Solubilization of the major outer membrane protein of *Rhodopseudomonas sphaeroides*, and subsequent isolation, has been achieved by both non-detergent- and detergent-based methods. The protein was differentially solubilized from other outer membrane proteins in 5 M guanidine thiocyanate which was exchanged by dialysis for 7 M urea. The urea-soluble protein was purified to homogeneity by a combination of DEAE-Sephadex chromatography and preparative electrophoretic techniques. Similar to the peptidoglycan-associated proteins of other Gram-negative bacteria, the protein was also purified by differential temperature extraction of the outer membrane in the presence of sodium dodecyl sulfate (SDS) followed by preparative SDS-polyacrylamide gel electrophoresis. Immunochemical analysis of the proteins isolated by the two techniques established the immunological identity and homogeneity of each preparation. Immunoblots of SDS-polyacrylamide gels revealed that antibody directed against the major outer membrane protein reacted with the three high molecular weight aggregates present in the outer membrane which we have previously shown to be composed of the major outer membrane protein and three nonidentical small molecular weight proteins.

Studies in many of the Gram-negative bacteria have revealed the outer membrane to be a complex structure involved not only in maintaining the structural integrity of the cell (1), but also actively involved in such physiological roles as pore formation (2-4), mediation of nutrient transport and binding activities (6-7), and phage and colicin receptor formation (8-10). Despite the molecular complexity of the outer membrane, there appears to be an underlying similarity among many genera studied, although subtleties in detail are well documented. Many similarities have been shown to exist in the proteins found in the outer membranes of various bacteria, such as the heat-modifiable proteins (11, 12), pore-forming proteins (2, 3), peptidoglycan-associated proteins (13-16), and lipoproteins (17-19).

Previous studies in our laboratory (20) have reported on the initial characterization of the isolated outer membrane of *Rhodopseudomonas sphaeroides* from chemoheterotrophically grown cells. These studies demonstrated that *R. sphaeroides* possesses a complex outer membrane protein array which has many of the characteristics commonly associated with other Gram-negative bacteria, but in addition this structure demonstrates more complex interactions between intramembranous proteins. These interactions center around the formation of complexes between the major outer membrane protein and each of three nonidentical, smaller molecular weight polypeptides (20). In addition, the major outer membrane protein was suggested to contain covalently attached lipid (20). Although there is as yet no definite proof of the function of the major outer membrane protein of *R. sphaeroides*, the fact that it is present as a common subunit in each of the aggregates together with its abundance would suggest an important role for it in the outer membrane. To further investigate the physical and chemical characteristics of this protein, as well as its physiological role, studies were initiated to isolate this protein in homogeneous form.

Studies involving the intracytoplasmic membrane proteins of *R. sphaeroides* by Cohen and Kaplan (21) have demonstrated the applicability of disrupting the membrane with the chaotropic salt guanidine thiocyanate as initially described by Moldow *et al.* (22), followed by its replacement by dialysis against concentrated urea solutions, to obtain proteins in solubilized aqueous form, which are then amenable to further purification procedures. This technique was suggested to be of general use in the isolation of membrane proteins. In this paper, we report the application of this method to the isolation of the major outer membrane protein of *R. sphaeroides*. In addition, since initial studies suggested a possible porin-related function for the major outer membrane protein (20), we have applied standard techniques of isolation in the anionic detergent sodium dodecyl sulfate to the outer membrane of *R. sphaeroides* in order to compare the behavior of this protein to that reported for peptidoglycan-associated proteins identified in outer membranes of other genera of Gram-negative bacteria (13-16).

**MATERIALS AND METHODS AND RESULTS**

**DISCUSSION**

While there have been reports in the literature on the separation and characterization of the cytoplasmic membrane...
Other outer membrane proteins are not retained significantly by peptidoglycan with the SDS extraction conditions employed. The behavior demonstrated by the major outer membrane protein is analogous to that reported for Protein H of Pseudomonas aeruginosa by Mizuno and Kageyama (25) and for the matrix protein of Escherichia coli by Rosenbusch (13) in that the protein is SDS-insoluble at low temperatures but SDS-soluble at higher temperatures, although the exact temperatures of solubilization vary with the bacterial species. By analogy to the observations in other Gram-negative bacteria (13-16), we would suggest the possibility of a porin function for the major outer membrane protein; however, the unambiguous proof of this function may be more complicated than for other Gram-negative bacteria. If the apparent molecular weight of the three aggregates on SDS-polyacrylamide gels (20) is reflective of the in vivo molecular weight, then it could be suggested that the pore is a dimer, or multiple thereof, and composed of two heterologous subunits. Achieving the proper ratio of subunits in reconstitution studies of pore formation could be difficult due to the heterogeneous nature of the aggregates (20), as opposed to the homogeneous trimers observed in E. coli (43, 44).

Although the apparent molecular mass of 47,000 Da on 10% SDS-PAGE is larger than that reported for the peptidoglycan-associated proteins of other genera (33,000-44,000 Da), this is due to the anomalous electrophoretic mobility in SDS-gels of this protein, as will be reported, and its molecular mass is actually closer to that of the other proteins described. Further work utilizing the antibodies directed against the major outer membrane polypeptide is being directed at investigating the interaction(s) of these polypeptides with the outer membrane of R. sphaeroides.

The major outer membrane protein was purified by a combination of DEAE-Sepharose chromatography and preparative polyacrylamide gel electrophoresis. The procedure resulted in a homogeneous polypeptide preparation whose purity has been demonstrated on SDS-PAGE (Fig. 4) which allows examination by size, as well as on 8 M urea-PAGE and isoelectric focusing (Fig. 4) which allows examination by charge. In addition, the preparation was homogeneous when analyzed by the immunological techniques of crossed immunoelectrophoresis and Western immunoblotting of SDS-polyacrylamide gels (Fig. 5). When the isolation procedure was applied to outer membranes derived from phototrophically grown cells, the resulting homogeneous polypeptide was identical with the polypeptide isolated from chemoheterotrophically grown cells by all of the above criteria. The further physical and chemical characterization of this isolated polypeptide will be reported.

Although examination of the chemical nature of the polypeptide is important in defining the chemical properties of the outer membrane, in addition, we wanted to correlate our observations with those reported for other Gram-negative bacterial outer membranes. For these reasons and the initial observations suggesting a porin function for this protein (20), we examined the solubilization of the outer membrane proteins by the anionic detergent SDS, which has been extensively used by other workers (13-16) to investigate peptidoglycan-associated lipoproteins and matrix or porin proteins. In addition to being a method by which to compare the R. sphaeroides protein to peptidoglycan-associated proteins identified in other Gram-negative bacteria, this approach yielded a detergent-based method for purification of the major outer membrane protein.

The major outer membrane protein was purified by a combination of SDS extraction of the outer membrane at room temperature and 75 °C (see “Materials and Methods”) combined with preparative SDS-PAGE (Table II). This resulted in a homogeneous polypeptide which behaved identically with the protein isolated by the GuSCN-urea procedure (Fig. 4) when examined on SDS-PAGE and on 8 M urea-PAGE and isoelectric focusing following removal of SDS. By immunological criteria, the polypeptide isolated by the SDS procedure was shown to be analogous to the polypeptide isolated by the GuSCN-urea procedure (Fig. 5).

Similar to the behavior of outer membrane proteins of other Gram-negative bacteria (13-16), the solubilization of the R. sphaeroides outer membrane in SDS revealed that the major outer membrane protein was retained by the peptidoglycan fraction at room temperature, but was released during 75 °C solubilization. This suggested that the major outer membrane protein is tightly associated with, but not covalently bound to, the peptidoglycan. The noncovalent nature was additionally supported by the solubility of the protein in GuSCN-urea. Other outer membrane proteins are not retained significantly by peptidoglycan with the SDS extraction conditions employed. The behavior demonstrated by the major outer membrane protein is analogous to that reported for Protein H of Pseudomonas aeruginosa by Mizuno and Kageyama (25) and for the matrix protein of Escherichia coli by Rosenbusch (13) in that the protein is SDS-insoluble at low temperatures but SDS-soluble at higher temperatures, although the exact temperatures of solubilization vary with the bacterial species. By analogy to the observations in other Gram-negative bacteria (13-16), we would suggest the possibility of a porin function for the major outer membrane protein; however, the unambiguous proof of this function may be more complicated than for other Gram-negative bacteria. If the apparent molecular weight of the three aggregates on SDS-polyacrylamide gels (20) is reflective of the in vivo molecular weight, then it could be suggested that the pore is a dimer, or multiple thereof, and composed of two heterologous subunits. Achieving the proper ratio of subunits in reconstitution studies of pore formation could be difficult due to the heterogeneous nature of the aggregates (20), as opposed to the homogeneous trimers observed in E. coli (43, 44).

Although the apparent molecular mass of 47,000 Da on 10% SDS-PAGE is larger than that reported for the peptidoglycan-associated proteins of other genera (33,000-44,000 Da), this is due to the anomalous electrophoretic mobility in SDS-gels of this protein, as will be reported, and its molecular mass is actually closer to that of the other proteins described. Further work utilizing the antibodies directed against the major outer membrane polypeptide is being directed at investigating the interaction(s) of these polypeptides with the outer membrane of R. sphaeroides.

REFERENCES
6526

*Outer Membrane Protein Purification*

SOLUBILIZATION, ISOLATION AND IMMUNOCHEMICAL CHARACTERIZATION OF THE OUTER MEMBRANE PROTEINS OF RHODOPOSPHORA SYNOXODIUM

Carolyn D. Deal and Samuel Kaplan

MATERIALS AND METHODS

Bacteria and Growth Conditions. Rh. sulfatovorans strain 2.4.1 was grown on 1.1% Bacto-Novobiocin agar plates in the presence of 50 mM NaSCN. One-hundred microliters of cell suspension (2) to a density of 1 x 10^8 cells per ml was added to 10 ml of growth medium, as described by Schlegel et al. (23), which contains 0.3 M NaSCN, 0.05 M KSCN, 0.02 M NaHCO_3, and 0.01 M glucose. The medium was incubated at 30°C under constant illumination with a 25-W bulb. The bacteria were collected after 48 h by filtration through a 5-μm glass fiber filter followed by centrifugation and stored as a cell paste at -7°C. For [14C]acetate labeling of outer membranes with actinomycin D, shaken cultures were grown in an oxygen-free medium containing 50 mM NaSCN and 1 μCi/ml [14C]acetate [final concentration 40 μCi/ml containing 0.5 μCi of [14C]acetate (58.2 mCi/mmol) per ml].

Outer Membrane Isolation. Outer membrane fractions were prepared by the method of Deal and Kaplan (20), with the following modifications. Outer membranes were dialyzed extensively against glass distilled H_2O and lyophilized. Lyophilized outer membranes (120 mg) were suspended in 10 mM Tris adjusted to pH 7.2 with Na_2CO_3 and to pH 7.4, 10 mM sodium phosphate buffer, and 2 mM EDTA and 5 M mercaptoethanol at a protein concentration of 5 mg/ml and stirred for 30 min. The solution was dialyzed against two changes (50:1, v/v) 10 to 12 mM mannitol buffers and agents in the initial solubilization in GuSCN. The insoluble protein was pelleted by centrifugation at 150,000 x g for 3 h (Beckman 50 Ti rotor at 4°C).

The resulting pellet was solubilized for 30 min at room temperature in 1 M GuSCN containing the same buffers and agents as in the initial solubilization at a protein concentration of 5 mg/ml. The solution was dialyzed against 1 M urea containing 100 mM Tris pH 7.2 and 5 M mercaptoethanol. This dialysis was repeated until no UV absorption at 280 nm was discernible. The solution was then dialyzed against distilled H_2O.

Analytical Polyacrylamide Gel Electrophoresis. Polyacrylamide gels (120x160x0.8mm) were run essentially as described by Davis (21), with the following modifications. Stock solution 10 mM sodium phosphate buffer, pH 7.2, 50 mM Na_2EDTA, 1 M glycerol and 0.014 bromophenol blue for 15 min at 4°C or 30 min at 23°C. After electrophoresis, outer membrane proteins stained with Coomassie Blue were analyzed by 2-d common (20), and then further purified by sucrose gradient centrifugation (21) or by immunoblotting.

RESULTS

Solubilization of Outer Membrane Proteins. The preparation of SDS soluble and insoluble proteins was based on the method of Mono (26) with the following modifications. Highly purified outer membranes were extracted in 2x, pH 7.4, 100 mM Tris buffer containing 1 M urea at 4°C for 1 h (Beckman 50 Ti rotor at 4°C), centrifuged (1 h, 150,000 x g) and the supernatant dialyzed further against 50 mM Tris pH 7.2 containing 2 M urea (and 5 M mercaptoethanol if NaSCN was present) at 4°C for 12 h each) of 7 M urea in mixing the sample in GuSCN. The insoluble protein was solubilized in 28 mM GuSCN containing 50 mM NaSCN. The insoluble protein was solubilized by the method of Deal and Kaplan (20) and was subjected to electrophoresis as described previously (20). Prior to electrophoresis, samples were diluted 1:20 v/v in 10% SDS. Electrophoresis was performed essentially as described by Davis (21). The membranes were run against distilled H_2O containing 150,000 x g for 3 h (Beckman 50 Ti rotor at 4°C).

In the present study, the insoluble protein was further solubilized in 2 M GuSCN containing 1 M NaSCN (as above) and was used immediately for further purification procedures.

Figure 1. SDS Polyacrylamide electrophoresis of outer membrane proteins. SDS-polyacrylamide gel electrophoresis of proteins solubilized in 2 M GuSCN. Lane 1, outer membrane protein; lane 2, outer membrane protein following 1 M GuSCN solubilization; lane 3, outer membrane protein following 4 M GuSCN solubilization. Lane 1 contains 50 μg protein; lanes 2 and 3 contain 25 μg protein. Lanes were stained with Coomassie Blue.

Additionally, it was observed (Fig. 2, lane 3-4) that the behavior of several of the 1 M GuSCN-insoluble polypeptides could be altered by lysosyme treatment of the 1 M GuSCN-insoluble protein, but not prior to solubilization with 5 M GuSCN as described under "Materials and Methods." In particular the 20, 24, 25, and 29 kilodalton polypeptides which were previously insoluble under these conditions were partially solubilized by treatment with 100 μg polypeptide lysozyme per mg of protein (lanes 2, 3 and 4). This suggests that these polypeptides can be solubilized by lysozyme treatment with which they would (20), and thus form an association with the peptidoglycan.

Immunoblotting. Transfer of proteins from SDS-polyacrylamide gel to nitrocellulose was carried out according to the method of L neste (32) with modifications as described by Kears (33). Protein A-containing from Sigma Chemical Corp. was isolated by the Chloroform technique (36). Synthesis of Quinoline Aldehyde. Quinoline-8-carboxaldehyde was synthesized according to the method of Cohen and Kaplan (34).

Analysis of Proteins. Protein concentrations were determined by a modified biuret reaction described by Nursey and Richards (35).

Chemicals. Polyacrylamide was purchased from Bio-Rad Laboratories Inc. and was used without further purification. Sodium dodecyl sulfate was obtained from Sigma Chemical Co. Silica and polyacrylamide gels were obtained from Bio-Rad Laboratories Inc. Gel electrophoresis and polyacrylamide gel electrophoresis were performed essentially as described by Davis (21). Immunoblotting was performed essentially as described by Towbin et al. (33). Protein A-containing from Sigma Chemical Corp. was isolated by the Chloroform technique (36). Synthesis of Quinoline Aldehyde. Quinoline-8-carboxaldehyde was synthesized according to the method of Cohen and Kaplan (34).

Analytical Procedure. Protein concentrations were determined by a modified biuret reaction described by Nursey and Richards (35).
Isolation of the Urea-Soluble Major Outer Membrane Protein

The major urea solubilized polypeptide (Fig. 1, lane 3) was further resolved by DEAE-Sephadex column chromatography as detailed in Table I. Aliquots of fractions 5-15 were pooled and eluted with 0.2 M NaCl, as revealed by SDS-PAGE. In contrast, the bulk of the polypeptide, as revealed by SDS-PAGE, was shown to be homogenous when examined in SDS polyacrylamide gels according to the method of Burdette and Sambrook (41). As shown in Table I, lanes 1-3, the protein was shown to be homogenous when examined in SDS polyacrylamide gels. In all instances examined, the polypeptide isolated by SDS extraction behaved identically to the polypeptide isolated by GSHCN urea solubilization as shown in Figure 4. This indicated that the two preparations represented isolation of the same polypeptide and therefore could be used interchangeably in further studies depending on the state of the polypeptide necessary for the technique to be employed.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer membrane</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Delipidated protein</td>
<td>41.2</td>
<td>96</td>
</tr>
<tr>
<td>1 M GSHCN extracted-urea solvable protein</td>
<td>16.6</td>
<td>39</td>
</tr>
<tr>
<td>5 M GSHCN extracted-urea solvable protein</td>
<td>8.8</td>
<td>21</td>
</tr>
<tr>
<td>Urea-insoluble protein</td>
<td>10.7</td>
<td>25</td>
</tr>
<tr>
<td>Protein eluting at 0-2 M NaCl from DEAE-Sephadex</td>
<td>5.5</td>
<td>13</td>
</tr>
<tr>
<td>Through urea pH 6.7-PAGE</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

Figure 4: Analysis of the purified major outer membrane protein by polyacrylamide gel electrophoresis. Lanes 1 and 2, 36 C. succinogenes outer membrane protein on SDS-PAGE; lanes 3 and 4, urea-insoluble outer membrane protein on SDS-PAGE (pH 3-5.5); lane 5, 15 C. succinogenes outer membrane protein on SDS-PAGE (pH 3-5.5); lanes 6 and 7, 36 C. succinogenes outer membrane protein on SDS-PAGE (pH 3-5.5).
Aggregate Formation of the Major Outer Membrane Protein. We have previously shown by two dimensional polyacrylamide gel electrophoresis (20) that the major outer membrane protein forms aggregates with each of three nonidentical smaller molecular weight polypeptides (21.3, 24.7, and 29 kilodaltons). These high molecular weight aggregates (68, 71, and 75 kilodaltons) are stable during SDS solubilization at room temperature prior to electrophoresis (Fig. 6, lane 1). As described above, we have isolated the major outer membrane protein and have generated monoclonal antibody directed against that protein. Outer membrane samples solubilized at room temperature and at 75°C were electrophoresed on SDS 10% polyacrylamide gels, then transferred to nitrocellulose for immunoblotting with the major outer membrane protein antibody as described under “Materials and Methods.” The antibody reacted with the high molecular weight aggregates in the heated and unheated samples. The antibody reacted with the major outer membrane protein in the heated sample (Fig. 6, lane 4), thus unambiguously demonstrating the presence of the major outer membrane protein in the three high molecular weight aggregates in the unheated sample. The reaction of the antibody with the heated and unheated outer membrane samples revealed that the protein we have isolated is the major outer membrane protein and that it demonstrated the aggregation properties we originally observed by two-dimensional polyacrylamide gel electrophoresis and was the common subunit in the three high molecular weight aggregates.

Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer membrane</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>Room temperature SDS soluble</td>
<td>3.6</td>
<td>33</td>
</tr>
<tr>
<td>75°C SDS soluble</td>
<td>5.8</td>
<td>53</td>
</tr>
<tr>
<td>SDS insoluble</td>
<td>1.4</td>
<td>13</td>
</tr>
<tr>
<td>75°C SDS soluble fraction through preparative SDS-PAGE</td>
<td>4.2</td>
<td>38</td>
</tr>
</tbody>
</table>

Figure 6 SDS polyacrylamide electrophoresis of outer membrane solubilized at room temperature and 75°C and autoradiogram of an immunoblot of the gel. Lanes 1 and 2, SDS-PAGE of outer membrane solubilized at room temperature and 75°C, respectively; lanes 3 and 4, SDS-PAGE of outer membrane samples solubilized at room temperature and 75°C, respectively, probed with major outer membrane protein antisera. Lanes 1 and 2 contained 50 μg protein; lane 3, 20 μg; and lane 4, 4 μg protein.
Solubilization, isolation, and immunochemical characterization of the major outer membrane protein from Rhodopseudomonas sphaeroides.

C D Deal and S Kaplan


Access the most updated version of this article at http://www.jbc.org/content/258/10/6524

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/10/6524.full.html#ref-list-1