The characterization of the major outer membrane protein of *Rhodopseudomonas sphaeroides* is described. Molecular weight estimations using Ferguson plots derived from sodium dodecyl sulfate and urea-polyacrylamide gels were 39,500 and 32,200, respectively, in good agreement with the value of 39,800 obtained from amino acid compositional studies. NH₂-terminal amino acid determinations of the major outer membrane protein revealed a blocked NH₂ terminus. Gas chromatography of the acid-hydrolyzed protein confirmed the presence of fatty acid covalently associated with the protein presumably through an amino linkage. Peptide mapping of tryptic and chymotryptic digestions of the major outer membrane protein contained covalently attached lipid (1). To further investigate this protein, studies were initiated to physically and chemically characterize the protein, particularly the nature of the fatty acyl association. Knowledge of the structural characteristics of the protein is important to further investigations as to the physiological role of the protein in the outer membrane of *R. sphaeroides*.

This report presents the initial characterization of the major outer membrane protein isolated as previously described from *R. sphaeroides* (2). Analysis of the physical characteristics of membrane proteins has been complicated by their anomalous behavior with respect to standard techniques of analysis of water-soluble polypeptides (3-6). Isolation of the major outer membrane protein by both non-detergent- and detergent-based techniques (2) has permitted the application of methods adapted to both aqueous and nonaqueous systems for the analysis of the major outer membrane protein. The data obtained on the fatty acid composition and the analysis of peptides confirmed our previous identification of the major outer membrane protein as a lipoprotein (1). This is discussed in terms of the relationship between this protein and lipoproteins previously identified in other species.

**MATERIALS AND METHODS**

**Isolation of the Major Outer Membrane Protein**—The major outer membrane protein was purified from isolated outer membrane as described (2). [¹³C]Acetate-labeled protein was isolated from cells grown on media supplemented with [¹³C]acetate as described (2). For [¹⁴C]glycerol labeling of cells, medium A was supplemented with 0.5% Casamino acids, 0.1% yeast extract, and glycerol (final concentration, 10 μg/ml) containing 30 μCi/ml of [²⁻¹⁴C]glycerol (100 Ci/mmol).

**Molecular Weight Determinations**—Molecular weight of the purified polypeptide was determined from the electrophoretic mobility in SDS-polyacrylamide gels (7) and in 8 M urea-polyacrylamide gels (8). The polypeptide and standard proteins of known molecular weight were electrophoresed on gels of 6, 8, 10, and 12% polyacrylamide in each of the two gel systems. Molecular weight determinations were based on the retardation coefficients (K values) obtained from unweighted linear regression analysis of Ferguson plots of *R. sphaeroides* acrylamide concentration. The K values of the standard proteins were used to define a linear relationship with the known molecular weights. Standard proteins used were bovine serum albumin (68,000), ovalbumin (44,500), pepsin (35,000), a-chymotrypsinogen (25,700), a-casein (23,500), soybean trypsin inhibitor (20,500), and lysozyme (13,700).

**Amino Acid Analysis**—Individual aliquots of 100 μg of protein were hydrolyzed in vacuo for 24, 48, and 72 h at 110 °C in 6 N HCl. An additional 24-h hydrolysis was conducted in the presence of dimethyl sulfoxide to determine cysteine and cysteic acid (9). Methionine was determined as methionine sulfone from the same oxidative hydrolysis. Norleucine was included as an internal standard to 24-h hydrolysis to correct for losses. Valine and isoleucine values were extrapolated to zero hydrolysis time to correct for losses. Valine and isoleucine values were determined from the 72-h time point as they displayed a time-dependent release from the polypeptide (10). Amino acid analysis was performed on a Beckman 119CL amino acid analyzer with a single column of Beckman W3H resin. Tryptophan was determined spectrophotometrically in guanidine hydrochloride as described by Eidelberg (11). Peptide compositions were determined from a single 24-h hydrolysis in 6 N HCl.

**NH₂-terminal Determinations**—NH₂-terminal amino acid determinations were conducted on protein isolated in urea and in SDS by the dansylation conditions of Zanetta et al. (12) and Gray (13), respectively. The dansylated polypeptides were hydrolyzed (6 N HCl, 115 °C, 4 h) and the residue was resuspended in acetone/acetic acid (3:2, v/v). The dansylated amino acids were resolved by chromatography on polyamide sheets (5 × 5 cm) utilizing the three solvent systems described.
Fatty Acid Analysis—Phospholipids were extracted from the isolated polypeptide according to the method of Inouye et al. (15), followed by fatty acid analysis conducted as described by Mizuno and Kamin. For fatty acid methyl esters, the Dansylated derivatives were resolved as described above. Known peptides were eluted from the cellulose thin layer plates and counting in a Triton/toluene-based scintillation fluid. Gas chromatography was in butanol/pyridine/acetic acid/H2O (50:33:1:40). Peptides were eluted from the cellulose with 20% pyridine as described by Kates (18). The fatty acid methyl esters were obtained by hydrolysis in sealed tubes containing 2-4 mg of protein in 2.5 ml of 3 N HCl in methanol with 0.1 ml of benzene for 3 h at 100 °C. Alternatively, fatty acid methyl esters were obtained by mild alkaline hydrolysis in 0.5 N sodium hydroxide in anhydrous methanol as described by Kates (18). The fatty acid methyl esters were extracted with hexane three times. Gas chromatography to determine the fatty acid composition was conducted on a Hewlett-Packard 5890A gas chromatograph using a 6-foot column with a 2-mm inner diameter. The column was filled with Supelco resin 3% SP-2100 DOD at 4°C/min. The identification of fatty acid methyl esters, authentic fatty acid methyl esters obtained from Supelco Chemical Co. were used as standards. The molar ratio of fatty acid to protein was determined by adding pentaenoic acid as an internal standard prior to hydrolysis. Gas chromatography of [14C]acetate-labeled methyl esters was conducted on a Packard 428 gas chromatograph. Phospholipids from whole cell and isolated membrane fractions were extracted by the method of Bligh and Dyer (19) as modified by Ames (20). Fatty acid composition was determined as described.

Enzymatic Digestions—Lyophilized protein was suspended in 0.5 M ammonium bicarbonate buffer, pH 8.3, at a concentration of 500 µg/ml. Tryptsin or chymotrypsin (100 µg of enzyme/ml of stock) was added to the protein suspension to a final concentration of 4% (w/w) in two aliquots 3 h apart. The digestion was carried out at 37 °C for 6 h. Amberlite (100 µg of enzyme/ml of stock) was added to the protein suspension to a final concentration of 4% (w/w) in four aliquots 6 h apart. The digestion was carried out at 37 °C for 24 h. Upon completion of the digestion, the solution was lyophilized.

Peptide Maps—The lyophilized peptides were resuspended in 2% ammonium hydroxide at a concentration of 5 mg/ml. The peptides were resolved in two dimensions on cellulose thin layer plates by electrophoresis in the first dimension followed by chromatography in the second dimension (15, 21). Electrophoresis was conducted on 5-15 µg of digest at pH 3.7 or pH 6.5 at 500 V utilizing the pyridine/acetic acid/H2O system described by Gracy (15). Chromatography was in butanol/pyridine/acetic acid/H2O (50:33:1:40). Peptides were visualized with fluorescamine (21). The amount of radioactivity in each peptide was determined by scraping spots of the plates and counting in a Triton/toluene-based scintillation fluid. Peptides were eluted from cellulose thin layer plates by electrophoresis and by a modified biuret reaction described by Munkres and Richards (22).

Chemicals—Acrylamide was purchased from Eastman Kodak Co. and purified by mixed-bed ion exchange using Bio-Rad AG 501-X8 (D). Sequential grade urea, dapsylchloride, and constant boiling HCl were purchased from Pierce. Micropolyacrylamide sheets (5 x 5 cm) were obtained from Schleicher & Schuell. Cellulose thin layer sheets (20 x cm) were purchased from Kodak. Tosylphenylalanyl chloromethyl ketone-treated trypsin and α-chymotrypsin were purchased from Worthington. Fluorescamine was obtained from Sigma. Pronase was purchased from Calbiochem. Sodium [1-14C]acetate (58.3 µCi/mmol), [2-3H]glycerol (10.0 Ci/mmol), and carrier-free 32P, were purchased from New England Nuclear.

RESULTS

Molecular Weight Determinations—Previous results in our laboratory had suggested the covalent association of fatty acid with the major outer membrane protein of R. sphaeroides (1). In order to quantitate the stoichiometry of the fatty acid bound to the protein, it was necessary to obtain an accurate molecular weight for the major outer membrane protein. Molecular weight determinations based on a linear relationship between the log molecular weight and relative electrophoretic mobility (Rf) in SDS-polyacrylamide gels were conducted as described under “Materials and Methods.”

To determine if the major outer membrane protein displayed typical behavior in relationship to SDS binding, we initially compared the electrophoretic mobility (Rf) of the protein to a series of water-soluble polypeptides in SDS-gels of varying acrylamide concentrations. A Ferguson plot of Rf versus acrylamide concentration should yield an extrapolated mobility value at 0% acrylamide (Mw) of narrow range for all of the polypeptides examined (24-26). This held true for the water-soluble polypeptides; however, the major outer membrane protein proved to display a Mw value that differed from the anticipated value and was similar to that demonstrated by pepsin, a protein known to bind abnormally low amounts of SDS (27).

Thus, the molecular weight of the protein solubilized and purified in SDS (2) could not accurately be determined by a log molecular weight versus Rf plot in SDS-polyacrylamide gels. Instead, the molecular weight of the major outer membrane protein and standard proteins was determined from the retardation coefficient (Kvc) calculated by unweighted linear regression analysis of Ferguson plots of Rf versus acrylamide concentration. This analysis yielded a value of 39,300 ± 5%.

We had previously reported a method for the solubilization and isolation of the major outer membrane protein which is not based on detergent solubilization (2), but instead employs solubilization by the chaotropic salt guanidine thiocyanate followed by dialysis into urea. Use of this isolation procedure

Fig. 1. Molecular weight determinations of the major outer membrane (om) protein by electrophoretic mobilities. A shows the standard curve obtained for the molecular weight determinations by electrophoretic mobility in SDS-polyacrylamide gels. The line is defined by the equation Kvc = 549 + 0.079 Mw with a correlation coefficient of 0.996. B shows the standard curve obtained for the molecular weight determinations by electrophoretic mobility in 8 M urea-polyacrylamide gels at pH 8.7. The line is defined by the equation Kvc = 4.13 + 0.0485 Mw with a correlation coefficient of 0.988. All procedures were performed as described under “Materials and Methods.”
allowed us to determine the molecular weight of the protein by its electrophoretic mobility in 8 M urea-polyacrylamide gels of varying concentration and the subsequent use of Ferguson plots in the calculation of the retardation coefficients (Fig. 1B). The molecular weight of the polypeptide derived from this technique was 32,200 ± 5%. Combined with the value of 33,800 ± 3% obtained from the amino acid composition (Table I), the three methods yielded an average molecular weight of 35,100, therefore confirming our initial concerns as to the accuracy of the apparent molecular weight of 47,000 determined from the electrophoretic mobility in 10% SDS-polyacrylamide gels. Although averaging the three methods yields a value of 35,100, it could be suggested that a value of 33,000 averaged from the two non-SDS methods is closer to the actual molecular weight due to possible undocumented effects of varying acrylamide concentration on SDS binding, thus altering the extrapolated value of M. This possibility would, as a worse case, represent a difference of approximately 6% in the estimated molecular weight.

Amino Acid Composition and NH₂ Terminus Determination—The amino acid composition of the major outer membrane protein was determined to further characterize the protein in particular as to the presence of a glyceride-cysteine residue (33) as well as to provide another method of molecular weight determination. Table I presents the amino acid composition as mole per cent and mole number (mole of amino acid/mol of protein). The molecular weight was calculated from the mole per cent data (Table I) assuming a common denominator of 1 Arg and 2 Lys. Although no attempt was made to take into account the amide content, examination of the composition reveals that the protein is relatively high in acidic residues. This is in accord with its low isoelectric point of 4.4 (2). Additionally, the protein contains no cysteine or glyceride-cysteine (33). Although it is possible that glyceride-cysteine, due to its lability, may not have been observed during amino acid analysis, the absence of cysteine or its oxidation products is strong evidence for the absence of the glyceride-cysteine residue in the major outer membrane protein. The protein contains 73.4% neutral and hydrophobic amino acids as based on the hydrophobicity scale of Nozaki and Tanford (28). The polarity of the polypeptide is 38.1% as calculated by the method of Capaldi and Vanderkooi (29).

Both of these values are in accord with expected values for an integral membrane protein.

Various attempts to determine the NH₂-terminal amino acid were unsuccessful suggesting that the NH₂ terminus was blocked. Resolution on polyamide thin layer chromatograms as described under "Materials and Methods" of 2 and 10 nmol of hydrolyzed, dansylated major outer membrane protein, isolated by each of the two previously described methods (2), failed to detect any dansyl amino acid derivatives. Although this method does not exclude all possible NH₂-terminal residues, such as tryptophan or proline which are hydrolyzed under acidic conditions, the failure to detect any breakdown products makes this unlikely. The suggested nature of the blocking group will be described later.

Fatty Acid Composition—Previous observations in our laboratory (1) have suggested that the major outer membrane protein contained covalently attached fatty acid. To further investigate this possibility, the protein was exhaustively extracted with chloroform/methanol (2:1) to remove noncovalently attached phospholipid and the hydrolyzed protein was analyzed by gas chromatography. To confirm that potentially contaminating phospholipid was removed, protein isolated from cells whose phospholipids had been labeled with [14C]orthophosphoric acid was subjected to the same procedure. The isolated protein contained no detectable 14C at a level of labeling that would have detected less than 0.5 mol of phospholipid/mol of protein. The protein was hydrolyzed in methanolic sodium hydroxide resulting in the release of 0.17 mol of fatty acid per mol of protein. Hydrolysis of the protein with methanolic sodium hydroxide resulted in the release of 0.17 ± 0.09 mol of fatty acid/mol of protein, indicating that the fatty acid is bound to the protein via a more alkali-resistant

### Table I

Amino acid composition of the major outer membrane protein

| Amino acid | Mole per cent | Mole No. |
|------------|---------------|---------|
| Aspartic acid | 13.2 | 44.7 |
| Threonine | 6.1 | 20.8 |
| Serine | 5.6 | 19.1 |
| Glutamic acid | 7.9 | 26.6 |
| Proline | 1.0 | 3.5 |
| Glycine | 17.5 | 59.2 |
| Alanine | 11.0 | 37.4 |
| Valine | 7.0 | 23.6 |
| Methionine | 0.4 | 1.3 |
| Isoleucine | 4.6 | 5.6 |
| Leucine | 8.8 | 29.9 |
| Tyrosine | 5.5 | 18.5 |
| Phenylalanine | 6.9 | 20.4 |
| Histidine | 1.8 | 6.1 |
| Lysine | 2.3 | 7.8 |
| Arginine | 1.2 | 4.0 |
| Tryptophan | 5.6 | |
| Cysteine | 0 | 0 |

### Table II

Fatty acid composition of the major outer membrane protein and the phospholipids of *R. sphaeroides*

| Per cent of the total fatty acid | 16:0 | 16:1 | 18:0 | 18:1 |
|-------------------------------|------|------|------|------|
| Whole cell phospholipid | 3.5 ± 0.2 | 1.0 ± 0.4 | 6.5 ± 1.0 | 80.0 ± 1.9 |
| Cytoplasmic membrane phospholipid | 8.0 ± 0.5 | 1.0 ± 0.3 | 6.0 ± 0.9 | 85.0 ± 1.6 |
| Outer membrane phospholipid | 6.5 ± 1.0 | 6.0 ± 0.9 | 12.0 ± 1.6 | 82.4 ± 1.7 |
| Major outer membrane protein | 7.8 ± 0.4 | 0 | 17.4 ± 3.4 | 74.5 ± 3.9 |
| Moles fatty acid/mol protein | 1.22 ± 0.42 | | | |

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amide linkage rather than through an alkali-sensitive ester linkage (18, 30).

Several attempts to label the cells with [2-3H]glycerol as described under "Material and Methods" resulted in the label being primarily localized in the phospholipid of the outer membranes (Fig. 2). Although label was detectable in a protein in the outer membrane which could be analogous to the Braun lipoprotein (1), as well as polypeptides at 15, 17, and 20 kD which could be analogous to the peptidoglycan-associated lipoproteins identified in Escherichia coli by Mizuno and Kageyama (31) and Mizushima and co-workers (32), no label was detectable in the major outer membrane protein above general background levels. Although not conclusive, these results together with the absence of cysteine would suggest that a glycerol moiety was not attached to the protein.

Previous studies in our laboratory (1) employing a [14C]acetate label have indicated the localization of fatty acyl material in the major outer membrane protein. To characterize the label for future studies, fatty acid methyl esters were prepared from the major outer membrane protein isolated from cells grown on [14C]acetate as described under "Materials and Methods." As shown in Fig. 3, 10 major peptides were resolved by two-dimensional mapping on cellulose thin layer plates and eluted from the cellulose with 20% acetic acid-containing peptide. Electrophoresis was performed in the horizontal direction (cathode to the right) at pH 6.5 for the tryptic peptides pH 3.7 for the chymotryptic peptides, and chromatography in the vertical direction. Fifteen µg of each digest was resolved.

Peptide Analysis—In order to localize the fatty acid moiety on the major outer membrane protein, peptide analysis was employed. Tryptic and chymotryptic digests of the major outer membrane protein purified from cells grown on [14C]acetate were resolved by two-dimensional mapping on cellulose thin layer plates as described under "Material and Methods." As shown in Fig. 3, 10 major peptides were resolved from the digestion of the major outer membrane protein in the presence of 2% trypsin (w/w) for 6 h at 37 °C. Although not in exact accord with the number of peptides predicted from the amino acid composition (Table I), the agreement is good due to the increased probability in a protein of this size for the occurrence of adjacent residues, such as carboxyl proline, aspartic acid, or glutamic acid, to modify the specificity or rate of hydrolysis by the enzyme (10). Digestion of the protein with 4% chymotrypsin for 6 h at 37 °C resulted in the chromatographic resolution of 22 peptides. Accurate prediction of the number of peptides expected is difficult since the action of chymotrypsin is dependent on factors outside the immediate environment of the peptide bond cleaved (10). Quantitation of the amount of radioactivity in each peptide of both digests (Table IV) localized the bulk of the [14C]acetate label in one peptide in each digest, thus indicating that the fatty acid is covalently attached to a unique position on the protein. In the case of the tryptic peptides, the average background radioactivity derived from [14C]acetate was 4965 cpm/peptide and for chymotrypsin 960 cpm/peptide. In both cases, this is significantly lower than that in the presumptive fatty acid-containing peptide.

Chymotryptic peptides were scraped from the thin layer plates and eluted from the cellulose with 20% pyridine. Dansylation of the peptides as described under "Materials and Methods" failed to detect a dansyl derivative from the [14C]acetate peptide, as opposed to other peptides derived from the protein which gave rise to dansylated amino acid derivatives under the same conditions (Table IV). Although this was not a rigorous cataloging of the amino peptide termini for many of these chymotryptic peptides, it demonstrated that under these conditions we could detect an NH2-terminal residue. The inability to detect an NH2-terminal residue in the fatty acid-containing peptide suggested that further investigation of the NH2-terminal structure was essential and clearly established the possibility that the fatty acid was the blocking

![FIG. 2. Autoradiogram of an SDS-polyacrylamide gel of R. sphaeroides outer membrane isolated from cells grown on [3H]glycerol as described under "Materials and Methods." Lanes 1 and 2, outer membrane solubilized at room temperature and 75 °C, respectively. Each lane contained 5 x 10^6 cpm. Numbers on the left are molecular weights (×10^5). PL, phospholipids.](http://www.jbc.org/)
Recovery of amino acids from the aqueous phase and hexane. Amino acid analysis showed the presence of tyrosine, and phenylalanine residues, but also after a variety of fatty acids. The chymotryptic peptide, which presumably lacks a primary amine, is detectable at the 1-5 nmol level, whereas the fatty acid-hydrolyzed pronase-digested peptide was hydrolyzed in 6 N HCl for 4 h at 110 °C. Partitioning between the aqueous phase and hexane resulted in 93.2% of the radioactivity being recovered in the organic phase (Table VI). Dansylation and resolution on polyamide plates of the aqueous phase as described under “Materials and Methods” gave rise to a dansyl derivative which co-migrated with authentic dansylalanine, the identity and amount of which was determined by amino acid analysis. Quantitation of the methylated organic phase by gas chromatography identified 0.9 mol of fatty acid/mol of amino acid with the fatty acid co-migrating with C16:0 standard.

Taken together these results indicated that the major outer membrane protein contains a covalently associated fatty acid, 1 mol of which is present per mol of protein. The fatty acid was determined to be amide-linked to an NH-terminus of a fatty acid containing the protein. Because the protein was found to contain 1.22 ± 0.42 mol of fatty acid/mol of protein, the possibility of a second fatty acid being associated with some fraction of the protein was considered.

### Table IV

| Peptide | Counts/min | Recovery of applied counts | Peptide | Counts/min | Recovery of applied counts |
|---------|------------|----------------------------|---------|------------|----------------------------|
| 1       | 29,783     | 71.6%                      | 1       | 1,010      | 67%                        |
| 2       | 4,080      |                            | 2       | 1,001      |                            |
| 3       | 6,192      |                            | 3       | 906        |                            |
| 4       | 1,836      |                            | 4       | 1,364      |                            |
| 5       | 4,266      |                            | 5       | 1,271      |                            |
| 6       | 7,722      |                            | 6       | 765        |                            |
| 7       | 3,871      |                            | 7       | 1,517      |                            |
| 8       | 8,151      |                            | 8       | 1,096      |                            |
| 9       | 7,007      |                            | 9       | 1,096      |                            |

### Table V

| Amino acid | Mole per cent | Mole No. |
|------------|---------------|----------|
| Aspartic acid | 0.2 | 0.01 |
| Threonine | 0.3 | 0.01 |
| Serine | 18 | 0.97 |
| Glutamic acid | 0.2 | <0.1 |
| Proline | 0.03 | <0.1 |
| Glycine | 57.9 | 3.1 |
| Alanine | 13.8 | 0.74 |
| Valine | 0.05 | <0.1 |
| Isooleucine | 0.05 | <0.1 |
| Leucine | 9.3 | 0.5 |
| Tyrosine | 0.05 | <0.1 |
| Phenylalanine | 0.07 | <0.1 |
| Histidine | 0.03 | <0.1 |
| Lysine | 0.05 | <0.1 |
| Arginine | 0 | <0.1 |
| Moles fatty acid/mol peptide | 0.99 ± 0.17 |

### Table VI

| Peptide                    | Total recovery of counts/min in fatty acid-containing fraction | Per cent of total recovered counts/min |
|---------------------------|---------------------------------------------------------------|---------------------------------------|
| Protein                   | 100                                                          | 100                                   |
| Chymotryptic peptide      | 40                                                           | 42.8                                  |
| Organic extractable pronase-digested peptide | 90.8 | 41.3 |
| Organic extractable acid-hydrolyzed pronase peptide | 90.3 | 31.4 |
| Moles fatty acid/mol aqueous extractable acid-hydrolyzed pronase peptide | 0.9 ± 0.2 |
major outer membrane protein cannot be completely excluded. However, the data make it unlikely. As a worse case, if the 33% of the nonrecovered counts from the chymotryptic peptide map were localized in one peptide which we failed to identify, this could result in an additional fatty acid on only a fraction of the protein molecules. This is a worse case situation since a number of the counts not accounted for must also exist as background labeling of the peptides also unaccounted for, as we see in Table IV a low level of radioactivity is present in all the peptides examined. Although unlikely, this possibility has not been completely excluded. Nonetheless, it is clear that we can conclude that each molecule of the major outer membrane protein has an NH₂-terminal fatty acid linked by an amide bond to the NH₂-terminal L-alanine residue.

DISCUSSION

Previous investigations from our laboratory have led to the isolation of the major outer membrane protein of R. sphaeroides (1). This report presents the initial physical and chemical characterization of that protein with particular emphasis on the covalently associated lipid. Isolation of the protein by two methods (2), guanidine thiocyanate-urea solubilization and SDS solubilization, allowed us to analyze the protein by a variety of procedures, both in the presence and absence of detergent.

Examination of the electrophoretic mobility of the protein in SDS-polyacrylamide gels of varying acrylamide concentrations revealed that the protein demonstrated anomalous mobility in comparison to water-soluble protein standards, thus indicating a nonlinear relationship between the electrophoretic mobility of the protein and the log of the molecular weight. This is in agreement with the anomalous SDS binding that has been described for a variety of acidic and membrane proteins (3-6, 27). Determination of the molecular weight by alternative methods (see “Results”) confirmed that the protein exhibited a retarded mobility on 10% SDS-polyacrylamide gels, thus giving an apparent molecular weight (47,000) that was significantly higher than the actual molecular weight (35,100).

An expected for a membrane protein, the amino acid composition was primarily neutral and hydrophobic amino acids (Table I), with a correspondingly low polarity (38.1%). Fatty acid analysis of the isolated protein confirmed our initial suggestion (1) that the protein contained a covalently associated fatty acid which was present in a molar ratio of 1.22 ± 0.42 mol of fatty acid/mol of protein (Table II). The fatty acid composition reflected that of the whole cell phospholipids (Table II), which in agreement with previous studies (34, 36), contain a high proportion of cis-vaccenic acid. The acid lability, combined with the general alkaline resistance of the fatty acid linkage indicated the presence of an amide-linked fatty acid, since the conditions employed for alkaline hydrolysis have previously been shown to be specific for ester-linked acyl chains (30).

Two-dimensional peptide maps (Fig. 3) of tryptic and chymotryptic digests of the [¹⁴C]facetate-labeled major outer membrane protein revealed the fatty acid to be associated with one peptide in both digests (Table IV). Inability to detect a dansylated derivative from either the isolated protein or the isolated fatty acid-containing peptide, coupled with the general resistance to alkaline cleavage of the fatty acid linkage, indicated a possible amide linkage involving the NH₂-terminal amino acid. Amino acid analysis of the fatty acid-containing peptide (Table V) which revealed the presence of serine, glycine, alanine, and leucine residues supported this conclusion due to the lack of an available amino acid side chain to form an amide linkage. Further, because of the general alkali stability of the fatty acyl linkage, it was unlikely that the fatty acid was ester-linked to the hydroxyl of serine. The fatty acid was present in a molar ratio of 0.99 mol of fatty acid/mol peptide (Table V). The similarity of the molar ratio to that of the intact protein (1.22 ± 0.42 mol of fatty acid/mol of protein) indicated that the fatty acid present in the major outer membrane protein is essentially fully recovered in the NH₂-terminal peptide.

Pronase digestion of the fatty acid-containing peptide yielded an organic extractable residue. Attempts to dansylate this residue failed to detect a dansyl amino acid derivative. However, following acid hydrolysis of the chloroform/methanol extract of the pronase-digested isolated chymotryptic peptide and partitioning between the aqueous phase and hexane, chromatography of the dansylated aqueous phase on polyamide plates resulted in the detection of dansylalanine. This result was confirmed and quantitated by amino acid analysis. Gas chromatography of the methylated organic phase revealed the presence of fatty acid in a molar ratio of 0.9 mol/mol of amino acid.

Although the possibility of a second fatty acid covalently associated with a fraction of the major outer membrane protein has not been completely excluded, taken together these results indicate that the major outer membrane protein contains 1 mol of fatty acid/mol of protein with the fatty acid covalently attached via an amide linkage to an NH₂-terminal L-alanine residue, thus explaining the inability to detect a dansylated NH₂-terminal amino acid from the protein.

Amide linkage of fatty acids to protein have previously been identified in the E. coli lipoprotein by Hantke and Braun (33). Proteins analogous to the Braun lipoprotein have been identified in a variety of other Gram-negative bacteria (3, 37). Additionally, a series of peptidoglycan-associated lipoproteins in E. coli, P. aeruginosa, Proteus mirabilis, and other Gram-negative bacteria which contain both NH₂-terminal amide-linked fatty acids and a glyceride-cysteine moiety have been identified (32, 38, 39). Initial results with glycerol labeling (Fig. 2) indicate the presumptive identification of this type of lipoprotein in R. sphaeroides as well.

The occurrence of N-acylphosphatidylserine which contains a fatty acid amide linked to the serine head group has been recently reported by Donohue et al. (34) in the phospholipids of R. sphaeroides. It has been suggested that this phospholipid and the major outer membrane protein could be amidated by the same mechanism or that the N-acylphosphatidylserine could serve as an intermediate in the acylation of the major outer membrane protein (34).

Fatty acids covalently attached to proteins have been reported by Schlesinger and co-workers (40-43) in Sindbis virus and vesicular stomatitis virus, as well as in the membranes of chicken embryo fibroblasts. These fatty acids are ester-linked to an amino acid residue, such as serine or threonine, within the protein with the molar ratio varying from protein to protein. Covalently attached fatty acids have also been reported in the membrane proteins obtained from human red cells and polymorphonuclear cells, as well as several rat tissues (44). These appear to be mainly ester-linked fatty acids, although a small proportion may be amide-linked (44); however, the exact nature of the linkages has not been reported. In both instances (40-44), the post-translational attachment of fatty acids at specific sites on the protein has been hypothesized to direct, insert, and anchor the proteins into the membrane. This finding suggests that this class of lipoproteins may be widespread in nature, serving as a structural entity to stabilize the interaction of proteins in biological membranes.

Similarities exist between the major outer membrane protein of R. sphaeroides, the matrix protein of E. coli, and...
Protein F of *P. aeruginosa* (31, 45). Both of these latter proteins have been shown to be peptidoglycan-associated on the basis of differential SDS solubility (31, 45). The *R. sphaeroides* major outer membrane protein demonstrated differential SDS solubility, which is similar to the matrix proteins, and by analogy has been suggested to be peptidoglycan-associated (2).

The data provided here suggest that the major outer membrane protein of *R. sphaeroides* is a new class of lipoprotein which is more analogous to the porin proteins than to the peptidoglycan-associated lipoproteins. This is supported by the similarities in properties of the matrix proteins and the major outer membrane protein and the lack of glycerol-cysteine moiety in the *R. sphaeroides* protein, as found in the peptidoglycan-associated lipoproteins (31–33). The major outer membrane protein could represent a *R. sphaeroides* protein which contains a covalently associated fatty acid to stabilize its interaction with the hydrophobic regions of the membrane or possibly with hydrophobic regions of the small molecular weight polypeptides with which it has been demonstrated to interact (1). Further characterization of the major outer membrane protein which is in progress in this laboratory, as well as the polypeptides with which it interacts, should extend our knowledge of *R. sphaeroides* outer membrane physiology and structure.

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