Nine Amino Acid Residues at the NH$_2$-terminal of Lipoprotein Are Sufficient for Its Modification, Processing, and Localization in the Outer Membrane of *Escherichia coli*

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John Ghrayeb and Masayori Inouye

From the Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

We have examined the structural requirements at the NH$_2$-terminal region of the lipoprotein for its assembly in the outer membrane of *Escherichia coli* by constructing a hybrid protein consisting of an NH$_2$-terminal portion of the prolipoprotein, consisting of the signal peptide and 9 amino acid residues of lipoprotein, and the entire β-lactamase sequence. The results from this study indicate that the hybrid protein is modified with glyceride, processed in a globomycin-sensitive step, and localized in the outer membrane. The translocation of the hybrid protein across the cytoplasmic membrane occurs post-translationally and is inhibited by carboxyl cyanide m-chlorophenylhydrazo. Our results, therefore, indicate that the signal peptide and 9 amino acid residues of prolipoprotein are sufficient for its modification, processing, and localization in the outer membrane.

The major lipoprotein of the *Escherichia coli* outer membrane, the most abundant protein in the cells, is produced through several complex post-translational modification steps (1). The lipoprotein is first synthesized as a precursor form, prolipoprotein, which has a 20-amino acid long signal peptide (2). Maturation of the prolipoprotein requires several successive modification and processing steps which result in the assembly of lipoprotein in the outer membrane of *E. coli* (2, 3). The NH$_2$-terminal portion of lipoprotein consists of a glyceride cysteine to which two fatty acids are attached by two ester linkages and one fatty acid attached by an amide linkage (4). A cyclic peptide antibiotic, globomycin, was found to inhibit the cleavage of the signal peptide, resulting in the accumulation of glyceride-modified prolipoprotein in cells treated with this antibiotic (5, 6).

We have previously reported on the effects of changing or deleting amino acid residues in the signal peptide of lipoprotein on the secretion of lipoprotein (7–10). In this paper, we attempted to examine the structural requirements of the lipoprotein for its assembly in the outer membrane of *E. coli*. In particular, we examined the role of the unique NH$_2$-terminal structure on the localization of the lipoprotein in the outer membrane. For this purpose, we have constructed a hybrid protein containing the signal peptide and 9 amino acid residues of the NH$_2$-terminal of lipoprotein linked to the entire sequence of β-lactamase of pBR322. Our results indicate that the hybrid protein is modified, processed, and localized in the outer membrane. We, thus, conclude that a lipid-modified cysteine and 8 more amino acid residues of lipoprotein are sufficient for its interaction with the outer membrane.

**MATERIALS AND METHODS**

*bacterial Strains and Plasmids—* *E. coli* strain JA221 (Δpp^−^ hisdM^−^ trpE5 leuB6 lacI^q^ recA1 F′ lacF^−^ proC^−^)^{11} (11) was used in all experiments as the host cell. Cells were grown in the following media depending on the experiment: M9 media supplemented with glucose (4 mg/ml), tryptophan (20 µg/ml), leucine (20 µg/ml), methionine (2 µg/ml), thiamine (2 µg/ml), MgSO$_4$.7H$_2$O (200 µg/ml), chloramphenicol (10 µg/ml), and ampicillin (50 µg/ml), and 1 broth supplemented with ampicillin (50 µg/ml) and/or chloramphenicol (10 µg/ml).

Plasmids pMH014, pIN-II-B3 Cm$^R$, pIN-III-A-Cm$^R$ (12), and pTG206 (a generous gift from J. Knowles, Harvard University) were used in the plasmid constructions.

**DNA Manipulations—** Isolation of plasmid DNA and various manipulations were carried out as previously described (11). Restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories. DNA polymerase, Klenow fragment, was from New England Nuclear. An EcoRI 12-mer linker oligonucleotide (dCCGGAATTCGCG) was purchased from New England Biolabs. DNA sequencing was carried out by the method of Maxam and Gilbert (13).

**Expression of the Hybrid Lipoprotein-β-Lactamase Gene—** For labeling experiments, 10-ml cultures were grown in M9 media to a Klett reading of 50 (blue filter) at which time IPTG (Sigma) was added to a final concentration of 2 mM. After 20 min, 50 µCi of [35S]methionine (Amersham Corp., 1000 Ci/nmol) were added for 5 min. For labeling with [3H]palmitic acid (New England Nuclear, 250 µCi, 17.6 Ci/nmol), the induction with IPTG was for 10 min and labeling was for 20 min. When globomycin (obtained from Dr. Arai, Sankyo Pharmaceutical Co., Tokyo, Japan) was used, it was added to a final concentration of 100 µg/ml, using 100% ethanol as the vehicle (final concentration 0.05%), 5 min prior to labeling. As a control, ethanol was used without globomycin. Labeling was stopped by quick chilling to 0 °C.

For pulse-chase experiments, after induction for 20 min with IPTG, [35S]methionine (100 µCi/ml) was added. The chase was begun after 10 s of pulse by the addition of nonradioactive methionine to a final concentration of 0.4 mg/ml. 1 ml samples were removed at various times and added to 1 ml of ice-cold stopping solution (7). When CCCP (Sigma) was used, it was added in 100% ethanol (0.05% final concentration) to a final concentration of 30 µM. Ethanol was added without CCCP for control experiments.

Cell disruption was accomplished using a Heat Systems-Ultrasonics Cup Horn Sonicator. Preparation of membrane and soluble fractions and SDS-PAGE analysis were as previously described (7). Osmotic shock was performed according to the method of Neu and Heppel (14) as modified by Koshland and Botstein (15). Fractionation

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1. M. Inukai, J. Coleman, and M. Inouye, manuscript in preparation.
2. J. Coleman and M. Inouye, unpublished data.
3. The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide m-chlorophenylhydrazo.
of cytoplasmic and outer membranes was accomplished by using sucrose density gradient centrifugation (16) or by using sodium lauryl sarcosinate (17). TEM1 β-lactamase was purified according to published procedures (18). Antiserum to β-lactamase was prepared as previously described (19). Immunoprecipitation using anti-β-lactamase serum was performed as previously described (19) except that the carrier was prepared as follows. A 100-ml culture of JA221 lpp−/ Es was grown in L broth to a Klett reading of 30 at which time IPTG was added to a final concentration of 2 mM. After 3 h, the cells were collected, and the membrane fraction was prepared and solubilized in 0.5 ml of 10 mM phosphate, pH 7.0, containing 1% SDS and 10% glycerol.

RESULTS

Fig. 1 shows the construction of a plasmid carrying the DNA sequence coding for a hybrid protein consisting of the signal peptide and 9 amino acids of lipoprotein linked to β-lactamase. The lpp gene was derived from plasmid pH014 where an EcoRI site was introduced in the lpp gene such that digestion with EcoRI removes a major portion of the lpp gene leaving a portion, lpp', coding for the signal peptide and 9 amino acid residues of the lipoprotein. The plasmid was first made amp R, cm R, in order to simplify the later selection of transformants, by inserting the cm R gene into the PstI site of the bla gene in pH014. The source of the β-lactamase DNA was plasmid pTG206 (a generous gift from J. Knowles), which is a derivative of pBR322. One feature of pTG206 is a single base change in the bla gene which created a BstEII site in the region coding for the signal peptide cleavage site. Thus, digestion of pTG206 with BstEII and HaeII released a DNA fragment coding for the mature β-lactamase structure without the signal peptide. This fragment was made blunt-ended and ligated, with the use of 12-mer EcoRI linkers, to pH014 cm R to give pJG202 (Fig. 1). The cm R, amp R phenotype of the transformants indicated that the insertion of the bla DNA was in the correct orientation and reading frame. The plasmid pJG202 carries the lpp promoter and the lac promoter-operator region so that expression of the hybrid protein is controlled by a lac inducer such as IPTG. The addition of IPTG to the cells carrying pJG202 resulted in low level production of a new hybrid protein which migrated slower than authentic β-lactamase in SDS-PAGE, but was only detectable with pulse-labeling with [35S]methionine (data not shown). In order to increase the level of expression, the hybrid DNA between the XbaI and EcoRI site of pJG202 was transferred to an expression plasmid, pIN-III-A-Cm R, which carries a stronger lac promoter than the lac promoter in pJG202 and the lacI gene but is otherwise similar to pJG202. The addition of IPTG to the cells carrying the newly constructed plasmid, pJG310, resulted in the production of the hybrid protein of the same size as in the case of pJG202; however, the level of production was much higher. In fact, the product could be seen with Coomassie brilliant blue staining of SDS-PAGE gels and was the major component of total cellular protein (data not shown). DNA sequencing of the DNA fragment between the XbaI site and the HincII site in the inserted β-lactamase gene in pJG310 confirmed the predicted DNA sequence from Fig. 1 (data not shown). The amino acid sequence of the hybrid protein deduced from DNA sequence is shown in Fig. 2.

Expression of the Hybrid Product—The strain JA221 lpp−/F lacI was used as the host cell to study the expression of the hybrid lipoprotein-β-lactamase gene carried on plasmid pJG310. In the presence of IPTG, growth of the cells in L broth or M9 media was inhibited at 30, 37, and 42 °C, indicating the hybrid protein was lethal to the cells (data not shown).

In order to determine the products from pJG310, exponentially growing cells were grown in the presence or absence of IPTG for 20 min and labeled with [35S]methionine for 5 min at 37 °C. Cells were also labeled with [3H]palmitic acid for 20 min after induction with IPTG for 10 min. When globomycin was used, it was added 5 min prior to labeling. After labeling, the cells were collected and washed, the total membrane fraction was isolated, and a portion was treated with anti-β-lactamase serum. Membrane fractions and/or immunoprecipitates were submitted to SDS-PAGE and autoradiography as described under "Materials and Methods." As seen in Fig. 3A, the addition of IPTG caused the appearance of a new band in the membrane fraction (lane 2) which migrated slower than β-lactamase and was cross-reactive with the anti-β-lactamase serum. The amino acid sequence of the hybrid protein deduced from DNA sequence in Fig. 2 was found to be homologous to the signal peptide region of lipoprotein. The amino acid residues of the lipoprotein are shown in parentheses.

Fig. 2. Amino acid sequence of the hybrid lipoprotein-β-lactamase product deduced from the DNA sequence. LPP, lipoprotein.
Nine Amino Acids of Lipoprotein Are Sufficient for Processing

Localization of lipo-a-lactamase and Its Precursor.—To determine the cellular localization of lipo-a-lactamase more precisely, cells were induced with IPTG for 20 min and labeled with [35S]methionine for 5 min in the presence or absence of globomycin, which was added 5 min prior to labeling. After labeling, the washed cells were fractionated into periplasm, cytoplasm, and cytoplasmic and outer membrane fractions. The various fractions were then subjected to SDS-PAGE and autoradiography as described under “Materials and Methods.”

Neither the lipo-a-lactamase or its precursor was found in the periplasmic fraction (data not shown). However, after sonication of intact cells, a fraction of both products started to appear in the total soluble fractions to a degree which depended on the sonication time (data not shown). Effective conditions of sonication were thus chosen to minimize the release of membrane-associated proteins. When the total membrane fractions from cells labeled in the presence or absence of globomycin were separated into cytoplasmic and membrane fractions using either sucrose density gradient centrifugation (Fig. 4) or sodium lauryl sarcosinate (data not shown), the bulk of the mature lipo-a-lactamase was found in the outer membrane fraction using both methods. This result indicated that a lipid-modified NH2-terminal cysteine plus 8 more amino acid residues of the NH2 terminus of the mature lipoprotein are sufficient to direct the hybrid protein to the outer membrane. The globomycin-accumulated precursor of the hybrid protein, however, was found mainly in the cytoplasmic membrane fraction by detergent fractionation (data not shown) and mainly in the outer membrane fraction using sucrose density gradient centrifugation (Fig. 4). It is likely that the precursor, which was overproduced in these cells, may be loosely associated with the periplasmic face of the cytoplasmic membrane; but due to the apparent strong affinity of the lipid-modified cysteine for the outer membrane, the precursor was found with the outer membrane fraction by the sucrose density centrifugation technique.

Pulse-Chase Analysis of the Maturation of Lipo-a-lactamase.—In order to examine in more detail the secretion of the hybrid protein across the cytoplasmic membrane, cells were induced with IPTG for 20 min followed by pulse labeling with [35S]methionine for 10 sec at 37°C and chasing for 0.5, 1, and 2 min with nonradioactive methionine. The cells were then collected and washed, the total soluble and membrane fractions were isolated and treated with anti-a-lactamase serum, and the immunoprecipitates were then submitted to SDS-PAGE and autoradiography as described under “Materials and Methods.”

As can be seen from Fig. 5, there was an initial accumulation of the precursor, of a higher molecular weight than lipo-a-lactamase, in the cytoplasmic fraction which disappeared during the chase period with a concomitant appearance of the mature lipo-a-lactamase in the membrane fraction. The con-
version of the precursor was virtually complete within 2 min after the initial pulse. A closer examination of the soluble fraction during the pulse (Fig. 5, lane 1) revealed the presence of several bands with faster mobility than the mature product and which disappeared during the chase (Fig. 5, lanes 3 and 4). These bands most likely represent nascent chains which are completed during the chase. This result would explain the observed increase in the level of soluble precursor during the first 30 of the chase (Fig. 5, lane 2). The processing of the hybrid protein is clearly different from that of the lipoprotein, where no accumulation of any soluble precursor is observed (10). In fact, the maturation mechanism of lipo-β-lactamase was more like that of β-lactamase, where an initial accumulation of a soluble precursor was observed during a pulse-chase experiment similar to the one described here (18).

The Effects of CCCP on the Secretion of Lipo-β-lactamase— To examine the energy requirements for the maturation of the lipo-β-lactamase precursor, a pulse-chase experiment was carried out exactly as above, except that the energy poison, CCCP, was added to a final concentration of 30 μM, 5 min prior to the start of the pulse. Preincubation with CCCP resulted in a 10-fold reduction of [15S]methionine incorporation into total cellular protein including lipo-β-lactamase when compared to labeling in the absence of CCCP (data not shown). The effect of CCCP on the maturation of lipo-β-lactamase is shown in Fig. 6. CCCP appeared to slow down dramatically the conversion of the soluble precursor to the mature product. In effect, there was a steady increase in the level of soluble precursor during the chase period, while only a small amount of mature product appeared only after the end of the 2-min chase period. In addition, the precursor started to accumulate in the membrane fraction during the chase. The appearance of the membrane-associated precursor was accompanied by the slow appearance of the mature product. Thus, it is likely that CCCP may have interfered with the translocation across the cytoplasmic membrane as well as the modification and/or processing of the lipo-β-lactamase precursor.

DISCUSSION

To study the structural requirements at the NH₂-terminal region of lipoprotein for its assembly in the outer membrane of E. coli, we have constructed a hybrid protein consisting of an NH₂-terminal portion of the prolipoprotein and the entire β-lactamase sequence.

The results from this study indicate that the precursor form of the hybrid protein is translocated across the cytoplasmic membrane using the prolipoprotein signal peptide and is subsequently modified with glyceride as in the case of lipoprotein. The signal peptide of the glyceride-modified precursor is then cleaved by the prolipoprotein signal peptidase followed by its assembly into the outer membrane. The overproduction of this hybrid protein was found to be lethal to the cells. In addition, the glyceride-modified precursor can be accumulated when cells are treated with globomycin, a specific inhibitor of the prolipoprotein signal peptidase (5). Furthermore, this precursor is most likely loosely associated with the cytoplasmic membrane. Little of the mature hybrid protein or its precursor was found in the periplasmic fraction where β-lactamase is located. Therefore, the presence of the entire β-lactamase sequence in the hybrid protein did not maintain it in soluble form in the periplasmic space. Instead, the lipid-modified NH₂-terminal fragment derived from the lipoprotein seems to have a predominant effect on the localization of the hybrid protein in the outer membrane. One could argue that this result is simply due to the presence of the lipid moiety at the NH₂-terminal end of the hybrid protein which could anchor the protein in the hydrophobic lipid bilayer of the membrane. However, the facts that the hybrid protein was not found in the cytoplasmic membrane and that it existed exclusively in the outer membrane indicate that the specific interaction with the outer membrane may be caused by the NH₂-terminal sequence derived from the lipoprotein in addition to the lipid moiety. In addition, it is known that certain lipoprotein mutants accumulate unmodified prolipoprotein in the outer membrane (8, 9), indicating that although the lipid moiety may greatly stabilize the binding of lipoprotein to the outer membrane, its presence is not essential. It should also be noted that the β-lactamase portion of the hybrid protein is fully active, indicating that the β-lactamase is in the correct conformation for catalytic activity.

Our data indicate that the translocation of the hybrid protein across the cytoplasmic membrane occurs post-translationally since there was accumulation of a soluble precursor which was then converted to the mature product which was assembled in the outer membrane. In addition, the translo-
A similar result has been observed with certain signal peptide mutants of lipoprotein (10), although CCCP did not affect translocation of the wild type lipoprotein (10). On the other hand, CCCP has been shown to completely inhibit the maturation of β-lactamase (18). It appears, therefore, that the translocation mechanism of hybrid precursor bears more resemblance to that of pro-β-lactamase than to that of the wild type lipoprotein.

The presence of the lipoprotein signal sequence and the presence of a short NH2-terminal sequence of the mature lipoprotein are insufficient for directing the mode of translocation, although they are sufficient for the final localization of the hybrid protein. The present data are consistent with our recent results which indicated that the insertion of 8 amino acid residues between the positions 9 and 10 of the wild type lipoprotein sequence did not affect its assembly in the outer membrane (20). In this regard, it would be interesting to construct similar hybrid proteins with even shorter NH2-terminal sequences in order to determine the minimum structural requirement for modification and/or localization of the hybrid protein. In addition, hybrid proteins containing increasingly longer portions of the lipoprotein sequence fused to β-lactamase could be constructed in order to study the effects on translocation. These studies are now in progress and will shed more light on the mechanism of protein secretion across membranes and assembly of the membrane proteins.

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