THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 8, Issue of April 25, pp. 3707-3712, 1980
Printed in U.S.A.

Accumulation of Glyceride-containing Precursor of the Outer Membrane Lipoprotein in the Cytoplasmic Membrane of *Escherichia coli* Treated with Globomycin*

(Received for publication, November 7, 1979)

Musaddeq Hussain, Shigeyuki Ichihara, and Shoji Mizushima‡

From the Laboratory of Microbiology, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464 Japan

The protein accumulated in the cell envelope of *Escherichia coli* treated with globomycin was identified as the precursor of the outer membrane lipoprotein. The prolipoprotein was almost exclusively localized in the cytoplasmic membrane. The prolipoprotein could be immunoprecipitated with antilipoprotein immunoglobulin and could be chased to the lipoprotein both *in vivo* and *in vitro*. Globomycin inhibited the chase. The prolipoprotein contained glycerol and fatty acid residues, whereas no free sulfhydryl group was detected in it. From these results, it is concluded that the prolipoprotein possesses a glyceride which is covalently bound to the cysteine residue in the peptide as the lipoprotein does and that the removal of signal peptide takes place after the modification.

The inhibition of bacterial growth with increasing concentrations of globomycin was accompanied by a gradual increase in the accumulation of the prolipoprotein. Furthermore, growth of the lipoprotein-negative mutant was highly resistant to globomycin. These results strongly indicate that the accumulation of the prolipoprotein in the cytoplasmic membrane causes the death of cells.

The structure of the outer envelope of Gram-negative bacteria is quite intricate. It has cytoplasmic and outer membranes, having a network of peptidoglycan in between (1). The mechanisms of transportation and assembly of the outer membrane proteins are of much interest. The most abundant outer membrane protein of *Escherichia coli*, the lipoprotein, is first synthesized as a precursor form (2). The complete chemical structure of this lipoprotein (3, 4) and the amino acid sequence of its precursor (5, 6) have been determined. The lipoprotein consists of 58 amino acid residues with an unusual amino acid, glycerylcysteine (*S*-(propane-2',3'-diol)-3-thio-2-aminopropionic acid), at the NH_2-terminal position. The precursor contains 20 additional amino acid residues extending from the NH_2 terminus of the lipoprotein (5). The lipoprotein is localized in the outer membrane (7, 8) in two different forms (9, 10): one-third as the bound form covalently linked to the peptidoglycan and the other two-thirds as the free form. However, the mode of transport of the lipoprotein across the cytoplasmic membrane and its assembly into the outer membrane still remain unclear.

* This work was supported by grants from the Ministry of Education, Science and Culture of Japan, and the Nisshin Seifun Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.

Recently a cyclic peptide antibiotic called globomycin was shown to inhibit the growth of some Gram-negative bacteria (11, 12). It was also suggested that in the presence of globomycin a precursor form of the lipoprotein accumulates in the cell membrane (13). With the help of fluorography in polyacrylamide gel electrophoresis, we have found that a precursor of the outer membrane lipoprotein exclusively accumulated in the cytoplasmic membrane of *E. coli* treated with globomycin. We have also found that this precursor contained both glycerol and fatty acid(s) as the mature lipoprotein does. These findings help in the understanding of the mode of processing, transport, and assembly of the lipoprotein.

EXPERIMENTAL PROCEDURES

Materials—Globomycin was a gift from Sankyo Co. Ltd., Tokyo. L-[4,5-^{3}H]Leucine (specific activity, 136 Ci/mmol), L-[4,5-^{3}H]lysine (specific activity, 70 Ci/mmol), [2-^{3}H]glycerol (specific activity, 500 mCi/mmol), [9,10-^{3}H]palmitic acid (specific activity, 500 mCi/mmol), and iodo[2-^{14}C]acetic acid (specific activity, 54 mCi/mmol) were purchased from the Radiochemical Centre, Amersham. Acrylamide was from Eastman Kodak Co. All other reagents were of reagent grade.

Bacterial Strains and Media—*Escherichia coli* B (the wild type strain) and MH61, a lipoprotein-negative mutant, were used. The mutant was spontaneously isolated from the B strain as a globomycin-resistant mutant in nutrient broth containing 5 μg/ml of globomycin. The following *E. coli* K12 strains were also used; JE5512 (Hfr Cavalli *lpp*$^+$ *man-1 pps*), JE5513 (*lpp-2*, formerly *lpo*); a lipoprotein-negative mutant derived from JE5512 (14), E613 (*lpp*$^+$ *thi-1 argE3 his-4 proA2 lacY1 galK2 mtl-1 xyl-5 tsx-29 supE44*? λ^-), E614 (*lpp-3*, formerly *mlpA*) derived from E613 (15), and YA21 (F$^-$ *met leu* λ^-) (16). Strains E613 and E614 were kindly given by H. Wu, University of Connecticut. Cells were grown either in M-9/glucose (0.2% w/v) medium or glycerol/casamino acids medium (16) at 37°C with shaking.

Preparation of Envelope Fractions—To cells growing at the logarithmic phase (3.5×10^8 cells/ml) was added 0.5% (w/v) solution of globomycin in methanol to a final concentration of 5 μg for B strains and 160 μg for K12 strains/ml of culture medium. After a 5-min incubation, [^{3}H]leucine (2 μCi/50 nmol/ml), [^{3}H]lysine (2 μCi/14 nmol/ml), [^{3}H]glycerol (10 μCi/20 nmol/ml), or [^{3}H]palmitic acid (10 μCi/20 nmol/ml) was added. After 15 min of labeling, cells were chilled and harvested. Parallel control experiments were carried out without globomycin. Envelope fractions were prepared by sonication and differential centrifugation as described by Inouye and Guthrie (17). Envelope fractions labeled with [^{3}H]glycerol or [^{3}H]palmitic acid were washed three times with chloroform/methanol (2:1) as described by Hirashima *et al.* (10).

Immunoprecipitation—Antiserum against the lipoprotein of *E. coli* K12 was a gift from Sankyo Co. Ltd., Tokyo. The lipoprotein used as the antigen was purified in our laboratory by the method of Inouye *et al.* (18). The immunoglobulin fraction was prepared from the antiserum by ammonium sulfate precipitation (50% saturation) (19). The final preparation was dialyzed overnight against distilled water and the precipitate formed was removed by centrifugation.

For immunoprecipitation, 100 μl of the envelope fraction solubilized with 1% SDS[1] at 100°C for 5 min was mixed with 1.25 ml of 0.12 M

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

sodium phosphate buffer (pH 7.1), 0.12% SDS and 100 μl of the immunoglobulin preparation. The immunoprecipitation was carried out as described by Halegoua *et al.* (20).

Determination of Amino Acid Sequence—Envelope fractions labeled with [^{3}H]lysine or [^{3}H]leucine were prepared from cells grown in 30 ml of M-9/glucose medium, dissolved with SDS, and immunoprecipitated with antilipoprotein immunoglobulin as described above. The immunoprecipitates were washed twice with 67% acetone, lyophilized, and subjected to the sequential Edman degradation (21) without further purification. Thiazorinone derivative extracted with ethylene chloride from each step of the degradation was dried with a jet of nitrogen and the radioactivity was measured in Bray's scintillator with an Aloka liquid scintillation counter.

Paper Electrophoresis and Paper Chromatography of Amino Acids—Envelope fractions labeled with either [^{3}H]lysine or [^{3}H]leucine were dissolved in 1% SDS at 100°C for 5 min and the peptidoglycan was removed by centrifugation at 100,000 × *g* for 30 min. Proteins were precipitated with 67% acetone and hydrolyzed with 6 N HCl at 115°C for 18 h. The hydrolysate of [^{3}H]lysine-labeled sample (4,700 cpm) was mixed with authentic amino acids (0.1 μmol each), applied on a 60-cm-long strip of Toyo No. 51 paper, and subjected to high voltage electrophoresis with pyridine/acetic acid/water (5:0.2:95, pH 6.5) at 3,000 V for 35 min. Spots visualized with ninhydrin were cut out and the radioactivity was measured in toluene scintillator with an Aloka liquid scintillation counter. The hydrolysate of [^{3}H]leucine-labeled sample (5,100 cpm) was mixed with authentic amino acids (0.1 μmol each), spotted on Toyo No. 51 paper, and developed one-dimensionally with 1-butanol/pyridine/acetic acid/water (15:10:3:12) for 40 h. Spots were visualized and the radioactivity in each spot was counted as described above.

Separation of Outer and Cytoplasmic Membranes—E. coli B growing at the logarithmic phase (3.5 × 10^8 cells/ml) in 200 ml of M-9/glucose medium was pretreated with globomycin (5 μg/ml) for 2 min and then incubated with [^{3}H]leucine (400 μCi in 3 nmol) for 3 min in the presence of globomycin. Longer treatment with globomycin resulted in poor separation of the outer and cytoplasmic membranes. The outer and cytoplasmic membranes were separated by the method of Mizushima and Yamada (16). In case of the globomycin-treated cells, globomycin (5 μg/ml) was also added to the reaction mixture for spheroplast formation to prevent conversion of the prolipoprotein into the mature form.

Carboxymethylation of Envelope Proteins—Cells were grown in glycerol/casamino acids medium and the envelope fraction was isolated (17). The envelope fraction from 2 × 10^{10} cells was dissolved in 0.4 ml of 10 mM sodium phosphate buffer, pH 7.1, containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and incubated at 37°C for 2 h. Envelope proteins then were carboxymethylated as described by Wu *et al.* (22) by using 3 μmol of iodo[^{14}C]acetic acid containing 10 μCi of radioactivity. Envelope proteins were precipitated with 80% (v/v) acetone and analyzed by polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis and Fluorography—Gel electrophoresis in 0.5% SDS with 7.5% (w/v) acrylamide gels as described by Inouye and Guthrie (17) was carried out in a slab gel system with a modification, that is, 0.16% (w/v) *N,N'*-methylenebisacrylamide was used in place of ethylenediacrylate as the cross-linking reagent. After electrophoresis, gels were washed with dimethyl sulfoxide and impregnated with 2,5-diphenyloxazole for fluorography as described by Bonner and Laskey (23). Kodak X-Omat R films were used.

Other Analytical Methods—The amount of protein was determined by the method of Lowry *et al.* (24). The amount of 2-keto-3-deoxyoctonate was determined by the method described by Osborn *et al.* (25).

RESULTS

Identification of a Protein Accumulated in Globomycin-treated Cells as the Precursor of the Outer Membrane Lipoprotein—It has previously been suggested that globomycin induces the accumulation of the precursor of the lipoprotein of the outer membrane (13). Here we proved it to be true. Fig. 1 shows the polyacrylamide gel electrophoretic profiles of envelope proteins labeled with [^{3}H]leucine. The lowest major band in the JE5512 and B strains was identified as the lipoprotein from the following evidence: (i) this band co-migrated with the authentic lipoprotein purified from YA21 according to the method of Inouye *et al.* (18); (ii) this band specifically reacted with antilipoprotein immunoglobulin; and (iii) JE5513 that lacks the lipoprotein did not have this band. Strain MH61 did not have this band either. In globomycin-treated cells a new band appeared slightly above the position of the lipoprotein at the expense of the lipoprotein. This was the supposed position of the prolipoprotein reported by Halegoua *et al.* (26). The new band did not appear in globomycin-treated samples of the two lipoprotein-negative mutants (JE5513 and MH61). In addition, the protein in the new band as well as the lipoprotein specifically reacted with antilipoprotein immunoglobulin (Fig. 1). Fig. 1 also shows that the effect of globomycin was almost completely confined to the lipoprotein. Although some differences in quantity were observed in other major bands, these were not reproducible.

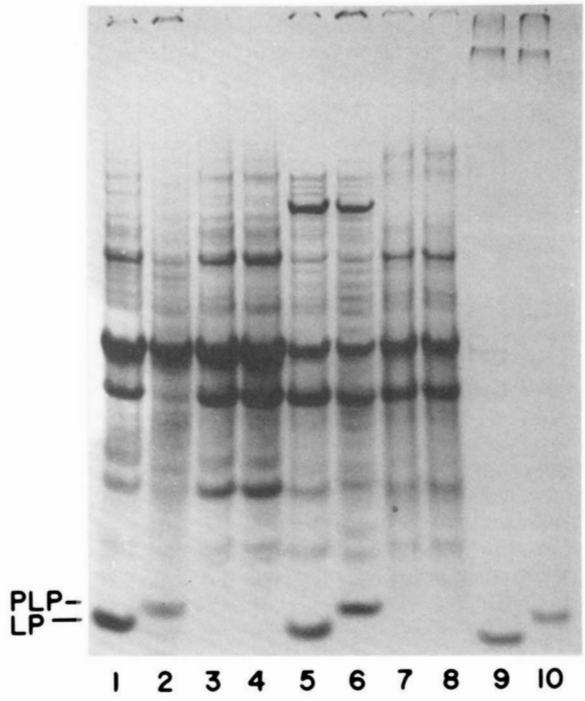

FIG. 1. **Identification of the protein accumulated in globomycin-treated cells as prolipoprotein.** Cells being grown in M-9/glucose (0.2% w/v) medium with or without globomycin (160 μg/ml for K12 strain and 5 μg/ml for B strain) were labeled with [^{3}H]leucine. Envelope fractions were isolated, solubilized in 10 mM sodium phosphate buffer (pH 7.1), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol at 100°C for 5 min, and analyzed on polyacrylamide gel (17). The gel then was processed for fluorography (23). *Slots 1* and *2*, JE5512; *Slots 3* and *4*, JE5513 (*lpp-2*); *Slots 5* and *6*, B strain; *Slots 7* and *8*, MH61. *Slots 1, 3, 5,* and *7*, controls; *Slots 2, 4, 6,* and *8*, globomycin-treated samples; *Slots 9* and *10*, antilipoprotein immunoprecipitates of *Slots 5* and *6*, respectively. The x-ray film was exposed to the gel at −70°C for 7 days. Each *slot*, except *Slots 9* and *10*, contained 30,000 cpm. *Slots 9* and *10* contained 4,000 cpm. *LP*, lipoprotein; *PLP*, prolipoprotein.

In order to determine the NH_2-terminal sequence of this new protein the following experiment was carried out. The envelope fraction prepared from cells grown in the presence of either [^{3}H]lysine or [^{3}H]leucine was solubilized in SDS solution and antilipoprotein immunoprecipitates were obtained and analyzed for the NH_2-terminal sequence according to the method of Edman (21). The amino acid analyses of the total envelope protein revealed that more than 90% of [^{3}H]lysine and [^{3}H]leucine incorporated into the envelope protein existed as lysine and leucine, respectively (data not shown). Fig. 2 clearly shows that the second and the fifth residues were lysine. It also suggests that the sixth and the eighth residues were leucine. Under the conditions employed

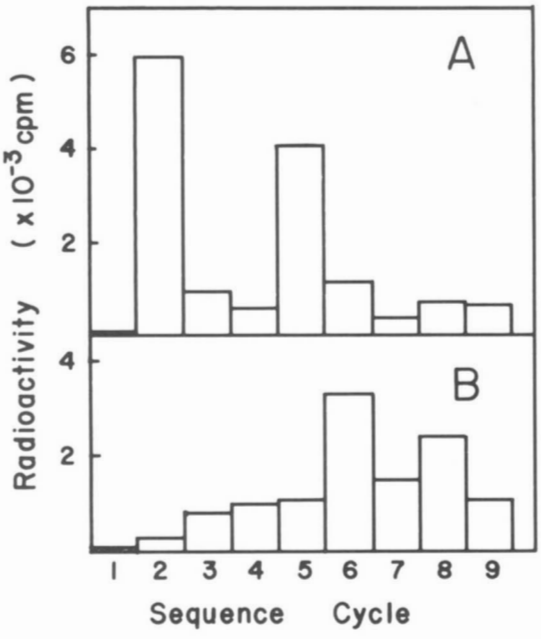

FIG. 2. **Sequential Edman degradation of antilipoprotein immunoprecipitates of cell envelopes from the globomycin-treated wild type B strain labeled with [^{3}H]lysine (*A*) and [^{3}H]leucine (*B*).** Cultivation of cells in the presence of globomycin (5 μg/ml) with either [^{3}H]lysine or [^{3}H]leucine, preparation of envelope fractions, isolation of antilipoprotein immunoprecipitates, and the Edman degradation were carried out as described under "Experimental Procedures." The total radioactivity used for the sequencing was 10^5 cpm in both cases.

incorporation of the radioactivity into the lipoprotein was almost negligible. In addition, the NH_2 terminus of the mature lipoprotein is blocked by a fatty acid residue (3). Therefore, the sequence determined here must represent that of the new protein. This sequence was identical to that of the NH_2 terminus of the prolipoprotein (5, 6).

When cells of *E. coli* B labeled with [^{3}H]leucine in the presence of globomycin were transferred to fresh M-9/glucose medium containing nonradioactive L-leucine and chased, the new band disappeared, giving rise to the lipoprotein within one-fourth of a generation (Fig. 3). Addition of globomycin (5 μg/ml) during the chase inhibited the conversion. When the envelope fraction from cells treated with globomycin was washed with water and incubated in 10 mM sodium phosphate buffer (pH 7.1) at 37°C for 4 h, the band partially disappeared with the simultaneous appearance of the lipoprotein band (Fig. 3). The appearance of the lipoprotein band was again inhibited by globomycin. The recovery of the lipoprotein from the precursor after the chase seemed to be low. This may be accounted for by the facts that (i) the numbers of leucine residues in the prolipoprotein and lipoprotein are 9 and 4, respectively, and (ii) small amounts of radioactivity produce disproportionately faint fluorographic images (27).

From these results we conclude that the new protein was a precursor of the lipoprotein and globomycin inhibited the processing of the precursor into the mature lipoprotein.

Localization of Prolipoprotein in Membranes—The lipoprotein is exclusively localized in the outer membrane (7, 8). Its precursor form could not be detected in the cytoplasmic membrane even by very short pulse-labeling (28). On the other hand, a mutant prolipoprotein having a single amino acid replacement of glycine with aspartic acid in the signal sequence has been reported to be distributed in the cytoplasmic and outer membranes (29). To determine the localization of prolipoprotein in the globomycin-treated cells, [^{3}H]leucine-labeled membranes of *E. coli* B were fractionated into the outer and cytoplasmic membranes. The distribution of 2-keto-3-deoxyoctonate in the outer and cytoplasmic membrane subfractions in relation to protein contents was 0.62 and 0.67 in the outer membranes and 0.1 and 0.09 in the cytoplasmic membranes of globomycin-treated and nontreated samples, respectively. Outer and cytoplasmic membrane fractions gave almost completely different polyacrylamide gel profiles (Fig. 4). These results indicate that the separation of the two membranes was good. The good separation was also confirmed by the evidence that the lipoprotein was exclusively found in the outer membrane fraction. In contrast to the lipoprotein, the prolipoprotein in the globomycin-treated cells was almost exclusively localized in the cytoplasmic membrane (Fig. 4). It should be noted here that, in spite of the localization in the cytoplasmic membrane, the prolipoprotein could not be extracted either with 1% Triton X-100, 10 mM $MgCl_2$ (30) or with 0.5% sodium sarcosinate (31) (data not shown).

Nature of Prolipoprotein—The NH_2-terminal amino acid residue of the lipoprotein is a cysteine which is attached to a diglyceride through its sulfhydryl group (3). To see whether the sulfhydryl group of the cysteine residue, which is the 21st amino acid residue of the prolipoprotein, was free or bound to glycerol or glyceride in the prolipoprotein, cells of *E. coli* B were labeled with [^{3}H]glycerol in the presence of globomycin and the envelope fraction was prepared. After exhaustive

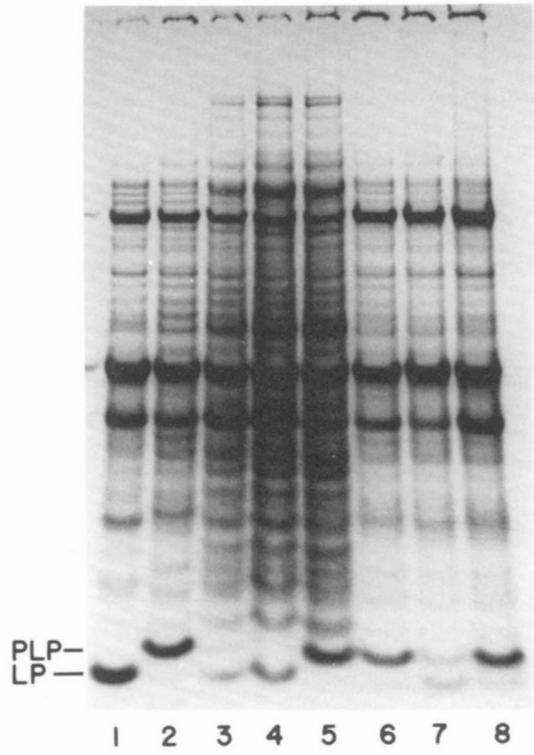

FIG. 3. ***In vivo* and *in vitro* chase of [^{3}H]leucine-labeled prolipoprotein.** Cells of *E. coli* B grown in the presence of globomycin (5 μg/ml) (*Slot 2*) were washed with and suspended in 4 volumes of M-9/glucose medium containing 70 μM L-leucine and chased for one-quarter generation (*Slot 3*) or one generation (*Slot 4*). In one sample, 5 μg/ml of globomycin was added during the chase (*Slot 5*). Envelope fractions were isolated and analyzed as mentioned in Fig. 1. *Slot 1* shows a control sample without globomycin treatment. For the *in vitro* chase, envelope fraction labeled with [^{3}H]leucine in the presence of globomycin (5 μg/ml) (*Slot 6*) was suspended in 10 mM sodium phosphate buffer (pH 7.1) and incubated at 37°C for 4 h in the absence (*Slot 7*) or presence (*Slot 8*) of globomycin (50 μg/ml). The x-ray film was exposed for 3 days. Each *slot* contained 50,000 cpm. *LP*, lipoprotein; *PLP*, prolipoprotein.

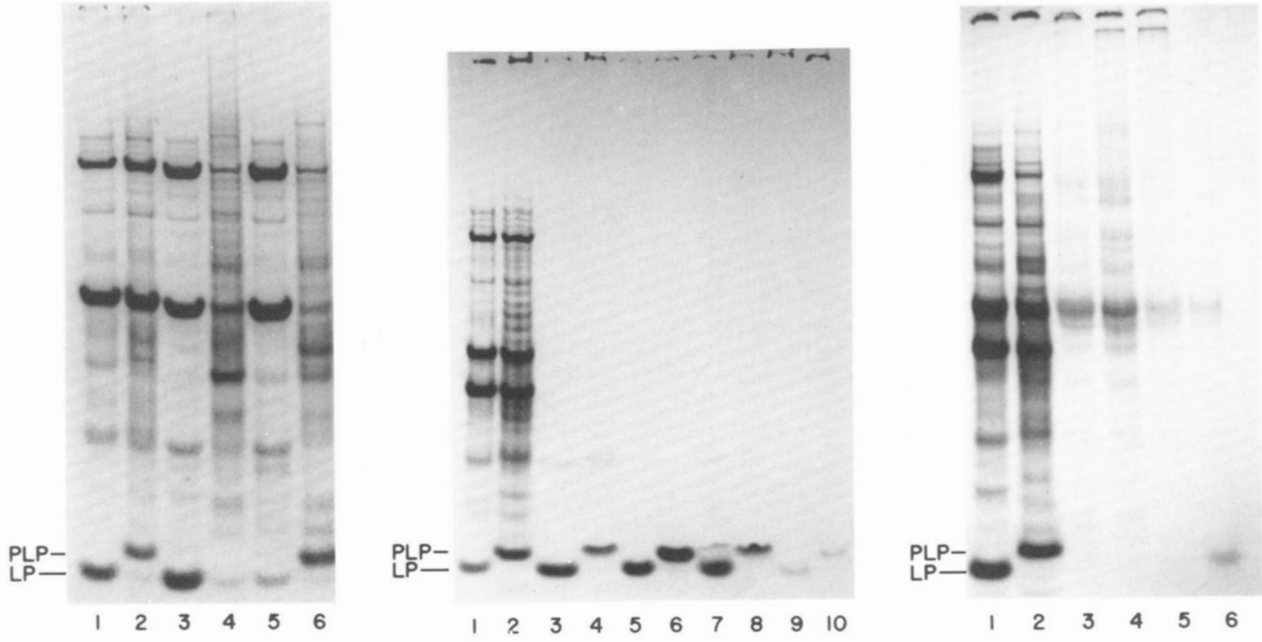

FIG. 4 (*left*). **Localization of prolipoprotein in membrane subfractions.** Outer and cytoplasmic membranes were prepared from *E. coli* B labeled with [^{3}H]leucine in the presence or absence (control) of globomycin and analyzed on SDS-polyacrylamide gel as mentioned in Fig. 1. *Slots 1* and *2*, unseparated envelope fractions of control and globomycin-treated (5 μg/ml) cells, respectively; *Slots 3* and *4*, outer and cytoplasmic membrane fractions from control cells, respectively; *Slots 5* and *6*, outer and cytoplasmic membrane fractions from globomycin-treated cells, respectively. Each *slot* contained 50,000 cpm. The x-ray film was exposed for 5.5 days. *LP*, lipoprotein; *PLP*, prolipoprotein.

FIG. 5 (*center*). **Electrophoretic profiles of cell envelopes labeled with [^{3}H]glycerol or [^{3}H]palmitic acid.** Envelope fractions of *E. coli* B labeled with [^{3}H]glycerol or [^{3}H]palmitic acid in the presence or absence of globomycin (5 μg/ml) were isolated and analyzed on SDS-polyacrylamide gel as mentioned in Fig. 1. *Slots 1* and *2*, [^{3}H]leucine labeling without or with globomycin (20,000 cpm each); *Slots 3* and *4*, [^{3}H]glycerol labeling without or with globomycin (6,000 cpm each); *Slots 5* and *6*, antilipoprotein immunoprecipitates of *Slots 3* and *4* (4,000 cpm each); *Slots 7* and *8*, [^{3}H]palmitic acid labeling without or with globomycin (7,500 cpm each); *Slots 9* and *10*, antilipoprotein immunoprecipitates of *Slots 7* and *8* (2,000 cpm each). The x-ray film was exposed for 8 days. *LP*, lipoprotein; *PLP*, prolipoprotein.

FIG. 6 (*right*). **SDS-polyacrylamide gel electrophoresis of carboxymethylated envelope fractions.** The envelope fractions from 2×10^{10} cells grown in glycerol/casamino acids medium in the presence (*Slots 2* and *4*) or absence (*Slots 1, 3, 5,* and *6*) of globomycin (5 μg/ml) for one generation were treated with iodo[^{14}C]acetic acid as mentioned under "Experimental Procedures." The acetone-precipitated proteins were dissolved in 100 μl of the solubilizing buffer and analyzed on SDS-polyacrylamide gel as mentioned in Fig. 1. The amount of envelope fraction in *Slots 3* to *6* was adjusted so that individual slots contained nearly equal numbers of lipoprotein/prolipoprotein molecules. *Slot 3*, B strain (20 μl, 5,000 cpm); *Slot 4*, globomycin-treated B strain (20 μl, 6,500 cpm); *Slot 5*, E613 (20 μl, 3,500 cpm); *Slot 6*, E614 (5 μl, 2,000 cpm). *Slots 1* and *2*, [^{3}H]leucine-labeled envelope fractions of control and globomycin-treated B strain, respectively; no iodo[^{14}C]acetic acid treatment. *LP*, lipoprotein; *PLP*, prolipoprotein.

removal of lipids with chloroform/methanol (2:1), envelope proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). The ^{3}H label was seen specifically on the prolipoprotein as well as lipoprotein. Phospholipid migrating much faster than the lipoprotein on this gel was hardly seen with the lipid-free samples. A similar experiment with [^{3}H]-palmitic acid showed that the prolipoprotein also contained palmitic acid (Fig. 5). Lipopolysaccharide of the B strain was reported to migrate to almost the same position as the lipoprotein does (32). Under the conditions we employed, lipopolysaccharide migrated slightly faster than the prolipoprotein, making it possible to identify both substances separately. The amounts of glycerol and palmitic acid incorporated into the prolipoprotein were comparable to those incorporated into the lipoprotein in the control cells. The fact that there was no incorporation of [^{3}H]glycerol and [^{3}H]palmitic acid in the other bands, except a few minor bands, indicates that glycerol and palmitic acid were directly incorporated into the prolipoprotein without passing through their catabolized products. Immunoprecipitation experiments confirmed that both glycerol and fatty acid were incorporated into the prolipoprotein (Fig. 5).

As the fluorographic technique used here did not allow accurate estimation of radioactive substances (27), it was uncertain whether the sulfhydryl groups of all of the prolipoprotein molecules were modified by glycerol or not. The prolipoprotein contains only 1 cysteine residue that becomes the NH_2-terminal cysteine residue of the lipoprotein (4, 5). Therefore, the presence of free sulfhydryl group in the prolipoprotein was examined by carboxymethylation with iodo[^{14}C]acetic acid. Strain E614 that possesses the glycerol-free prolipoprotein was used as control. The envelope fractions were isolated, treated with iodo[^{14}C]acetic acid, and analyzed by gel electrophoresis. The amount of envelope fraction used was adjusted so that nearly equal numbers of lipoprotein/prolipoprotein molecules could be applied to individual slots. As shown in Fig. 6, the prolipoprotein of E614 was extensively carboxymethylated as had been shown previously (22), whereas no carboxymethylation was observed of prolipoprotein accumulated in the globomycin-treated B strain as well as the lipoprotein of nontreated B and E613 strains.

All results described here strongly support the view that the protein in question is a prolipoprotein to which glyceride has already been linked covalently through the sulfhydryl group of the cysteine residue. However, the number of fatty acids bound to the glycerol residue has not been determined yet.

Concentration of Globomycin and Accumulation of Prolipoprotein—Fig. 7 shows the effect of different concentrations of globomycin on the cell growth and the accumulation of the

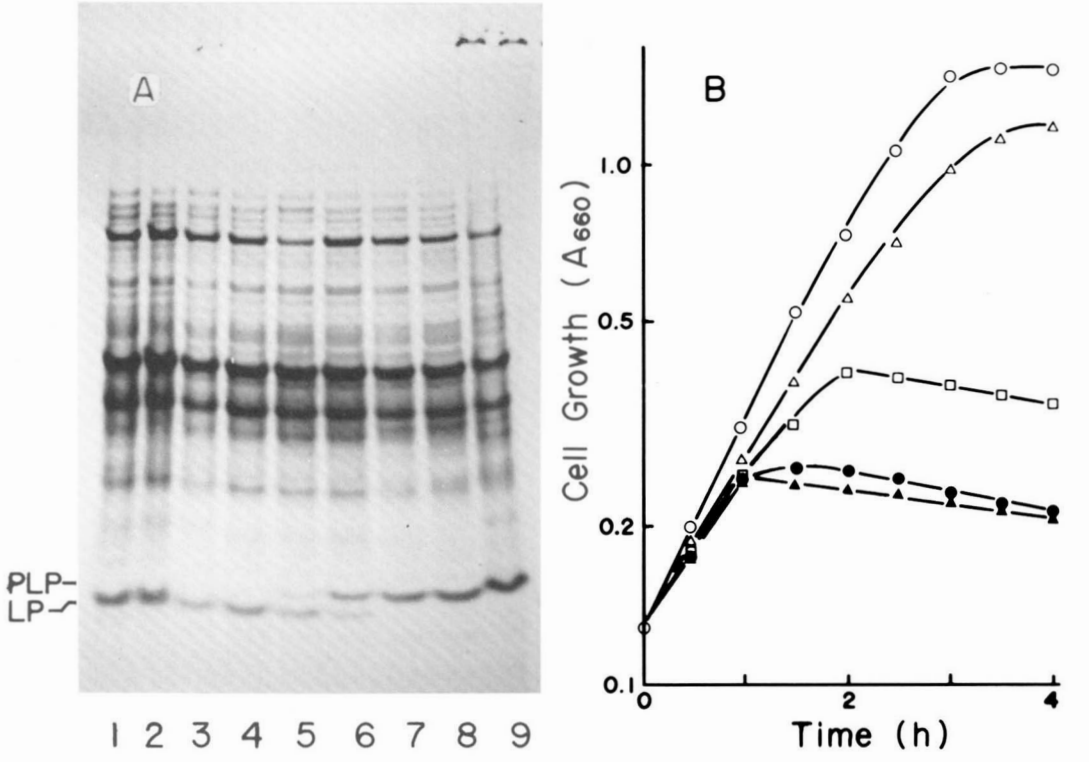

FIG. 7. **Effect of globomycin concentrations on the growth and lipoprotein of *E. coli* B.** *A*, cells being grown in M-9/glucose medium were treated with globomycin and [^{3}H]leucine as described under "Experimental Procedures." The envelope fractions were isolated and analyzed on SDS-polyacrylamide gel as described in Fig. 1. Concentrations of globomycin (in micrograms/ml) were: *Slot 1*, 0.0; *Slot 2*, 0.2; *Slot 3*, 0.4; *Slot 4*, 0.5; *Slot 5*, 0.8; *Slot 6*, 1.0; *Slot 7*, 2.0; *Slot 8*, 4.0; *Slot 9*, 5.0. Each slot contained 20,000 cpm and the gel was exposed to an x-ray film for 7 days. *LP*, lipoprotein; *PLP*, prolipoprotein. *B*, cells were grown in M-9/glucose medium containing the following concentrations of globomycin (in micrograms/ml): ○, 0.0; △, 0.2; □, 0.4; ●, 1.0; and ▲, 5.0. Growth was monitored.

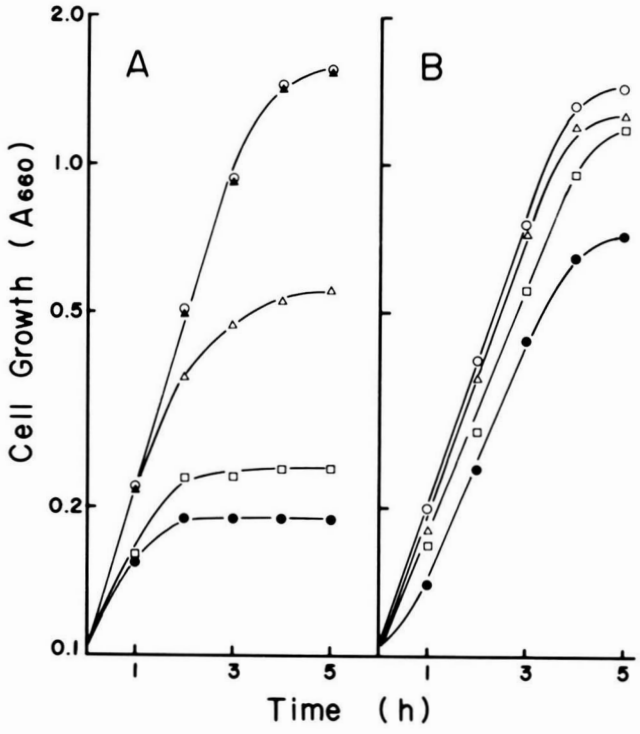

FIG. 8. **Effect of globomycin on growth of JE5512 and JE5513.** Cells were grown in M-9/glucose medium and the growth was monitored. Concentrations of globomycin (in micrograms/ml) were: ○, 0; ▲, 10; △, 40; □, 80; and ●, 160.

prolipoprotein in *E. coli* B. The inhibition of growth with increasing concentrations of globomycin was accompanied by a gradual increase in the accumulation of the prolipoprotein. A similar relationship was also observed with a K12 strain (JE5512), although higher concentrations of globomycin were required (data not shown). These results support the idea that the accumulation of the prolipoprotein in the cell envelope causes the death of cells (13). This idea was further supported by the finding that the lipoprotein-negative mutant JE5513 was highly resistant to globomycin (Fig. 8) and that a globomycin-resistant mutant isolated from the wild type *E. coli* B lacked the lipoprotein (Fig. 1).

DISCUSSION

In this work we showed that the treatment of *E. coli* with globomycin results in the accumulation of the prolipoprotein to which glyceride has already been linked covalently through the sulfhydryl group of the cysteine residue. Although results obtained in this work support the view that the prolipoprotein identified here is a precursor of the lipoprotein, the following possibility cannot be ruled out completely, that is, globomycin induces the accumulation of an abnormally large amount of glyceride-free prolipoprotein and the accumulation of the glyceride-containing prolipoprotein is a result of unusual secondary reactions. However, this is unlikely since, in spite of the accumulation of a large amount of the glyceride-containing prolipoprotein, the prolipoprotein having a cysteine residue with free sulfhydryl group could not be detected (Fig. 6).

One of the interesting findings in this work is that the prolipoprotein was exclusively found in the cytoplasmic membrane. The fact that the prolipoprotein can be chased to the

lipoprotein suggests that this localization of the prolipoprotein is physiologically normal. Therefore, one can claim that the processing of the peptide bond is essential for the transfer of the lipoprotein to the outer membrane. Contrary to this, more than 50% of the mutant prolipoprotein, having an aspartic acid as a substitute for glycine in the signal sequence and possessing no glyceride, was reported to be located in the outer membrane with some in the cytoplasmic membrane (29). Provided that both prolipoproteins are precursors of the lipoprotein, the mutant prolipoprotein must be before the glyceride-containing one in the biosynthetic pathway of the lipoprotein. At the moment it is difficult to explain why more than 50% of the glyceride-free prolipoprotein was found in the outer membrane. The lack of glyceride and the replacement of glycine with aspartic acid in the mutant prolipoprotein make this molecule more hydrophilic. This would help the release of the prolipoprotein from the cytoplasmic membrane.

Results shown in Figs. 7 and 8 strongly indicate that the accumulation of prolipoprotein is the cause of death of cells. It is probable that the accumulation of a large amount of unusual protein in the cytoplasmic membrane disturbs the molecular arrangement of this membrane and causes the death of cells. This idea is supported by the finding of Bassford *et al.* that an *E. coli* strain having a maltose-inducible *malE-lacZ* hybrid protein in the cytoplasmic membrane is maltose-sensitive, *i.e.* the strain cannot grow on maltose medium (33). Bassford *et al.* (33) proposed that this phenomenon can be explained by blocking by the hybrid protein of sites where the *malE* product is normally exported which in turn prevents the secretion of a number of other proteins, causing the inhibition of growth. However, in the present work the accumulation of the prolipoprotein was the only major unusual event in the cell envelope. Therefore, we would rather conclude in our case that the accumulation of the prolipoprotein itself caused the death of cells. Contrary to that of the glyceride-containing prolipoprotein, accumulation of the glycerol-free prolipoprotein is not lethal for cells (29). This may be accounted for by its predominance in the outer membrane. The mode of existence in the cytoplasmic membrane may also be different between these two prolipoproteins.

It should be noted that the prolipoprotein studied here possessed a glyceride residue. This residue is most probably covalently linked to the cysteine residue where the processing of the signal peptide takes place. This suggests that the processing enzyme can cleave the glycyl-glycerylcysteine peptide linkage. Furthermore, this might indicate that the modification of the cysteine residue with glyceride is essential for the processing. In the mutant lipoprotein, the replacement of glycine with aspartic acid was thought to prevent the removal of the signal peptide (29). However, the present work suggests that the following explanation may also be possible; the amino acid replacement prevents the modification of the cysteine residue with glyceride, which is indispensable for the processing of the signal peptide.

Acknowledgements—We thank Dr. M. Arai of Sankyo Co., Ltd. for globomycin and antilipoprotein serum, Dr. N. Takahashi for her help in NH_2-terminal sequence analysis, Mr. K. Watabe for preparation of the lipoprotein, and Drs. H. C. Wu and Y. Hirota for bacterial strains.

REFERENCES

1. Costerton, J. W., Ingram, J. M., and Cheng, K. J. (1974) *Bacteriol. Rev.* **38,** 87–110
2. Halegoua, S., Sekizawa, J., and Inouye, M. (1977) *J. Biol. Chem.* **252,** 2324–2330
3. Hantke, K., and Braun, V. (1973) *Eur. J. Biochem.* **34,** 284–296
4. Braun, V. (1975) *Biochim. Biophys. Acta* **415,** 335–377
5. Inouye, S., Wang, S., Sekizawa, J., Halegoua, S., and Inouye, M. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1004–1008
6. Pirtle, R. M., Pirtle, I. L., and Inouye, M. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2190–2194
7. Braun, V., and Bosch, V. (1973) *FEBS Lett.* **34,** 302–306
8. Lee, N., and Inouye, M. (1974) *FEBS Lett.* **39,** 167–170
9. Inouye, M., Shaw, J., and Shen, C. (1972) *J. Biol. Chem.* **247,** 8154–8159
10. Hirashima, A., Wu, H. C., Venkateswaran, P. S., and Inouye, M. (1973) *J. Biol. Chem.* **248,** 5654–5659
11. Nakajima, M., Inukai, M., Haneishi, T., Terahara, A., and Arai, M. (1978) *J. Antibiot.* **31,** 426–432
12. Inukai, M., Nakajima, M., Ōsawa, M., Haneishi, T., and Arai, M. (1978) *J. Antibiot.* **31,** 421–425
13. Inukai, M., Takeuchi, M., Shimizu, K., and Arai, M. (1978) *J. Antibiot.* **31,** 1203–1205
14. Hirota, Y., Suzuki, H., Nishimura, Y., and Yasuda, S. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1417–1420
15. Yem, D. W., and Wu, H. C. (1978) *J. Bacteriol.* **133,** 1419–1426
16. Mizushima, S., Yamada, H. (1975) *Biochim. Biophys. Acta* **375,** 44–53
17. Inouye, M., and Guthrie, J. P. (1969) *Proc. Natl. Acad. Sci. U. S. A.* **64,** 957–961
18. Inouye, S., Takeishi, K., Lee, N., DeMartini, M., Hirashima, A., and Inouye, M. (1976) *J. Bacteriol.* **127,** 555–563
19. Mayer, R. J., and Walker, J. H. (1978) in *Techniques in the Life Sciences* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I., and Tipton, K. F., eds) Vol. B1/II, pp. B119/8, Elsevier/North-Holland, Amsterdam
20. Halegoua, S., Hirashima, A., and Inouye, M. (1974) *J. Bacteriol.* **120,** 1204–1208
21. Iwanaga, S., Wallén, P., Gröndahl, N. J., Henschen, A., and Blombäck, B. (1969) *Eur. J. Biochem.* **8,** 189–199
22. Wu, H. C., Hou, C., Lin, J. J. C., and Yem, D. W. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1388–1392
23. Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
25. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) *J. Biol. Chem.* **247,** 3962–3972
26. Halegoua, S., Hirashima, A., Sekizawa, J., and Inouye, M. (1976) *Eur. J. Biochem.* **69,** 163–167
27. Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* **56,** 335–341
28. Kanazawa, H., and Wu, H. C. (1979) *J. Bacteriol.* **137,** 818–823
29. Lin, J. J. C., Kanazawa, H., Ozols, J., and Wu, H. C. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 4891–4895
30. Schnaitman, C. A. (1971) *J. Bacteriol.* **108,** 545–552
31. Filip, C., Fletcher, G., Wulff, J. L., and Earhart, C. F. (1973) *J. Bacteriol.* **115,** 717–722
32. Loeb, M. R., and Kilner, J. (1978) *Biochim. Biophys. Acta* **544,** 676–679
33. Bassford, P. J., Jr., Silhavy, T. J., and Beckwith, J. R. (1979) *J. Bacteriol.* **139,** 19–31