JOURNAL OF BACTERIOLOGY, Dec. 1982, p. 1163–1168
0021-9193/82/121163-06$02.00/0

Vol. 152, No. 3

Temperature-Sensitive Processing of Outer Membrane Lipoprotein in an *Escherichia coli* Mutant

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Received 14 June 1982/Accepted 31 August 1982

A mutant of *Escherichia coli* that accumulated prolipoprotein, a secretory precursor of the outer membrane lipoprotein, was isolated. The prolipoprotein accumulated in this mutant was modified by glyceride, but the in vitro cleavage of the signal peptide of the accumulated prolipoprotein was found to be temperature sensitive. The mutation appears to be located outside the gene for the lipoprotein, thus suggesting that the gene for the signal peptidase for the prolipoprotein was mutated.

Many membrane and secretory proteins in both procaryotic and eucaryotic cells are synthesized in precursor form with a signal peptide of 15 to 30 amino acid residues at the NH_2 terminus (12). Signal peptides are essential for the translocation of proteins across membranes. Signal peptidases that cleave off signal peptides are considered to play a key role in the process of protein secretion across membranes. Nevertheless, little is known about the nature of these enzymes or their mechanism of action in vivo. No signal peptidase except that for M13 phage coat protein (20) has been purified. The isolation of a mutant of altered or defective signal peptidase will provide a clue to the elucidation of the molecular mechanism of protein secretion across membranes.

Lipoprotein is one of the most thoroughly investigated membrane proteins in *Escherichia coli*. The protein is the most abundant in the cell in terms of number of molecules and has many unique features (10). The NH_2-terminal portion of the lipoprotein consists of glycerylcysteine to which two fatty acids are attached by two ester linkages and to which one fatty acid is attached by an amide linkage (4). The DNA sequences coding for the lipoproteins of the *Enterobacteriaceae* show extremely high homology, suggesting the biological importance of the structure of lipoproteins in gram-negative bacteria (18). The signal peptidase that cleaves off the signal peptide from the precursor of the lipoprotein (prolipoprotein) seems to exist in the envelope fraction (13). However, the enzyme has not yet been purified, and its mechanism of action is unknown. The glyceride and fatty acid moieties of the lipoprotein might be necessary for the correct assembly of the protein into the outer membrane and also for the action of the signal peptidase. Recently, a cyclic peptide antibiotic, globomycin, was found to inhibit the cleavage of the signal peptide, resulting in the accumulation of the prolipoprotein in the cell envelope (13). The accumulation of the prolipoprotein in the cytoplasmic membrane is believed to cause cell death (7). Provided that the target of globomycin is the signal peptidase, some of the globomycin-resistant mutants might have an altered signal peptidase unstable at high temperatures. We report here the isolation of an *E. coli* mutant that accumulates the prolipoprotein in the cell envelope. The signal peptidase of the mutant seems to be temperature sensitive.

MATERIALS AND METHODS

Bacterial strains and growth media. *E. coli* JE5506 F′ *pps his proA thi gal lac xyl mtl argE tsx* (6) and JE5505 F′ *lpp pps his proA thi gal lac xyl mtl argE tsx* (6) were used. Cells were grown in either M3 or M3su medium (17). For labeling with [^{35}S]methionine (0.5 μCi/0.5 μg per ml), M9 medium (15) with supplements of 4 mg of glucose, 2 μg of thiamine, 200 μg of $MgSO_4 \cdot 7H_2O$, 0.5 μg of methionine, 50 μg of arginine, 50 μg of histidine, and 50 μg of proline per ml was used. Tetracycline (8 μg/ml) was added for growth of the plasmid-harboring bacteria.

Plasmid construction. A plasmid carrying an inducible *lpp* gene was constructed by replacing the *lpp* promoter with a *lacUV5* promoter-operator fragment (see Fig. 1). pOP203-3 DNA carrying a *lacUV5* promoter-operator fragment (obtained from F. Fuller, Harvard University) was digested with restriction endonucleases *Eco*RI and *Hin*dIII. The 0.55-kilobase fragment was purified by polyacrylamide gel electrophoresis. The 95-base-pair *Alu*I fragment was obtained from the 0.55-kilobase fragment by *Alu*I digestion followed by polyacrylamide gel electrophoresis. pKEN111 (19) DNA cleaved by restriction endonucleases *Xba*I and *Xho*I was treated with T4 DNA polymerase in the presence of the four deoxynucleotides to

fill in cohesive ends and with calf intestine alkaline phosphatase. The plasmid DNA and the 95-base-pair *lacUV5* promoter-operator fragment were joined with T4 DNA ligase. The ligated DNA was used for the transformation of *E. coli* JE5505 *lpp*. Lipoprotein synthesis was inducible in 2 of the 24 tetracycline-resistant transformants examined.

Labeling experiments. Overnight cultures at 30°C of strain Y80 in M3su medium and of strains Y815 and Y821 in M3 medium containing 8 μg of tetracycline hydrochloride per ml were diluted 10-fold into 5 ml of M9 medium. The diluted cultures were incubated at 30°C with shaking. When the absorbance at 660 nm reached 0.3, 6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. When the absorbance at 660 nm reached 0.4, L-[^{35}S]methionine (0.5 μCi/0.5 μg per ml) was added, and shaking was continued for 30 min. For labeling at higher temperatures, the temperature was shifted to 42°C 30 min before labeling. The labeling was terminated by adding cold L-methionine at 500 μg/ml and cooling the cultures on ice. The membrane fraction was then prepared.

Labeling of the prolipoprotein with [^{3}H]glycerol and [^{3}H]palmitic acid was carried out as follows. Overnight cultures at 30°C of the mutant Y815 and wild-type strain JE5506 were diluted 10-fold into 30 ml of M9 medium. The diluted cultures were incubated at 30°C with shaking. When the absorbance at 660 nm reached 0.3, IPTG was added at a final concentration of 6 mM to the Y815 culture. When the absorbance at 660 nm reached 0.4, 10 μCi of [^{3}H]glycerol or [^{3}H]palmitic acid (20 ng/ml) was added, and shaking was continued for 30 min. Envelope fractions were prepared and washed with chloroform-methanol (2:1) as described by Hirashima et al. (5).

Other methods. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was carried out as described by Miller (15). Envelope fractions were prepared by sonication and differential centrifugation as previously described (11). Immunoprecipitation with antilipoprotein serum, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography were performed as previously described (3, 7). Globomycin was obtained from M. Arai, Sankyo Co., Ltd., Tokyo, Japan.

RESULTS

Construction of plasmid pHY001 and isolation of the mutants. *E. coli* JE5506 was mutated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and the mutated cells were plated onto M3su plates (17) containing 200 μg of globomycin per ml. The plates were incubated at 30°C for 5 days. The frequency of appearance of globomycin-resistant colonies on the plates was about 10^{-7}. Twenty percent of the mutants obtained showed temperature-sensitive growth. Mutant Y8 was one of the mutants thus obtained which showed temperature-sensitive growth in M3 medium (17) irrespective of the presence of globomycin. Only a minute amount of the lipoprotein was found in the envelope prepared from mutant Y8. To examine the expression of the lipoprotein gene (*lpp*) in the mutant, we constructed a small copy-numbered plasmid, pHY001, carrying the *lpp* gene, as described in Fig. 1. The promoter part of the *lpp* gene was replaced by a *lacUV5* promoter fragment so that the *lpp* gene could be induced by the addition of IPTG into the medium (Fig. 1). Mutant Y8 transformed by plasmid pHY001 was designated Y80 and was subsequently characterized. Strain Y80 was cultured in the presence of 6 mM IPTG and labeled with L-[^{35}S]methionine. The envelope fractions were analyzed by immunoprecipitation with antilipoprotein serum, followed by SDS-polyacrylamide gel electrophoresis and fluorography. A small amount of a protein migrating to the position of the prolipoprotein was detected in the gel when envelope fractions were prepared from cells cultured at 37°C in the presence of IPTG (Fig. 2, lane b). No such protein was detected when the cells were cultured at 30°C (data not shown). The amount of prolipoprotein was about 10% that of mature lipoprotein. To look for strains accumulating larger amounts of the prolipoprotein, we isolated temperature-resistant revertants from strain Y80. Y80 cells were plated onto M3 plates containing 8 μg of tetracycline hydrochloride per ml, and the plates were incubated at 40°C. The frequency of reversion was about 10^{-5}. A total of 200 revertants were examined

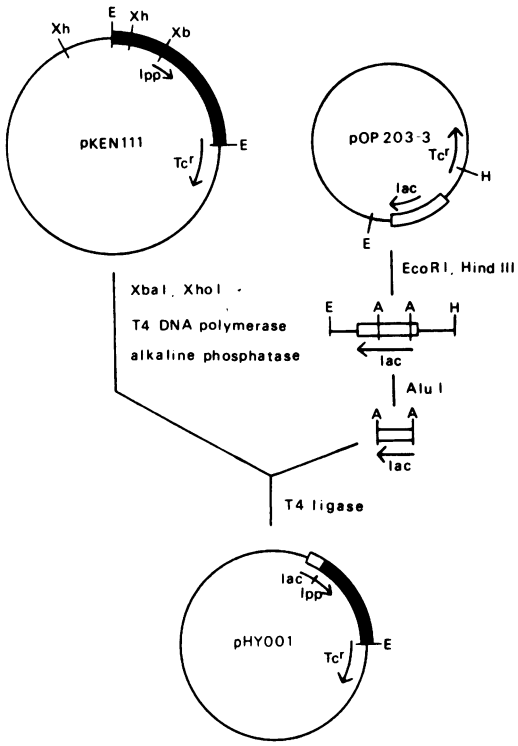

FIG. 1. Construction of plasmid pHY001. Restriction sites: E, *Eco*RI; Xb, *Xba*I; Xh, *Xho*I; H, *Hin*dIII; and A, *Alu*I.

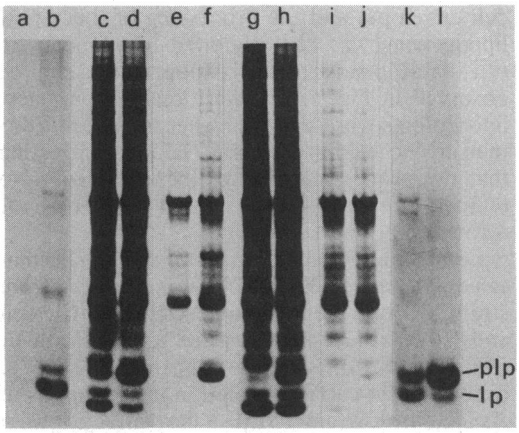

FIG. 2. Fluorogram of the membrane proteins of mutant cells fractionated by SDS-PAGE. Cells were labeled with [^{35}S]methionine as described in the text. The membrane fraction was prepared and treated with antilipoprotein serum (for lanes a, b, k, and l), and the immunoprecipitate was then analyzed by SDS-PAGE. Lanes a and b, Immunoprecipitated membrane proteins of strain Y80 labeled at 37°C in the absence (a) or the presence (b) of IPTG; lanes c through f, membrane proteins of strain Y815 labeled at 30°C (c and d) or at 42°C (e and f) in the absence (c and e) or the presence (d and f) of IPTG; lanes g through j, membrane proteins of strain Y821 labeled at 30°C (g and h) or at 42°C (i and j) in the absence (g and i) or the presence (h and j) of IPTG: lanes k and l, immunoprecipitated samples of proteins shown in lanes c and d. Samples equivalent to 1 ml of the original cultures were applied to the gels, except in lanes a, b, k, and l, where 2-ml equivalents were loaded. plp, Prolipoprotein; lp, mature lipoprotein.

for growth sensitivity to IPTG. Two strains (Y815 and Y821) showed IPTG-sensitive growth. Fig. 3 shows IPTG-sensitive and temperature-resistant growth of strain Y815, compared with growth of the parent strain Y80. The existence of plasmid pHY001 in strain Y815 was confirmed with the rapid alkaline extraction method of Birnboim and Doly (1). Upon addition of 6 mM IPTG into the medium at either 30 or 42°C, strains Y815 and Y821 accumulated the protein migrating to the position of the prolipoprotein in SDS-PAGE (Fig. 2, lanes c through j). The amount of this protein accumulated in strain Y815 was more than 5 times the amount of mature lipoprotein (Fig. 2, lanes d and f). Antilipoprotein serum reacted with the protein (Fig. 2, lanes k and l).

Temperature-sensitive processing in vitro of the prolipoprotein accumulated in the mutant envelope. The cell envelope was prepared from strain Y815 after labeling with [^{35}S]methionine in the presence of IPTG. The envelope was suspended in 10 mM sodium phosphate buffer (pH 7.1) and incubated at 30°C for 3 h. During incubation, the radioactivity of the protein corresponding to the prolipoprotein decreased, and that corresponding to the mature lipoprotein increased, (Fig. 4, lanes a and b), indicating the former protein is a precursor of the latter protein. The processing was inhibited when the incubation was carried out at elevated temperatures, such as 45 or 60°C (Fig. 4, lanes c and d). On the other hand, the prolipoprotein accumulated in wild-type strain JE5506 treated with globomycin was processed

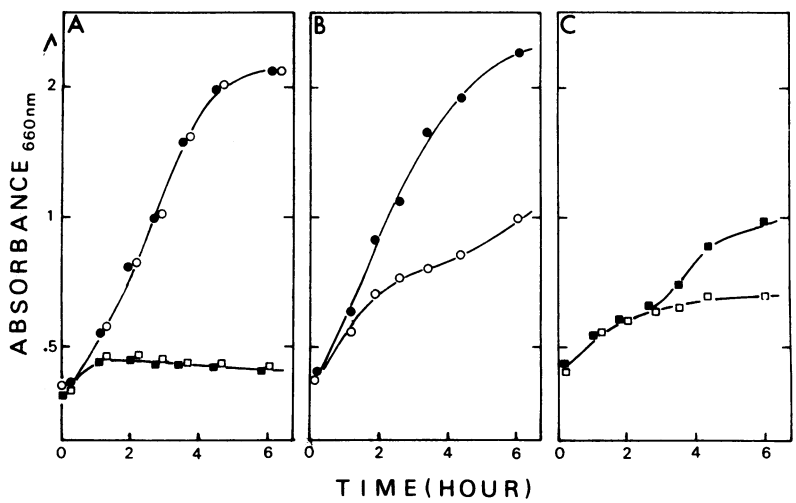

FIG. 3. Temperature sensitivity and IPTG sensitivity of the growth of mutant strains. IPTG (6 mM) was added to mutant cultures growing exponentially in M3 medium at 30°C (time 0). The temperature was also shifted at time 0. The absorbance at 660 nm was determined periodically. (A) Growth curves of the mutant Y80; (B and C) growth curves of the mutant Y815. Symbols: ○, IPTG present at 30°C; ●, IPTG not present at 30°C; □, IPTG present at 40°C; ■, IPTG not present at 40°C.

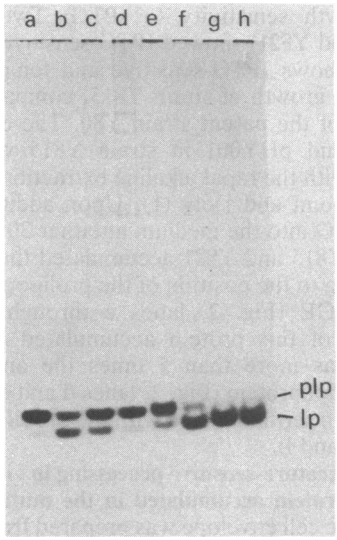

FIG. 4. Temperature-sensitive processing in vitro of the prolipoprotein accumulated in mutant Y815. The envelope fraction of mutant Y815 labeled with [^{35}S]methionine in the presence of 6 mM IPTG at 30°C was prepared as described in the text. As a control, the envelope fraction of the wild-type strain JE5506 labeled with [^{35}S]methionine in the presence of 50 μg of globomycin per ml was prepared. Membrane fractions were incubated at 30, 45, or 60°C for 3 h. All samples were immunoprecipitated with antilipoprotein serum and analyzed by SDS-PAGE. Lanes a through d, Y815 envelopes before incubation (a) and after incubation at 30°C (b), 45°C (c), or 60°C (d); lanes e through h, JE5506 envelopes before incubation (e) and after incubation at 30°C (f), 45°C (g), or 60°C (h). plp, Prolipoprotein; lp, mature lipoprotein.

at 30, 45, or 60°C (Fig. 4, lanes e through h), indicating that the effect of globomycin is reversible, as reported by Hussain et al. (8).

Characterization of the prolipoprotein accumulated in the mutant. The *E. coli mlpA* mutant that produced the glyceride-deficient prolipoprotein was found to be more resistant to globomycin than were the wild-type strains (14). Since our mutant was originally isolated as a globomycin-resistant mutant, the accumulation and the temperature-sensitive processing of the prolipoprotein in the mutant could have been due to a temperature-sensitive modification of the protein with glyceride. To examine this possibility, we labeled Y815 cells with [^{3}H]glycerol or [^{3}H]palmitic acid in the presence of IPTG. The membrane lipoproteins were analyzed as described by Hussain et al. (7). As shown in Fig. 5, both [^{3}H]glycerol and [^{3}H]palmitic acid were incorporated into the prolipoprotein. Ichihara et al. found several new lipoproteins in the *E. coli* cell envelope, and the processing of these new lipoproteins was also inhibited by globomycin (9). Faint bands of new lipoproteins can be observed in Fig. 5. The positions of some new lipoproteins were slightly higher in mutant Y815 than in the wild-type strain JE5506, suggesting that the processing of new lipoproteins is also temperature sensitive in mutant Y815 (Fig. 5, arrows).

Location of the mutation on the *E. coli* chromosome. Plasmid pHY001 DNA was prepared from strain Y815 by the method described by Clewell and Helinski (2) and was used to transform *E. coli* JE5505 (6), which is isogenic to JE5506, except that it carries a deletion in the *lpp* gene. As a control, strain Y8151, a derivative of strain Y815 cured of the plasmid, was transformed with the same DNA. The absence of the plasmid in recipient strains JE5505 and Y8151 and the presence of the plasmid in the transformants obtained were confirmed with the rapid alkaline extraction procedure of Birnboim and Doly (1) (data not shown). Neither of the recipient strains

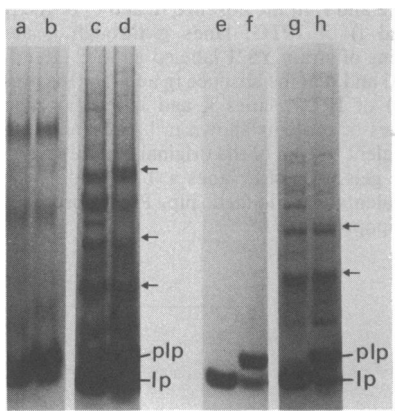

FIG. 5. Incorporation of [^{3}H]glycerol and [^{3}H]palmitic acid into the prolipoprotein. Overnight cultures at 30°C of mutant Y815 and wild-type strain JE5506 were labeled with [^{3}H]glycerol or [^{3}H]palmitic acid as described in the text. Envelope fractions were prepared and washed with chloroform-methanol (2:1) as described by Hirashima et al. (5). Immunoprecipitation, SDS-PAGE, and fluorography were done as described in the text. For the envelopes in lanes a through d, 7.5% acrylamide was used; for the envelopes in lanes e through h, 10% acrylamide was used. Lanes a through d, [^{3}H]glycerol-labeled envelopes of JE5506 (a and c) and Y815 (b and d) immunoprecipitated with antilipoprotein serum (a and b) or not immunoprecipitated (c and d); lanes e through h, [^{3}H]palmitic acid-labeled envelopes of JE5506 (e and g) and Y815 (f and h) immunoprecipitated with antilipoprotein serum (e and f) or not immunoprecipitated (g and h). Arrows indicate the positions of new lipoproteins migrating a little more slowly than those of JE5506. plp, prolipoprotein; lp, the mature lipoprotein.

synthesized any detectable amount of the lipoprotein in the presence of IPTG. Strain JE5505 transformed by plasmid DNA synthesized the mature lipoprotein, whereas strain Y8151 transformed by plasmid DNA accumulated the prolipoprotein (data not shown). It has also been found that the lipoprotein accumulated in strain Y815 was processed by the solubilized prolipoprotein signal peptidase prepared from the wild-type cells (H. Yamagata, manuscript in preparation). These results suggest that a chromosomal mutation located outside the *lpp* gene is responsible for the accumulation of the prolipoprotein because the active *lpp* gene is carried only on the plasmid in these strains.

DISCUSSION

Upon addition of IPTG into the medium, the mutant Y815 accumulated a large amount of the prolipoprotein in its envelope. The amount of the prolipoprotein was more than five times the amount of the mature lipoprotein. This amount is much larger than that reported in other *E. coli* mutants, such as the *secA*-containing mutant, in which a small amount of the precursor maltose-binding protein was detected by pulse labeling (16).

The temperature-sensitive processing in mutant Y815 is most probably due to an altered signal peptidase, for the following reasons. (i) Modification of the lipoprotein with glyceride is not altered in the mutant. (ii) The processing in vitro of the prolipoprotein is temperature sensitive. (iii) The mutation responsible for the accumulation of the prolipoprotein appears to be located outside the *lpp* gene.

The mutant Y815 accumulates the prolipoprotein at 30°C as well as at 42°C in the presence of IPTG, indicating that the activity of the signal peptidase is very weak even at a permissive temperature. The processing of some of the new lipoproteins seem to be affected in mutant Y815 as well. This supports the notion that the signal peptidase is altered in the mutant because new lipoproteins have been suggested to be processed by the same signal peptidase as is the original lipoprotein (9). The processing of other new lipoproteins does not seem to be affected in the mutant. Weak residual activity of the altered signal peptidase might be enough for the processing of these proteins or perhaps these new lipoproteins are processed by other signal peptidases. It should also be noted that the present mutation did not affect the processing of any other proteins, as judged by SDS-PAGE (data not shown).

It should be noted that mutant Y815 is a partial revertant, showing slower growth at 40°C than at 30°C even in the absence of IPTG. Inactivation of the signal peptidase at high temperatures might affect cell growth even in the absence of lipoprotein synthesis by inhibiting the processing of other new lipoproteins. Further genetic studies are necessary to examine whether the temperature-sensitive growth, globomycin resistance, and temperature-sensitive processing of the lipoprotein result from a single mutation.

ACKNOWLEDGMENTS

We thank M. Arai, Sankyo Co., Ltd., Tokyo, Japan, and F. Fuller, Harvard University, for supplying globomycin and plasmid pOP203-3, respectively. We also thank N. R. Movva, K. Nakamura, S. Inouye, S. Ichihara, and M. Hussain for suggestions. N.Y. is grateful to S. Mizushima for antilipoprotein serum and helpful discussion and to S. Udaka and N. Tsukagoshi for encouragement and discussion.

This work was supported in part by Public Health Service grant GM19043 from the National Institute of General Medical Sciences, grant NP-67 from the American Cancer Society, and grant R-RAM-0051 from the National Science Foundation.

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