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 immunochemical identity and homogeneity of each preparation. Immunoblotts 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 porin formation (2-4), mediation of nutrient transport and binding activities (5-7), 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 inner-membranous 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

* Portions of this paper (including "Materials and Methods," "Results," Figs. 1-6, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biochemical Chemistry, 960 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-2918, cite the authors, and include a check or money order for $8.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

1 The abbreviations used are: GuSCN, guanidine thiocyanate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
and outer membrane of members of the Rhodospirillaceae (20, 40–42), we report here, to our knowledge, the first isolation of an outer membrane protein from this genera and an initial effort to relate it to the outer membrane proteins identified in other Gram-negative bacteria. In addition, we have demonstrated the more general applicability of the GuSCN-urea solubilization procedure of Cohen and Kaplan (21) as utilized by Moldow et al. (22) to problems of membrane protein isolation. Although other interactions may affect polypeptide solubility, as seen with the alteration in the solubility of the 29-, 26.5- and 21.5-kDa polypeptides following lysozyme treatment (Fig. 2), the solubilization yields a polypeptide preparation in aqueous solution which is amenable to further purification, procedures and characterization.

The major outer membrane protein was purified by a combination of DEAE-Sephadex column 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 immunochemical 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 the 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 immunochemical 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 within the outer membrane of R. sphaeroides.

REFERENCES

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Outer Membrane Protein Purification

SOLUBILIZATION, ISOLATION AND IMMUNOCHEMICAL CHARACTERIZATION OF GuSCN

SOLUBILIZATION OF OUTER MEMBRANE PROTEINS FROM RHIZOPSEUDOMONAS SOLA

GuSCN Solubilization of Outer Membrane Proteins

The procedure employed for GuSCN solubilization of outer membrane proteins is a modification of the technique developed by Cohen and Kaplan (21) for the solubilization of chromatophore proteins. It is based on the differential solubility of outer membrane proteins when exposed to certain concentrations of the chaotropic salt GuSCN, followed by removal of the chaotropically reactive thiocyanate ion by dialysis against buffered aqueous solutions. As opposed to mildiplasmodium chromatophore proteins which exhibit no differential solubility in GuSCN (21), the outer membrane proteins of R. solanacearum exhibit differential solubility in GuSCN with several being soluble without prior treatments (Fig. 1).

Outer membrane proteins were obtained in urea soluble and insoluble fractions by exposure to 1 M and 5 M concentrations of GuSCN as described under "Materials and Methods" and illustrated in Figure 1. The GuSCN concentration of the initial exposure of outer membrane proteins to the chaotropic agent was optimized at 1 M to solubilize the maximum amount of contaminating protein with minimum solubilization of membrane proteins. After an initial exposure at a concentration of 2 M GuSCN resulted in the solubilization of the 47-kilodalton outer membrane protein (Fig. 1, lane 3), while the smaller polypeptide (25, 26, 27, and 29 kilodaltons) was solubilized at 5 M GuSCN (Fig. 1, lane 4). It should be noted that the outer membrane protein in the urea soluble fraction obtained at 1 M GuSCN was not observed in Figure 1 (lane 2). This fraction was immediately used for further purification procedures.

Figure 1. SDS Polyacrylamide electrophoresis of outer membrane, urea soluble and insoluble proteins following GuSCN solubilization, and urea soluble and insoluble proteins following lysozyme treatment. All lanes 1, outer membrane; 2, urea soluble protein following 1 M GuSCN solubilization; 3, urea soluble protein following 5 M GuSCN solubilization. Lane 1 contains 50 ng protein; lanes 2-4 contain 25 ng protein. Samples were dialyzed for 7 min at 75°C or for 7 min at 4°C until using the running buffers described previously. lanes 3 and 4. This suggests that these polypeptides, and possibly the remaining insoluble polypeptide with which they associate (20), could form an association with the peptidoglycan.

Additionally, it was observed (Fig. 2, lanes 3 and 4) that the behavior of several of the 5 M GuSCN-insoluble polypeptides could be altered by lysozyme treatment of the 5 M GuSCN insoluble proteins but prior to solubilization with 5 M GuSCN as described under "Materials and Methods." In particular the 25, 26, 27, and 29 kilodalton polypeptides which were previously insoluble under these conditions were solubilized by lysozyme treatment (Fig. 2, lanes 3 and 4). This suggests that these polypeptides, and possibly the remaining insoluble polypeptide with which they associate (20), could form an association with the peptidoglycan.
Isolation of the Ure-Soluble Major Outer Membrane Protein

The major ure-soluble polypeptide (Fig. 1, lane 3) was further resolved by SDS-polyacrylamide gel electrophoresis as detailed in Materials and Methods. A single polypeptide (Fig. 1, lane 3), 0.2 M NaCl, was noted to reproducibly cause a perturbation in the linearity of the gel gradient. The peak fractions were pooled and concentrated using an Amicon YM10 membrane.

**Final purification of the major outer membrane protein was achieved on preparative 8 M urea, 7 M polyacrylamide gel as described under "Materials and Methods." The polypeptide eluted from the prepared gel strip was shown to be homogeneous when run on 20% polyacrylamide gel. Additionally, it was demonstrated to be immunochemically homogenous. Recovery of the purified polypeptide was performed using both the urea-purified and SDS-polyacrylamide gel-electrophoresis and immunochemical methods.**

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</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 GdnCl-extracted-urea soluble protein</td>
<td>16.6</td>
<td>39</td>
</tr>
<tr>
<td>5 M GdnCl-extracted-urea soluble 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 0.2-2 M NaCl from DEAE-Sephacel</td>
<td>5.3</td>
<td>13</td>
</tr>
<tr>
<td>Though urea pH 6.7-PAGE</td>
<td>4</td>
<td>9</td>
</tr>
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</table>

**Figure 3** Protein profile obtained from DEAE-Sephadex column chromatography of 5 M GdnCl-extracted soluble protein. Approximately 0.1 mg of protein was applied to a DEAE-Sephadex column equilibrated in 10 mM Tris pH 7.0, 0.9 M NaCl-captocetanol. After elution with 2 column volumes of buffer at a flow rate of 15 ml/hr, the column was eluted with a 0-0.5 M NaCl gradient in 10 ml, containing the same buffer. 2.0 ml fractions were collected. ---, 2 mg SDS, conductivity (mV).

**Figure 6** Examples of the purified major outer membrane protein by polyacrylamide gel electrophoresis. Lanes 1 and 2, urea-PAGE; lanes 3 and 4, SDS-PAGE; lanes 5 and 6, isoelectric focusing PAGE (pH 3-5.5), and 7 and 8, isoelectric focusing PAGE (pH 4.5-6.5). 1. 8 M urea-PAGE, 10 and 25 μg respectively; 2 and 4, 8 M urea-PAGE 8.7-7.0, 10 and 25 μg respectively; 5 and 6, isoelectric focusing PAGE (pH 3-5.5), 10 and 25 μg respectively.
Aggregate Formation of the Major Outer Membrane Protein. We have previously shown by two-dimensional polyacrylamide gel electrophoresis (12) that the major outer membrane protein forms aggregates with each of three nonidentical smaller molecular weight polypeptides (21.3, 26.2, and 29 kilodaltons). These high molecular weight aggregates (48, 71, and 75 kilodaltons) are stable during SDS solubilization at room temperature prior to electrophoresis (Fig. 6, lane 1). These aggregates are solubilized and dissociated at 75°C (Fig. 6, lane 2). As described above, we have isolated the major outer membrane protein and have generated monospecific 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 unheated sample (Fig. 6, lane 3), but failed to react 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 unheated and heated 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>
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<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>
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<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; lane 3, autoradiogram of a nitrocellulose blot of lane 1; lane 4, autoradiogram of a nitrocellulose blot of lane 2. 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


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