Isolation and Partial Characterization of the Major Outer Membrane Protein of *Chromatium vinosum*

BENJAMIN C. LANE and RONALD E. HURLBERT

Immunology and Rheumatic Disease Section, Department of Medicine, School of Medicine, University of Southern California, Los Angeles, California 90033; and Department of Bacteriology and Public Health, Washington State University, Pullman, Washington 99164

The 42,000 major outer membrane protein of *Chromatium vinosum* was purified by a combination of ion-exchange chromatography, gel filtration, and isoelectric focusing. Upon isoelectric focusing, the final material produced four major bands. Three of the four bands were isolated and analyzed for similarity or differences. Protease peptide maps and cyanogen bromide maps of the three isoelectric species were identical. When the isolated isoelectric species were refocused, each produced multiple isoelectric species, suggesting that the procedure used was generating the multiple charged species. Protease treatment of the isolated outer membrane produced a 31,000 fragment from the 42,000 protein. This fragment was isolated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Although the amino acid compositions of the 42,000 protein and its 31,000 trypsin fragment were different, their polarity index was the same (45%). The amino-terminal sequences of the 42,000 protein and the 31,000 trypsin fragment were identical, and it was concluded that the amino-terminal was buried in the membrane.

The outer membranes of all gram-negative bacteria examined contain one to six dominant proteins (3, 6, 12, 15). A number of these dominant proteins have been isolated and partially characterized from *Escherichia coli* (4, 5, 22), *Proteus mirabilis* (17), *Salmonella typhimurium* (16), and *Pseudomonas aeruginosa* (15). In many cases the function of these proteins is known, but in others no physiological role has been found for them (3, 15).

Recently, we have isolated the outer membrane of *Chromatium vinosum* by several techniques, all of which yield identical protein profiles upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (13). Based on SDS-PAGE analysis in several systems, the outer membrane of *C. vinosum* appears to contain a single dominant protein with an apparent molecular weight of 42,000 (9, 13). However, when the outer membrane from a gram-negative species or strain is first isolated and characterized by SDS-PAGE, it is necessary to rigorously establish whether each band of interest in an acrylamide gel is composed of a single polypeptide species or whether it contains two or more comigrating proteins. As the early studies with the *E. coli* outer membrane show, confusion can result if this is not done (19, 20). Since the major outer membrane protein of *C. vinosum* produces multiple isoelectric species upon isoelectric focusing (13), it can be argued that these represent different (in vivo) proteins of very similar molecular weight or that they are artifacts of the technique, such as have been observed by others (14, 18, 24). Therefore, a study was undertaken to isolate the major outer membrane protein of *C. vinosum* so as to determine the nature of the multiple isoelectric species and to obtain material for basic chemical characterization, and for future studies on the physicochemical and functional aspects of the protein.

In addition, a comparison of the major outer membrane protein and its 31,000 molecular weight trypsin fragment (13) was undertaken to determine information on the orientation of the protein in the membrane and the nature of the exposed and buried portions.

MATERIALS AND METHODS

Growth and medium. *C. vinosum* was cultured photoanaerobically on malate as previously described (11). Cells were harvested during the late exponential phase and were stored at -70°C.

Outer membrane. Outer membranes were purified from French press-broken cells by the Triton X-100 procedure reported earlier (15) and were stored at -20°C in deionized water.

Purification of the 42,000-dalton outer membrane protein. A 100-mg amount of cell wall protein was suspended to 10 mg/ml in 2% Triton X-100-10 mM EDTA-10 mM Tris-hydrochloride (pH 7.5) and stirred for 30 min at room temperature. The superna-
tant obtained after centrifugation at 100,000 × g for 60 min was made 0.09 M in NaCl and was loaded onto a column (2 by 25 cm) of diethylaminoethyl-Sephadex equilibrated with 0.1% Triton X-100-0.09 M NaCl-0.01 M Tris-hydrochloride (pH 7.5). The column was washed with two volumes of the equilibration buffer, and the bound protein was eluted with four column volumes of a 0.1 to 1.0 M NaCl linear gradient in 0.1% Triton X-100-0.01 M Tris-hydrochloride (pH 7.5). The protein-containing fractions were pooled, dialyzed for 48 h against deionized water, and concentrated by flash evaporation. The protein was further concentrated, and residual detergent was removed by precipitation with five volumes of acetonitrile followed by standing at −20°C for 12 h.

Fractions which were enriched in the 42,000-dalton protein were pooled, and 30 mg of protein was solubilized in 3 ml of 2% SDS-5% 2-mercaptoethanol (2-ME)-10 mM Tris-hydrochloride (pH 7.5) by heating at 100°C for 3 min. This material was passed through a column (2.5 by 75 cm) of BioRad A (0.5 M) with 0.1% SDS-0.1% 2-ME-0.5 mM EDTA-0.02% sodium azide-10 mM Tris-hydrochloride (pH 7.5) as eluate. Protein fractions enriched in the 42,000 protein were pooled, dialyzed, and concentrated as described above and were further purified by isoelectric focusing. Up to 1 mg of protein was applied to each isoelectric focusing gel (6 mm by 11.5 cm). Focusing was performed for 6.5 h at 0.2 W per tube. The focused gels were extruded from the glass tubes and proteins were lightly stained in 0.01% Coomassie brilliant blue R250 in methanol-water-acetic acid (vol/vol/vol: 5:5:1) for 2 h. The four isoelectric bands of the 42,000 protein were sliced from the gel.

To recover the protein from the isoelectric gel slices, we packed it into glass tubes (6 mm by 12 cm) containing a 1-cm acrylamide plug on the bottom and to which a dialysis bag with a 5,000 molecular weight cutoff was attached with Parafilm. The proteins were eluted into the dialysis bag at 2.0 mA/tube for 16 h by using the running buffer from the Laemmli electrophoresis system (13). The eluted proteins were dialyzed 24 h at 4°C against deionized water to remove the buffer components, and the proteins were concentrated by acetone precipitation before storing frozen at −20°C in deionized water.

Purification of the 31,000 trypsin fragment of the 42,000 outer membrane protein. Outer membranes were suspended to 20 mg/ml of protein in 10 mM Tris-hydrochloride, pH 7.5. Trypsin was added to 10 mg/ml, and the membranes were incubated at 37°C for 60 min. After incubation, the membranes were washed three times with 10 mM Tris-hydrochloride, pH 7.5. The trypsinized membranes were solubilized in the SDS-PAGE sample buffer for 3 min at 100°C, and 3-ml portions of protein were separated by electrophoresis on a 12% polyacrylamide slab gel (3 mm by 10 cm by 14 cm) with a 1-cm stacking gel (13). After electrophoresis, the slab was lightly stained to locate the protein band. The region containing the 31,000 trypsin fragment was cut out of the gel, and the protein was recovered by electroelution as described above.

Chemical analysis. Protein, total carbohydrate, amino sugar, phosphorus, and amino acid analyses were performed as previously described (13).

Polyacrylamide gel analysis. SDS-PAGE was performed as described previously by Hui and Hurlbert (8).

The isoelectric focusing procedure of O’Farrell (18) was used with the following changes. Samples containing no more than 10 mg/ml of protein in 2% SDS-5% (wt/vol) 2-ME-0.05 M Tris-hydrochloride (pH 6.8) were heated to 100°C for 3 min in a capped tube. The cooled sample was made 2% (wt/vol) in Ampholines (LKB), 8% in Triton X-100, 8 M urea, and equal volumes of pH 3.5 to 5.5 and pH 4 to 6 Ampholines to a final concentration of 2%, and overlaid with sample overlay solution (18). Focusing was performed at 0.15 W/tube for 6.5 h at 20°C.

One-dimensional fingerprints. Partial protease digests of the purified 42,000 isoelectric species was done as described by Cleveland et al. (2). Pancreatic protease, ficin, and trypsin were incubated for 1 h at 37°C, with the purified proteins at protein-to-enzyme ratios of 500:1, 2,000:1, and 2,000:1, respectively. After heating at 100°C in the presence of SDS and 2-ME, the digests were separated on 15% polyacrylamide gels and stained with Coomassie brilliant blue R250.

Amino-terminal sequence analysis. The procedure of Weiner et al. (23) was used, with a single modification, to determine the amino-terminal sequence of purified proteins. When known proteins were analyzed, a fluorescent yellow spot due to SDS contamination was observed in the position of bis-trypsin for all proteins. To remove this spot, we extracted samples once with 80% acetonitrile after trichloroacetic acid precipitation of the dansylated protein. The protein was then washed with in 0.1 N HCl and acid hydrolyzed as described previously (22).

RESULTS

Purification of the 42,000 outer membrane protein. Ion-exchange chromatography appeared to separate the outer membrane proteins on the basis of size. Proteins with apparent molecular weights of 10,000 to 20,000 were observed in the material which washed through the DEAE column at 0.1 M NaCl and immediately after the NaCl gradient was begun. The majority of the 42,000 protein eluted in a protein peak between 0.15 and 0.35 M NaCl (Fig. 1A). The material eluting after the 42,000 protein peak was enriched in proteins with apparent molecular weights greater than the 42,000 protein. Based on spectrophotometric scans of Coomassie brilliant blue-stained gels, the proportion of 42,000 protein increased from 55 to 65% in the outer membrane to 70 to 75% of the DEAE protein fraction. In preliminary studies, an attempt was made to reuse the DEAE-Sephadex after washing in strong salts, but good separation could only be achieved when freshly prepared DEAE-Sephadex was used. The reason for this was not investigated.

The 42,000 protein eluted from agarose as the predominant peak (Fig. 1B, fractions 21 through
27). The small peak eluting at the void volume contained proteins with apparent molecular weights of 55,000 or greater (Fig. 1B), and the trailing peak (fractions 28 through 30) contained proteins of less than 42,000 molecular weight. Agarose gel filtration removed almost all of the protein with apparent molecular weights of 55,000 or greater from the 42,000 protein fraction and enriched the 42,000 protein to 80 to 85% of the total protein.

Isoelectric focusing of the 42,000 material from the agarose chromatography produced four major bands designated, respectively, as 42Ka, 42Kb, 42Kc, and 42Kd (Fig. 2A). The 42Kb, which was the predominant isoelectric species, was purified to 92 to 95% purity as judged by scans of the SDS gels (Fig. 2B). The 42Kd protein, which was the most contaminated isoelectric species (Fig. 2B), was recovered in the smallest amount.

Analysis of the 42,000 isoelectric species. To determine whether the four isoelectric species represented polypeptides with significantly different amino acid compositions, the three most pure proteins (42Ka, 42Kb, and 42Kc) were subjected to protease fingerprinting. In this procedure purified proteins are digested with a variety of proteases, each of which produces a unique pattern of peptide fragments on SDS-PAGE. If two proteins are alike, they will produce the same unique pattern with each particular protease used; whereas, if they are different, the patterns will be dissimilar. Pancreatic protease, ficin, and trypsin each generated a unique peptide map with the three isoelectric species, and the peptide maps of the three isoelectric species were identical with each individual protease (Fig. 3). Since the three proteases recognized different parts of the primary structure and yet the three isoelectric species produced identical peptide maps, it is clear that the isoelectric species are essentially the same and vary only slightly in charge.

Cyanogen bromide hydrolysis of 42Ka, 42Kb, and 42Kc confirmed that these isoelectric species were similar. Analysis of the hydrolysates by polyacrylamide gel electrophoresis revealed two broad smears with average molecule weights of 5,000 and 8,000 for each of the three isoelectric species (data not shown). Because of the large number of methionine residues present in the 42,000 protein (Table 1), it was not possible to
estimate the molecular weight from the cyanogen bromide fragments.

Since the three isoelectric species appeared to be very similar, and because the difference in these proteins can only be detected by isoelectric focusing, it seemed likely that the isoelectric species were the same protein with small charge differences. To determine whether these differences were inherent in the protein as synthesized by the cell or were induced by the isoelectric focusing procedures, we refocused each of the isolated species. In the former case each original species should give rise to a single band with the original pI; whereas in the second case, each species should produce multiple isoelectric species. When the 42K, 42Kb, and 42Ke isoelectric species were refocused, each produced multiple isoelectric species (Fig. 4). This shows that the isoelectric species of the 42,000 protein were a result of some modification caused by the isoelectric focusing procedure and are not naturally present in the cell.

Chemical analysis of the 42K species. Because the 42K isoelectric species was obtained in the largest amounts and with the least contamination, it was selected for further analysis of its primary structure. Phosphorus was undetectable in the purified protein, and only a trace of hexosamine (4.5 μg of hexosamine per mg of protein) was detected. Periodic Schiff staining of 42K after SDS-PAGE did not detect any carbohydrate in the protein band even when

**TABLE 1. Amino acid composition of the 42,000 outer membrane protein and its trypsin fragment**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>42,000 Protein</th>
<th>31,000 Trypsin fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol%</td>
<td>mol/mol of protein</td>
</tr>
<tr>
<td>Asp</td>
<td>17.0</td>
<td>69</td>
</tr>
<tr>
<td>Thr</td>
<td>6.0</td>
<td>25</td>
</tr>
<tr>
<td>Ser</td>
<td>6.8</td>
<td>28</td>
</tr>
<tr>
<td>Glu</td>
<td>6.9</td>
<td>28</td>
</tr>
<tr>
<td>Pro</td>
<td>2.3</td>
<td>10</td>
</tr>
<tr>
<td>Gly</td>
<td>13.8</td>
<td>56</td>
</tr>
<tr>
<td>Ala</td>
<td>10.6</td>
<td>43</td>
</tr>
<tr>
<td>½ Cys</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Met</td>
<td>3.0</td>
<td>12</td>
</tr>
<tr>
<td>Leu</td>
<td>7.2</td>
<td>29</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.7</td>
<td>19</td>
</tr>
<tr>
<td>Phe</td>
<td>3.6</td>
<td>15</td>
</tr>
<tr>
<td>Try</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lys</td>
<td>4.8</td>
<td>20</td>
</tr>
<tr>
<td>His</td>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>Arg</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>Mol wt</td>
<td>42,725</td>
<td>30,824</td>
</tr>
</tbody>
</table>

* Determined after performic acid oxidation.
* ND, Not done.
* Calculated from the amino acid composition.
* Based on Capaldi and Vanderkooi (1).
500 μg of protein was electrophoresed. However, a typical pattern for C. vinosum lipopolysaccharide (LPS) (10) was observed when as little as 20 μg of purified protein was electrophoresed, and this contaminating LPS may account for the hexosamine measured.

Amino-terminal analysis of the 42Ko protein showed that there was a single terminal amino acid. Beginning with the amino-terminal position, the sequence was: NH2-Asx-A1a-X-Leu-Bistyr. The third amino acid from the amino-terminal position chromatographed in the same region as did phenylalanine and methionine, but it did not cochromatograph exactly with either amino acid. The manual Edman degradation was not attempted beyond the fifth position.

The molecular weights calculated from the molar amino acid composition for 42Ko and 31,000 trypsin fragment agreed closely with the SDS-PAGE-determined molecular weights of 42,000 and 31,000, respectively (Table 1). The polarity of 42Ko and the 31,000 fragment, as calculated by the procedure of Capaldi and Vanderkooi (1), was 45% for both proteins.

**DISCUSSION**

The data presented here show that the four isoelectric species are artifacts of the isoelectric focusing process and verify that the outer membrane of C. vinosum contains only a single major protein. This conclusion is based upon the observations that each of the isoelectric species tested: (i) produces the same protease fingerprint pattern; (ii) produces the same cyanogen bromide hydrolysis pattern; and (iii) gives rise to multiple isoelectric species when refocused. The reason for the four isoelectric species of the 42,000 protein is unknown, but artistic charge heterogeneity has been observed to occur with samples stored frozen (18) or with proteins heated with SDS (27). Both treatments were applied to our samples. In addition, multiple isoelectric species of other outer membrane proteins have been observed (14). However, if this were the case it is difficult to explain why the isolated material from each band (which should represent a specific charge species) refocused as several bands, unless the isolated material lost the charge heterogeneity during isolation and only regained it during refocusing. Alternatively, the multiple isoelectric species may be the results of associations between the 42,000 peptide or between the polypeptide and the contaminating LPS (see below). Similar results have been reported for the II* outer membrane protein of E. coli K-12, and it has been suggested that the isoelectric heterogeneity is a result of both aggregation and interaction during isoelectric focusing of the protein with dodecyl sulfate, LPS, or phospholipid (7, 21). If this is the case, it is clear that such associations are not random, but are the result of specific interactions. Otherwise, the material would not have focused as distinct bands, either originally or after purification and refocusing.

Amino acid analysis of the 42Ko protein and the tryptic fragment of the 42,000 protein indicated that this integral membrane protein has a higher polarity than the majority of membrane proteins described by Capaldi and Vanderkooi (1). A similar observation was made by Garten et al. (5) for three outer membrane proteins of E. coli which had polarities of 43 to 44%. It is interesting to note that the 31,000 fragment has the same polarity index as the 42,000 protein. If it is assumed that the trypsin-sensitive portion extends out of the membrane into the hydrophilic environment and thus is hydrophilic in nature, it would follow that removal of this portion would result in the integral membrane portion (the 31,000 fragment) becoming less polar. However, as this is not the case, it appears likely that the amino acid sequence and the folding of the 42,000 protein are more responsible for its integral membrane nature than the relative ratios of polar or nonpolar amino acids.

Although the polarity of the 42,000 protein and its tryptic fragment were the same, there were some differences in their amino acid compositions. The mole percent contribution for most of the amino acids was only slightly changed. However, lysine, histidine, and arginine increased substantially in the tryptic fragment; whereas aspartic acids and asparagine were considerably lessened.

The fact that both the 42,000 protein and its tryptic fragment have the same amino-terminal amino acid sequence indicates that it is the carboxyl terminal end of the protein that is exposed to the environment. It is of course possible that the amino-terminal portion is also exposed but that it does not contain any sequences susceptible to the trypsin. However, data from a previous study which showed that Streptomyces griseus protease also produced an identical 31,000 fragment suggest that this is not likely (13). In this context it is of interest to note that the N-terminal portion of the II* outer membrane protein of E. coli K-12 also is protected from proteolytic digestion (7).

Even the most pure 42,000 protein isolated in this study is contaminated with LPS. All attempts to free the material of LPS were unsuc-
cessful, and it is not clear whether this is due to a tight association between the 42,000 protein and LPS or simply due to the co-purification of LPS along with the protein. We feel the latter is most likely since previous studies with C. vinosum LPS have shown that it spreads through the gel in both SDS-PAGE (10) and in isoelectric focusing (13).

ACKNOWLEDGMENT

We thank S. Gurusiddiaiah of the Analytical Lab for his technical assistance.

LITERATURE CITED