Mechanism of Localization of Major Outer Membrane Lipoprotein in *Escherichia coli*

**STUDIES WITH THE OmpF-LIPOPROTEIN HYBRID PROTEIN**

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Fujio Yu†, Hideo Furukawa‡‡, Kenzo Nakamura¶, and Shoji Mizushima††

From the Laboratories of ††Microbiology and ‡‡Biochemistry, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

A chimera gene consisting of the *ompF* promoter, the coding regions for the signal peptide and the NH₂-terminal 11 amino acid residues of outer membrane OmpF protein, and the coding region for the major outer membrane lipoprotein devoid of the NH₂-terminal 7 amino acid residues was constructed. *Escherichia coli* carrying the cloned chimera gene produced a hybrid protein with the predicted chemical structure. The protein was localized in the periplasmic space with an interaction with the peptidoglycan layer. These results indicate that the hybrid protein was expressed, secreted across the cytoplasmic membrane, and processed for the signal peptide normally. The hybrid protein, however, was not incorporated into the outer membrane, suggesting the importance of the lipid domain in the assembly of the lipoprotein into the outer membrane. Although a larger part of the protein was extractable with sodium dodecyl sulfate, a part of the hybrid protein was covalently bound to the peptidoglycan layer as the lipoprotein is. Upon treatment with lysozyme of the envelope the hybrid protein became water soluble. The solubilized protein most probably existed as a trimer.

These results most likely suggest that the major lipoprotein exists as a trimer in the periplasmic space with interactions with the peptidoglycan layer through the protein domain on one side and with the outer membrane through the lipid domain on the other side.

The cell envelope of Gram-negative bacteria consists of three layers. They are the outer membrane, the peptidoglycan layer, and the cytoplasmic membrane. The biogenesis of outer membrane proteins that are synthesized in the cytoplasm, therefore, involves export across the cytoplasmic membrane and translocation to the outer membrane through the peptidoglycan layer.

Among the outer membrane proteins, the major lipoprotein found by Braun (1) is especially interesting in terms of the biochemical events involved in its biogenesis. The lipoprotein is first synthesized as a precursor form containing the NH₂-terminal signal peptide (2). The precursor is then modified with glyceride at the cysteine residue that becomes the NH₂-terminus of the mature form (3). The glyceride-containing precursor accumulates in the cytoplasmic membrane when cells are treated with globomycin (3). The signal peptide is then cleaved off by lipoprotein signal peptidase localized in the cytoplasmic membrane (37), which is followed by acylation of the newly exposed amino group of the cysteine residue and then translation to the outer membrane (4, 5). A part of the lipoprotein is covalently linked through the COOH-terminal lysine to the peptidoglycan layer at a certain stage of the biosynthetic pathway (5–7). In spite of these biochemical studies, the mechanism of translocation and assembly into the outer membrane of the lipoprotein is still unclear.

In the present study, we constructed a chimera gene coding for a protein consisting of the signal peptide and the NH₂-terminal 11 amino acid residues of outer membrane protein OmpF and the COOH-terminal 51 amino acid residues of the lipoprotein (major part of the lipoprotein) to investigate its localization. The hybrid protein was processed for the signal peptide and secreted across the cytoplasmic membrane normally. However, it stayed in the periplasmic space without translocation into the outer membrane. We also found that the hybrid protein existed as a trimer that showed an interaction with the peptidoglycan layer. These findings are discussed in regard to localization and orientation of the major lipoprotein in the cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-[4,5-³H]Leucine (specific activity, 136 Ci/mmol) and L-[4,5-³H]lysine (specific activity, 70 Ci/mmol) were purchased from Amersham International Ltd. Dimethyl suberimidate was from Sigma, DEAE-cellulose from Serva Feinbiochemica GmbH. & Co. and acrylamide from Eastman Kodak Co. Restriction endonucleases *TaqI*, *Sau3AI*, *PvuII*, *BamHI*, *HindIII*, and *BglII* were from Takara Shuzo Co., *TrpI* (EC 3.4.21.4; bovine pancreas) and *lysozyme* (EC 3.2.1.17; hen egg white) were from P-L Biochemicals, Inc., *T4 ligase* from Takara Shuzo Co., and bacterial alkaline phosphatase from Worthington. A low molecular weight calibration kit (Pharmacia Fine Chemicals) was used as molecular weight standard. All other reagents were of reagent grade.

**Bacterial Strains and Media**—*Escherichia coli* J5512 (HfrC, man++, ttr), its lipoprotein-negative (lpp) derivative, J5513 (8), and T19 (K-12 F−, ttx ompB supE) (9) were used. Unless otherwise stated, cells were grown on M9, 0.4% glucose, 0.03% yeast extract (Difco) medium. For preparation of plasmids, cells were grown on L broth. Ampicillin (50 µg/ml) was added for growth of the plasmid-harboring bacteria.

**Plasmid Construction**—Purification of plasmid DNA was carried out as described by Birnboim and Doly (10). The outline of the plasmid construction is shown in Fig. 1. Plasmid pPHF02* was constructed from the Laboratories of Microbiology and Biochemistry, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan.

† Present address, Fermentation Research Laboratories, Sankyo Co., Ltd., Hitotsubashi, Shinagawaku, Tokyo 140, Japan.
‡ To whom correspondence should be addressed.

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†† Present address, Fermentation Research Laboratories, Sankyo Co., Ltd., Hitotsubashi, Shinagawaku, Tokyo 140, Japan.
§§ To whom correspondence should be addressed.
Fig. 1. Construction and structure of plasmid pHF100. A, pHF100 was constructed from pHF002 and pKEN125. represents the chromosomal DNA region carrying the ompF promoter (ompFp) and the coding regions for the signal peptide and the NH₂ terminus of the OmpF protein. represents the chromosomal DNA region carrying the promoter-operator region of the lac operon (lacp), and represents the chromosomal DNA region carrying the lpp gene. In pKEN125 the lpp gene is split into the promoter (lppp) and the coding regions, and the latter is under the control of the lac promoter-operator. The direction of the transcription is shown by arrows. For details of the nucleotide sequences of individual fragments, see Refs. 11 and 36. A', ampicillin resistant; T', tetracycline resistant. Restriction sites: E, EcoRI; S, Sau3AI; B, BglII; Ba, BarnHI; T, TthHB8I (TaqI); H, HincII. B, detailed structure of the ompF-lpp chimera gene in pHF100. The arrow shows the region that was sequenced for confirmation of the structure. indicates the position of 3²P-labeling at the 5'-end (HincII site) for sequencing. The first letter of the translation initiation codon is numbered +1. The Sau3AI site at the right end is approximately +500. lpp', transcription termination signal of the lpp gene. C, structure of the hybrid protein (precursor form) deduced from the DNA sequence. a.a, amino acid residues.

Preparation of Subcellular Fractions—Cells were grown to a density of 5 × 10⁸ cells/ml and treated with EDTA and lysozyme to form spheroplasts. EDTA-lysozyme treatment of JE5512 cells was performed according to the method of Mizushima and Yamada (15), and that of JE5513 and JE5513/pHF100 according to the same method modified by Mizuno and Kageyama (16). Spheroplasts were collected by centrifugation at 5000 × g for 10 min, and the supernatant that contained the outer membrane vesicles and periplasmic proteins was further centrifuged at 100,000 × g for 60 min to obtain the periplasmic fraction as the supernatant. The pellet (crude outer membrane) was suspended in water, dialyzed against 1 M Tris-Cl, and the preparation (precipitated by centrifugation) was subjected to 35–53% (w/w) sucrose density gradient centrifugation to obtain the purified outer membrane as described (15). The spheroplasts were fractionated into the cytosol and membrane fractions, and the cytoplasmic membrane was prepared from the latter fraction by 35–53% (w/w) sucrose density gradient centrifugation as described (15).

Purification of the ompF-lpp Hybrid Protein—The hybrid protein-containing cells (JE5513/pHF100) were sonicated, and the cell-free supernatant was treated with lysozyme (12 mg in 2.4 ml) at 30 °C for 1 h. After centrifugation at 100,000 × g for 30 min, the supernatant was dialyzed against 10
mm Tris-HCl (pH 8.0) and applied to a DEAE-cellulose column (0.8 × 20 cm) equilibrated with the same buffer. Protein was eluted with a 200-ml linear gradient of 0–0.2 M NaCl in the same buffer. The hybrid protein in the eluate was monitored by polyacrylamide gel electrophoresis. Fractions eluted with 0.05–0.1 M NaCl were combined, precipitated with acetone (final 80%), and dissolved in 1 ml of 10 mM sodium phosphate buffer (pH 7.2). It was further purified on a Sephadex G-75 column (2 × 40 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.2), 0.5 M NaCl. Fraction 30 to 37 shown in Fig. 6 were combined and used as the purified preparation.

Cross-linking—Protein cross-linking was carried out according to Davies and Stark (17). Samples (30 µg of protein/150 µl) were incubated with 450 µg of dimethyl suberimidate in 0.2 M triethanolamine-HCl (pH 8.5) at 25 °C for 60 min and dialyzed against 10 mM Tris-HCl (pH 8). Trypsin treatment of the cross-linked complexes was carried out in 10 mM Tris-HCl (pH 8) containing 50 µg/ml of trypsin at 37 °C for 2 h. The cross-linked preparations were analyzed by SDS-polyacrylamide gel electrophoresis.

Amino Acid Sequencing—Sequential Edman degradation was carried out as described (18). Amino acid phenylthiohydantoins were analyzed by high performance liquid chromatography with a Hitachi 638-50 equipped with a LS-410 detector. Amino acid phenylthiohydantoins were analyzed by high performance liquid chromatography with a Hitachi 638-50 equipped with a LS-410 detector.

RESULTS

Expression of the ompF-lpp Hybrid Protein—To confirm the structure of the chimera gene constructed in the present work, the TaqI-HincII fragment indicated in Fig. 1 was isolated from pHF100, and the DNA sequence that covers the ompF-lpp joint region was determined. The results were consistent with the gene structure given in Fig. 1 (data not shown), confirming that the chimera gene consists of the ompF promoter, the coding region for the signal peptide and the NH2-terminal 11 amino acid residues of the OmpF protein, and the coding region for the lipoprotein devoid of the NH2-terminal 7 amino acid residues.

Fig. 2 shows the expression of the ompF-lpp chimera gene. JE5513/pHF100 labeled with [3H]leucine (136 Ci/mmole) for 15 min. Cells were harvested, suspended in 1 ml of 10 mM sodium phosphate buffer (pH 7.2), and sonicated. After centrifugation at 5000 × g for 5 min to remove unbroken cells, the sonicate was further centrifuged at 100,000 × g for 30 min. The supernatant was designated as the soluble fraction. The precipitate, designated as the envelope fraction, was suspended in 200 µl of water. A portion corresponding to 1.2 × 10⁹ cells was diluted with water to 40 µl and mixed with an equal volume of 2% SDS, heated at 100 °C for 5 min, and immunoprecipitated with the anti-lipoprotein antiserum. The precipitates were dissolved in 10 mM sodium phosphate buffer (pH 7.1), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol at 100 °C for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. A, B, and C, envelope fractions from JE5512, JE5513, and JE5513/pHF100, respectively; D, E, and F, soluble fractions from JE5512, JE5513, and JE5513/pHF100, respectively; G, H, and I, immunoprecipitates of A, B, and C. Each lane contained envelope or soluble fraction from 0.6 × 10⁸ cells (170,000 dpm for an envelope fraction and 800,000 dpm for a soluble fraction). The x-ray film was exposed for 20 days. The position of the lipoprotein (LP) is indicated.

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The abbreviation used is: SDS, sodium dodecyl sulfate.

plasmic fraction, cytoplasmic membrane, and cytosol fraction after EDTA-lysozyme treatment and analyzed for the hybrid protein by SDS-polyacrylamide gel electrophoresis (Fig. 3). The hybrid protein was found only in the periplasmic fraction. These results indicate that the hybrid protein was processed...
and exported normally across the cytoplasmic membrane. Furthermore, the fact that the protein was not localized in the outer membrane suggests that the NH2-terminal portion of the lipoprotein is essential for assembly of the lipoprotein into the outer membrane.

**Noncovalent Interaction of the Hybrid Protein with the Peptidoglycan Layer**—The hybrid protein was found in the envelope fraction when cells were fractionated after sonication (Fig. 2), whereas it was recovered as a soluble periplasmic protein after EDTA-lysozyme treatment (Fig. 3). These results suggested that the solubilization of the protein is a result of EDTA-lysozyme treatment of the cell envelope. The envelope fraction was then treated with EDTA, lysozyme, and sucrose, individually or in combination (Fig. 4). Lysozyme caused the release of the hybrid protein from the envelope, whereas EDTA and sucrose did not. The results indicate that the holding of the hybrid protein to the envelope is due to an interaction with the peptidoglycan layer. Since the hybrid protein was extractable with SDS solution, as seen in SDS-polyacrylamide gel electrophoresis, the interaction was concluded to be noncovalent. The interaction was not interfered with in the presence of 0.2 M NaCl and 20 mM MgCl2.

The Hybrid Protein Can Form a Covalent Linkage with the Peptidoglycan Layer—A part of the lipoprotein exists as a bound form, being covalently linked to the peptidoglycan layer through the ε-NH2 group of its COOH-terminal lysine (1). To investigate whether the hybrid protein also forms a covalent linkage with the peptidoglycan layer or not, cells were labeled with [3H]leucine, and peptidoglycan was isolated from the envelope fraction by hot SDS extraction. The peptidoglycan fraction did not give any band on SDS-polyacrylamide gel except one of large molecular materials that remained on the top of the gel (Fig. 5, lane F). When the peptidoglycan fraction was treated with trypsin or lysozyme, the top band disappeared and new bands appeared at the bottom of the gel (Fig. 5, lanes G and H). The gel profiles were similar to those for the lipoprotein (Fig. 5, lanes A–D). Furthermore, the newly appearing bands were immunoprecipitated with the anti-lipoprotein antiserum (data not shown). Since trypsin specifically splits the arginyl-lysyl bond of the bound form of the lipoprotein to release it (26) and since the lysozyme digestion results in the appearance of bands representing the lipoprotein to which peptidoglycan chains of different lengths are attached (27), the results shown in Fig. 5 must show the presence of the bound form of the hybrid protein which was covalently linked to the peptidoglycan layer in the same manner as the lipoprotein is. The amount of the bound form of the hybrid protein, however, was much smaller than that of the free form (compare lanes E and G in Fig. 5).

**Purification and Characterization of the Hybrid Protein**—The hybrid protein was purified. Details of the purification procedure are given under “Experimental Procedures” and the legend to Fig. 6. The envelope obtained by sonication was incubated with lysozyme to release the hybrid protein, which was then successively purified on DEAE-cellulose and Sephadex G-75 columns. Fig. 6 shows the elution profile of the hybrid protein from the Sephadex G-75 column. The inset in Fig. 6 indicates that the final preparation was contaminated by several minor bands. Since these bands as well as the major band were immunoprecipitated with the anti-lipoprotein antiserum, they must represent the hybrid protein derived from the bound form. It should be noted that the hybrid protein was eluted faster than cytochrome c from the Sephadex G-75 column (Fig. 6). As the molecular weight of cytochrome c is 13,000 and that of the hybrid protein was calculated to be 6914, it is suggested that the hybrid protein exists as an
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Fig. 6. Purification of the hybrid protein by Sephadex G-75 column chromatography. The hybrid protein-containing fraction (15 mg of protein) from a DEAE-cellulose column was applied on a Sephadex G-75 column (2 x 40 cm), and 2.3-ml fractions were collected. Peak fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and the gels were stained with Coomassie brilliant blue (inset). V0, void volume; Vb, bed volume; Cyt, elution position of cytochrome c. The positions of the free form of the hybrid protein (HP) and lysozyme (Ly) in the gel are indicated.

Fig. 7. Cross-linking of the hybrid protein. The hybrid protein (30 μg) from the Sephadex G-75 column was cross-linked with dimethyl suberimidate and treated with trypsin as described under "Experimental Procedures." A, control (without cross-linking); B, cross-linked; C, cross-linked in the presence of 1% SDS; D, trypsin-treated without cross-linking; E, cross-linked and trypsin-treated; F, molecular weight standards: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), α-lactalbumin (14,400). The amount of protein in each lane (A–E) was 15 μg.

oligomer. The purified hybrid protein was cross-linked with dimethyl suberimidate and analyzed on SDS-polyacrylamide gel (Fig. 7). Three rather diffuse bands corresponding to the monomer, dimer, and trimer of the hybrid protein were observed. The band at the trimer position was stained most densely. When the cross-linked sample was subjected to trypsin treatment, the bands became sharper and clearer (Fig. 7, lane E). The SDS-treated sample could not be cross-linked. These results suggest that the hybrid protein exists as a trimer that is dissociable upon SDS treatment and that the diffuse profile was due to the presence in the trimer of the hybrid protein derived from the bound form.

The amino acid sequence of the NH2-terminal region of the hybrid protein was determined by the method of Edman (18). The amino acid sequence was NH2-Ala-Glu-Ile-Tyr-Asn-Lys-Asp-Gly-Asn-Lys-Val, being the same as that of the OmpF NH2 terminus. However, each step of the Edman degradation produced a minor amino acid. The sequence of the minor component was Asn-Lys-Asp-Gly-(Asn)-(Lys)-Val-Asp-, corresponding to that from the fifth amino acid of the hybrid protein. To determine whether the presumed minor peptide is an intrinsic one or an artificial one produced during purification, the hybrid protein labeled with [3H]lysine was directly obtained from SDS-dissolved cells by immunoprecipitation with the anti-lipoprotein immunoglobulin and subjected to sequential Edman degradation (Fig. 8). A peak of tritium first appeared at the sixth cycle and essentially no radioactivity was released before that indicating that the minor peptide was an artifact. Therefore, we conclude that the signal peptide cleavage takes place at the same site for both the hybrid protein and the OmpF protein. Egg white lysozyme was reported to show a certain proteolytic activity (28).

DISCUSSION

The promoter and the coding region for the signal peptide and the NH2-terminal 11 amino acid residues of the ompF gene were substituted for the corresponding region of the lpp gene containing the NH2-terminal 7 amino acid residues by means of in vitro DNA recombination. E. coli cells carrying the ompF-lpp chimera gene on a plasmid expressed a hybrid protein of the predicted chemical structure. The hybrid protein was localized exclusively in the periplasmic space; it was not found in the outer membrane (Fig. 3). These results indicate that the hybrid protein can be expressed, secreted across the cytoplasmic membrane, and processed for the signal peptide normally. A striking result was that the hybrid protein was not incorporated into the outer membrane. This indicates that a region essential for localization of the lipoprotein into the outer membrane is present in the lipid moiety and/or the NH2-terminal 7 amino acid residues of the lipoprotein. Since no significant difference was found in hydrophobicity between the 11 amino acid residues of the OmpF
protein and the 7 amino acid residues of the lipoprotein, it is highly probable that only the lipid domain is responsible for anchoring the lipoprotein in the outer membrane and the protein domain is entirely water soluble in nature. This view is consistent with that of McLachlan (29) who proposed a water-soluble nature of the protein domain based on the coiled-coil structure that was deduced from the primary and secondary structures of the lipoprotein. However, he proposed a dimeric form, this being inconsistent with our trimeric model which will be discussed later. Another possibility that should be examined further is that the lack of the lipid domain may induce a conformational change to the protein domain, which in turn affects the localization in the cell surface.

A part of the hybrid protein was found to be covalently linked to the peptidoglycan layer (Fig. 5). The mode of linkage was the same as that of the lipoprotein, indicating that the NH\textsubscript{2}-terminal region of the lipoprotein is not required for the covalent linkage of the COOH terminus to the peptidoglycan layer. As discussed above, this NH\textsubscript{2}-terminal region is essential for the assembly of the lipoprotein into the outer membrane. It is assumed, therefore, that the assembly into the outer membrane is not a prerequisite for the covalent binding of the lipoprotein to the peptidoglycan. Consistent with this, Ichihara et al. (7) showed that the prolipoprotein which is localized in the cytoplasmic membrane can be covalently linked to the peptidoglycan layer through the COOH-terminal lysine. Another interesting finding in this work was that the hybrid protein exists as a trimer that associates with the peptidoglycan layer (Figs. 4 and 7). It was proposed previously that one molecule of the bound form of the lipoprotein and two molecules of the free form may form a trimer on the peptidoglycan layer (30). The present work supports the presence of such a trimer. It also suggests, however, that a certain fraction of the free form of the hybrid protein forms a trimer by itself, since the amount of the bound form of the hybrid protein in the purified hybrid protein (Fig. 7) and in the envelope (Fig. 5) was seemingly insufficient for formation of such trimers with the free form. Provided this is true, a noncovalent interaction, in addition to the covalent one, must occur between the hybrid protein trimer and the peptidoglycan layer. It should be noted in this respect that another lipoprotein (peptidoglycan-associated lipoprotein) also noncovalently associates with the peptidoglycan (31, 32).

The OmpF protein exists as a SDS-stable trimer that associates with the peptidoglycan layer through the bound form of the lipoprotein (33–35). Therefore, one would assume that the trimeric structure and the interaction with the peptidoglycan layer of the hybrid protein are due to the NH\textsubscript{2}-terminal 11 amino acid residues derived from the OmpF protein. Although there is no direct evidence to exclude the possibility that OmpF protein and its stable association with the peptidoglycan layer. In conclusion, it is highly probable that the major lipoprotein exists as a trimer that associates with the peptidoglycan layer through the protein domain and with the outer membrane through the lipid domain.

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REFERENCES
Mechanism of localization of major outer membrane lipoprotein in Escherichia coli. Studies with the OmpF-lipoprotein hybrid protein.

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