small lipid bilayer vesicles and contain large amounts of both lipopolysaccharide and phospholipids. Much of these latter components can be removed by gel filtration in LDS or SDS, and the simplicity of the method and the relative purity of the final product make this an attractive method for preparation of porin from this organism. It should be emphasized, however, that the method is not applicable to all gram-negative species. For example, we found little release of porins from *E. coli* and *P. aeruginosa* cells with this technique (J. Weckesser, unpublished data).

The key observation that enabled us to isolate the porin in an oligomeric form was the sensitivity of the oligomeric structure to EDTA. Thus unlike the porin trimers from *E. coli* (14) and *Salmonella typhimurium* (18), the *R. sphaeroides* porin oligomer became dissociated into subunits upon treatment with EDTA. Presumably divalent cations are playing important roles in holding together the oligomeric structure. In fact, even the *E. coli* and *S. typhimurium* porin oligomers may be stabilized by a similar mechanism, as electrodialysis is reported to dissociate such oligomers into subunits (14, 18). Although the effect of alteration of pH during such a treatment cannot be neglected, it is tempting to assume that these oligomers are held together by di- or multivalent cations that are removed by electrodialysis but not with EDTA.

The oligomeric *R. sphaeroides* porin isolated from the saline extract migrated as a single band with an apparent molecular weight of 68,000. Upon heating or EDTA treatment, this complex dissociated into a population of single protein subunits with the apparent molecular weight of 47,000, and there was no evidence for the presence of subunits of lower molecular weight (Fig. 1). These observations form a contrast to earlier reports from another laboratory, which claim that the major 47-kdal protein of the outer membrane of the same strain existed as 1:1 heterodimeric complexes with 21.5-, 26.5-, and 29-kdal subunits (3, 4, 6). Scanning of the Coomassie brilliant blue-stained gels of the heated samples of our saline extract (such as those shown in Fig. 1, lanes B) revealed that proteins in the 21.5- to 29-kdal range existed in amounts of less than 3% of the amount of the 47-kdal protein, and we could not find any evidence for the presence of heterooligomers. Nor is it likely that rearrangement of the 47-kdal polypeptide from the heterooligomeric into the homooligomeric form took place during saline extraction, because oligomers prepared by direct extraction of the outer membrane also appeared to be homooligomers (Fig. 6). Although occasionally we did see multiple bands in the 70- to 90-kdal region in the SDS-PAGE analysis of both crude outer membranes and saline extracts (for example, see

Fig. 2B, lane A), we believe that they can be more easily explained by the presence of contaminating proteins, association of the complex with other components such as lipopolysaccharides, partial dissociation of the complex, or the formation of higher aggregates of porin oligomers, rather than by invoking the presence of heterooligomers. Thus we believe that at least most of the *R. sphaeroides* porin exists in a homooligomeric form, similar to the form in which porins of most other bacterial species so far studied are known to exist (15).

Although the oligomeric and monomeric porins of *R. sphaeroides* migrated on SDS-PAGE at positions expected for 68- and 47-kdal proteins, it is not possible to decide, from these data, the number of subunits within an oligomer. The stability of the oligomer in the presence of SDS obviously suggests that the oligomer has not been fully denatured by SDS, and thus its mobility does not necessarily reflect its molecular weight. Deal and Kaplan (5) also reported that the monomers show atypical behavior on SDS-PAGE.

The oligomeric porin of *R. sphaeroides* was at least as active as the *E. coli* porin in increasing the permeability of phospholipid bilayer to small solutes such as L-arabinose. The pore size appeared to be slightly larger than that of the *E. coli* OmpF porin (0.62 versus 0.58 nm in radius). The porin from *R. sphaeroides* UC was found to have a pore size close to 0.70 nm in radius (Zalman, Ph.D. Thesis).

An interesting feature of the *R. sphaeroides* porin was that the protein retained its pore-forming activity after dissociation into the monomeric form (Fig. 3 and 4). The overall secondary structure of the protein was also kept essentially unchanged upon dissociation, as judged from the circular dichroism spectra (Fig. 5). This rather high stability of the monomeric unit in SDS and the ability of the monomers to form channels upon insertion into liposomal bilayers are reminiscent of the properties of *P. aeruginosa* porin, studied in this laboratory (22). *P. aeruginosa* porin, however, was dissociated into monomeric units in SDS even without the addition of EDTA, and the *R. sphaeroides* porin oligomer seems to exhibit a degree of stability in between the easily dissociated *P. aeruginosa* porin oligomer and the stable porin oligomers of *E. coli* and *S. typhimurium* that cannot be dissociated even with EDTA. Finally, we emphasize that our data do not conclusively prove that the porins of *P. aeruginosa* and *R. sphaeroides* produce pores in their monomeric states. It is possible that they aggregate rapidly to form oligomers once within the lipid bilayer and that this process is essentially complete by the time that the swelling assay is performed. Further studies, such as cross-linking studies with liposomes reconstituted from monomeric porins, will be needed to establish this point.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI-09644 from the National Institute of Allergy and Infectious Diseases. J. Weckesser held a fellowship from the Deutsche Forschungsgemeinschaft.

We thank Gustin Ho and Emiko Y. Rosenberg for carrying out the experiment of Fig. 6.

LITERATURE CITED

1. **Adler, A. J., N. J. Greenfield, and G. D. Fasman.** 1973. Circular dichroism and optical rotatory dispersion of proteins and polypeptides. Methods Enzymol. **27**:675–735.
2. **Ames, B. N., and D. T. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage DNA. J. Biol. Chem. **235**:769–775.
3. **Baumgardner, D., C. Deal, and S. Kaplan.** 1980. Protein composition of *Rhodopseudomonas sphaeroides* outer membrane. J. Bacteriol. **143**:265–273.
4. **Deal, C. D., and S. Kaplan.** 1983. Solubilization, isolation, and immunochemical characterization of the major outer membrane protein from *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **258**:6524–6529.
5. **Deal, C. D., and S. Kaplan.** 1983. Physical and chemical characterization of the major outer membrane protein of *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **258**:6530–6536.
6. **Ding, D. H., and S. Kaplan.** 1976. Separation of inner and outer membranes of *Rhodopseudomonas sphaeroides*. Prep. Biochem. **6**:61–79.

6a. **Flammann, H. T., and J. Weckesser.** 1984. Characterization of the cell wall and outer membrane of *Rhodopseudomonas capsulata*. J. Bacteriol. **159**:191–198.

7. **Flammann, H. T., and J. Weckesser.** 1984. Porin isolated from the cell envelope of *Rhodopseudomonas capsulata*. J. Bacteriol. **159**:410–412.
8. **Hancock, R. E. W., G. M. Decad, and H. Nikaido.** 1979. Identification of the protein producing transmembrane diffusion pore in the outer membrane of *Pseudomonas aeruginosa* PAO1. Biochim. Biophys. Acta **554**:323–331.
9. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. **136**:381–390.
10. **Lane, B. C., and R. E. Hurlbert.** 1980. Isolation and partial characterization of the major outer membrane protein of *Chromatium vinosum*. J. Bacteriol. **143**:349–354.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Luckey, M., and H. Nikaido.** 1980. Specificity of diffusion channels produced by λ phage receptor protein of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:167–171.
13. **Lugtenberg, B., J. Meyers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major" outer membrane protein of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
14. **Nakae, T., J. Ishii, and M. Tokunaga.** 1979. Subunit structure of functional porin oligomers that form permeability channels in the outer membrane of *Escherichia coli*. J. Biol. Chem. **254**:1457–1461.
15. **Nikaido, H., and T. Nakae.** 1979. Outer membrane of Gram-negative bacteria. Adv. Microbiol. Physiol. **20**:163–250.
16. **Nikaido, H., and E. Y. Rosenberg.** 1981. Effect of solute size on diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*. J. Gen. Physiol. **77**:121–135.
17. **Renkin, E. M.** 1954. Filtration, diffusion, and molecular sieving through porous cellulose membranes. J. Gen. Physiol. **38**:225–243.

17a. **Salimath, P. V., J. Weckesser, W. Strittmatter, and H. Mayer.** 1983. Structural studies on the non-toxic lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023. Eur. J. Biochem. **136**:195–200.

18. **Tokunaga, M., H. Tokunaga, Y. Okazima, and T. Nakae.** 1979. Characterization of porins from the outer membrane of *Salmonella typhimurium*. II. Physical properties of the functional oligomeric aggregates. Eur. J. Biochem. **95**:441–448.
19. **Weckesser, J., G. Drews, and I. Fromme.** 1972. Chemical analysis of and degradation studies on the cell wall lipopolysaccharide of *Rhodopseudomonas capsulata*. J. Bacteriol. **109**:1106–1113.
20. **Weckesser, J., G. Drews, and R. Ladwig.** 1972. Localization and biological and physicochemical properties of the cell wall lipopolysaccharide of *Rhodopseudomonas capsulata*. J. Bacteriol. **110**:346–353.
21. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. **7b**:148–155.
22. **Yoshimura, F., L. S. Zalman, and H. Nikaido.** 1983. Purification and properties of *Pseudomonas aeruginosa* porin. J. Biol. Chem. **258**:2308–2314.