BIOGENESIS OF MEMBRANE LIPOPROTEINS IN *ESCHERICHIA COLI*

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ABSTRACT Globomycin-resistant mutants of *Escherichia coli* have been isolated and partially characterized. Approximately 2–5% of these mutants synthesize structurally altered Braun's lipoprotein. The majority of these mutants contain unprocessed and unmodified prolipoprotein. One mutant is found to contain modified, processed, but structurally altered lipoprotein. Mutants containing lipid-deficient prolipoprotein or lipoprotein also show increased resistance to globomycin. These results suggest that the inhibition of processing of modified prolipoprotein by globomycin may require fully modified prolipoprotein as the biochemical target of this novel antibiotic. Our failure to isolate mutant containing cleaved but unmodified lipoprotein among globomycin-resistant mutants is consistent with the possibility that modification of prolipoprotein precedes the removal of signal sequence by a unique signal peptidase. Recent evidence indicates that the minor lipoproteins in the cell envelope of *E. coli* are also synthesized as lipid-containing prolipoproteins and the processing of these prolipoproteins is inhibited by globomycin. These results suggest the existence of modifying enzymes in *E. coli* which would transfer glyceryl and fatty acyl moieties to cysteine residues located in the proper sequences of the precursor proteins. This speculation is confirmed by our demonstration that *Bacillus licheniformis* penicillinase synthesized in *E. coli* as well as in *B. licheniformis* is a lipoprotein containing glyceride-cysteine at its NH_2-terminus.

INTRODUCTION

The outer membrane of gram-negative bacteria contains abundant copies of a few proteins, the so-called major outer membrane proteins. One of the major outer membrane proteins in *Escherichia coli* and other gram-negative bacteria is the murein lipoprotein discovered by Braun and Rehn (1). The amino terminus of this lipoprotein consists of a novel lipoamino acid, *N*-acyl diglyceride-cysteine (2).

The primary translation product of lipoprotein m-RNA is a precursor form of lipoprotein called prolipoprotein which contains a peptide extension of 20 extra amino acids at the NH_2-terminus (Fig. 1) (3). Recently, a novel antibiotic, globomycin (Fig. 2), has been shown to inhibit selectively the processing of prolipoprotein (4). Studies by Mizushima and his co-workers on the mechanism of action of globomycin have led to two important observations: glyceride-containing prolipoprotein accumulates in globomycin-treated cells (5), and multiple, albeit minor, lipoprotein species are identified in both the inner cytoplasmic and outer membranes of the *E. coli* cell envelope (6). The processing of the lipid-containing precursor forms of these new lipoproteins is also inhibited by globomycin (6).

In this paper, we shall summarize our current knowledge on the biosynthesis and assembly of lipoproteins in bacteria. Some recent studies on the isolation and characterization of globomycin-resistant mutants will be presented. The implication of these findings on the specificity of the modification and processing of prolipoprotein will be discussed.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used in the present study include *E. coli* K-12 strain E609; globomycin-resistant mutant strains 3–2 and 6–23 derived from strain E609; strain JE5506, a *lpp*$^+$ transductant of strain JE5505; strain WB956 lysogenic with a λ phage containing pen gene from *B. licheniformis*; and strain IH 4715 of *B. licheniformis* which is derived from strain 749C. The relevant genotypes of these strains have been previously described (7, 9).

Growth Media and Culture Conditions

Media used in the present study include M9 minimal medium and proteose peptone beef extract (PPBE) broth.

Labeling studies were carried out employing these radioactive precursors: [^{3}H]palmitate (sp. act. 23.5 Ci/mmol); [2-^{3}H]glycerol (sp. act. 6.35 Ci/mmol); [^{14}C]leucine (sp. act. 150 mCi/mmol); [^{35}S]cysteine (sp. act. 866 Ci/mmol); and [^{35}S]methionine (300 mCi/mmol).

Preparation of Cell Envelope and Isolation of Labeled Proteins with Immunoprecipitation

The preparation of the crude cell envelope from *E. coli* and the extraction of phospholipids were carried out as described (10). Preparation of the crude cell envelope from *B. licheniformis* cells, extraction of phospho-

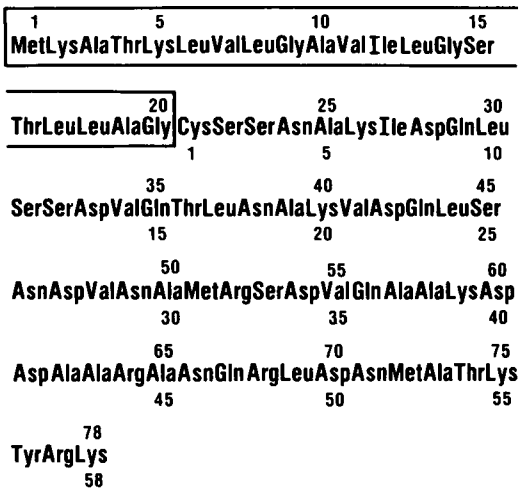

FIGURE 1 Primary structure of prolipoprotein in *E. coli*. The bracketed sequence represents the signal sequence which is removed during the processing of prolipoprotein to form mature lipoprotein.

lipids, isolation of membrane-bound penicillinase, and immunoprecipitation of labeled penicillinase with rabbit antiserum prepared against exopenicillinase of *B. licheniformis*, were carried out as described (9).

Isolation and Characterization of Globomycin-resistant Mutants

E. coli strain E609 grown in PPBE broth was mutagenized with nitrosoguanidine. Following phenotypic expressions, the mutagenized cells were treated with globomycin at a final concentration of 30–50 μg/ml at 30°C overnight. Single colonies were isolated and screened for temperature sensitivity in growth on nutrient agar plates, RNase leakiness and the presence or absence of lipoprotein antigen by the Ouchterlony double diffusion test using rabbit antisera against purified lipoprotein. Those mutants showing positive lipoprotein antigen and phenotypes suggestive of structural alteration in lipoprotein (7) were further analyzed biochemically by SDS polyacrylamide gel electrophoresis of lipoprotein immunoprecitates labeled with radioactive precursors. SDS polyacrylamide gel electrophoresis was carried out in the presence or absence of 2-mercaptoethanol to ascertain whether the mutant lipoprotein contained unmodified or extra cysteine residues.

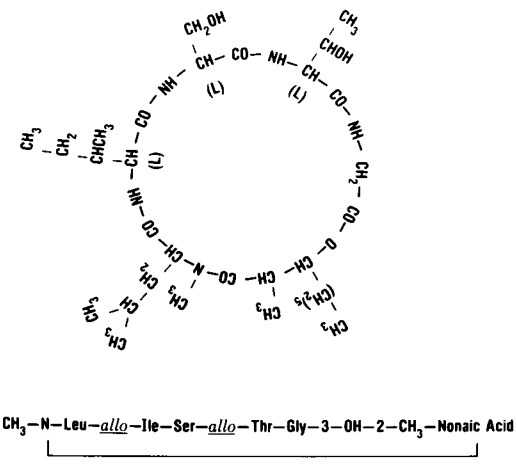

FIGURE 2 Structure of globomycin (4).

Other biochemical techniques

Sodium dodecyl sulfate/urea polyacrylamide gel electrophoresis was carried out as described previously (9, 11).

RESULTS

Post Translational Modifications of Prolipoprotein

The structure of prolipoprotein differs from that of mature murein lipoprotein in a number of ways. Consequently, a series of post translational modifications and processing occurs prior to the translocation of mature free-form lipoprotein to the outer membrane. These modifications include (*a*) the transfer of glycerol from the nonacylated glycerol moiety of phosphatidylglycerol to the sulfhydryl group of cysteine in prolipoprotein (12); (*b*) transacylation of both saturated and unsaturated fatty acids from phospholipids to the glycerolcysteine in prolipoprotein to give rise to the ester-linked fatty acids in lipoprotein (13, 14); (*c*) the proteolytic cleavage of the signal peptide at the NH_2-terminus of prolipoprotein; and (*d*) transacylation of palmitate from phospholipid to the α-NH_2 group of cysteine to *N*-acylate the NH_2-terminus of lipoprotein (13, 14). Finally, part of the mature and modified lipoprotein is covalently linked to peptidoglycan, presumably by transpeptidation.

The order of these modifications and processing reactions can be inferred from the recent work on the effect of globomycin on the processing of prolipoprotein (Fig. 3). If the processing of prolipoprotein occurs prior to the attachment of glycerol to the lipoprotein, the prolipoprotein accumulated in the globomycin-treated cells should be unmodified. However, if modifications by glyceryl transferase and transacylase take place before the processing by signal peptidase, the prolipoprotein found in the globomycin-treated cells would be modified to contain covalently linked glyceride. The latter possibility was shown to be the case (6) (Fig. 3 *a–e*). This observation, together with the simultaneous accumulation of several other glyceride-containing precursor forms of lipoproteins in globomycin-treated cells, strongly suggest that modification reactions as represented by the sequential transfer of glycerol and fatty acids to prolipoproteins can occur prior to the removal of signal peptides.

Lipid-deficient Lipoprotein Mutants Show Increased Resistance to Globomycin

We have recently shown that globomycin is a hydrophobic antibiotic which can enter the *E. coli* cells via the so-called hydrophobic channels (8). We have further shown that mutants synthesizing lipid-deficient lipoprotein or prolipoprotein are more resistant to globomycin than their corresponding wild-type parental strains (Table I). Although earlier workers have suggested the possibility that globomycin is a substrate analogue of the signal sequence in

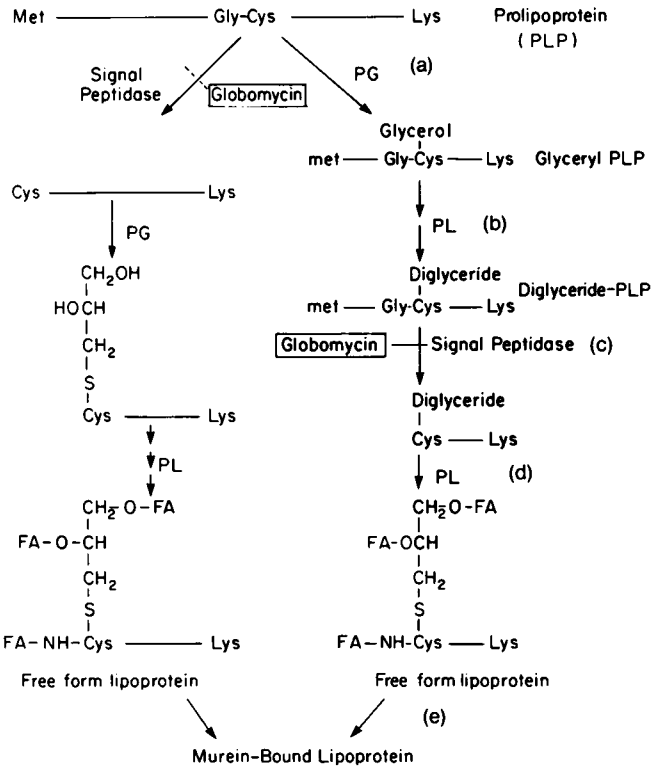

FIGURE 3 Alternative pathways for the post-translational modification and processing of prolipoprotein in *E. coli*. The pathway on the right is consistent with the data. *a*, glyceryl transferase; *b*, transacylase; *c*, signal peptidase; *d*, transacylase; and *e*, transpeptidase or ligase.

prolipoprotein (4), it seems plausible that globomycin may bind to the diglyceride moiety in prolipoprotein, thereby interfering with its processing and translocation. Inasmuch as the new lipoproteins may have signal sequences distinct from that of Braun's lipoprotein, the common effect of globomycin on the processing of these prolipoproteins of diverse primary structures may be attributed to the formation of prolipoprotein-globomycin complexes. The failure of glyceride-containing prolipoprotein to be translocated from the cytoplasmic membrane to the outer membrane might be responsible for the antibiotic action of globomycin (6). The increased resistance of lipid-deficient prolipoprotein mutant towards globomycin may be accounted for by the apparently normal translocation of unmodified uncleaved mutant prolipoprotein to the outer membrane (15).

We have isolated globomycin-resistant mutants from several *E. coli* K-12 strains. Approximately 2–5% of the globomycin resistant mutants contain dimeric forms of Braun's lipoprotein which could be converted into the monomeric forms by treatment with 2-mercaptoethanol. The majority of the lipoprotein-dimer mutants synthesize unprocessed and unmodified prolipoprotein, with the apparent molecular weights of these mutant lipoproteins identical to that of the *mlpA* prolipoprotein previously reported (16) (see Table I). One of the mutants, strain

TABLE I
GLOBOMYCIN RESISTANCE IN *E. COLI* MUTANTS CONTAINING LIPID-DEFICIENT LIPOPROTEIN

Strain	Lipoprotein	Nature of mutation	Relative sensitivity*
Wild type	normal structure	None	1
lpo mutant	none	Deletion	>4
lpm mutant	cleaved, underacylated lipoprotein	$Arg_{57} \rightarrow Cys_{57}$	3–4
mlpA mutant	uncleaved, unmodified	$Gly_{-7} \rightarrow Asp_{-7}$	2–4
Mutant 3-2	uncleaved, unmodified	?	4–5
Mutant 6-23	cleaved, modified	X → Cys	4–5

*Minimal effective concentration was determined by measuring plating efficiency as well as inhibition of growth in liquid cultures in the presence of varying concentrations of globomycin.

6–23, synthesizes a modified and processed lipoprotein which forms dimers due to the presence of an extra cysteine in lipoprotein (Table I). This is similar but not identical to the *lpm* (*lpp*-1) mutant previously reported by Hirota and his co-workers (17), although the exact location for the structural alteration in this new *lpp* mutation remains to be determined. Of the dimer-forming mutants obtained thus far, we have yet to isolate a mutant which synthesizes cleaved but unmodified lipoprotein. The existence of the latter class of lipid-deficient lipoprotein mutant might be expected if the processing of prolipoprotein by a signal peptidase precedes the modification of the cysteine residue. All mutants thus far isolated which are defective in the modification of lipoprotein synthesize uncleaved prolipoprotein; this observation suggests that the modification of prolipoprotein by glyceryl transferase and transacylases precedes and may indeed be a prerequisite of the processing of prolipoprotein by a unique signal peptidase specific for glyceride-containing prolipoproteins.

We have previously characterized a novel mutant altered in the structure of murein lipoprotein. Due to a single amino acid substitution of aspartic acid for glycine at the 14th position of the prolipoprotein, all of the posttranslational modifications of prolipoprotein are aborted. The amount of murein-bound lipoprotein found in this mutant is greatly reduced as compared to the wild-type strain. However, the murein-bound lipoprotein, present at 4% of the wild-type level, was found to be fully modified and processed, normal in size, containing both glyceride and amide-linked fatty acid (18). This observation is of considerable interest since it suggests that modification of lipoprotein may be required for the assembly of lipoprotein into the murein sacculi. This is consistent with the observation that glyceride-containing prolipoprotein is assembled into the peptidoglycan (19). The finding that the modified bound form of lipoprotein in the *mlpA* mutant is processed further suggests that the primary defect in the assembly of *mlpA* mutant prolipoprotein into

the peptidoglycan is the poor efficiency of the mutant prolipoprotein as the substrate for the glyceryltransferase. The fraction of *mlpA* mutant-prolipoprotein which is modified by the covalent attachment of glycerol is subsequently cleaved to form mature lipoprotein.

A Common Modification and Processing System for Structurally Different Lipoproteins in *E. coli*.

While Braun's lipoprotein is the most abundant protein and by far the major lipoprotein in *E. coli*, evidence is rapidly accumulating to indicate that the presence of glyceride-cysteine at the NH_2-terminus is not unique for Braun's lipoprotein. In fact, the number of newly recognized lipoprotein species is rapidly increasing (6, 20). These lipoproteins share several features in common with Braun's lipoprotein: they contain covalently linked glyceride, they are synthesized as precursor proteins presumably containing signal sequences at the NH_2-termini, and the processing of these glyceride-containing prolipoproteins is inhibited by globomycin (6).

The prevalence of various lipoproteins in *E. coli* suggests the modification of a number of structurally unrelated prolipoproteins by a common set of enzymes. These lipoproteins vary greatly in size and subcellular localization, and show no immunological cross-reactivity with antisera against Braun's lipoprotein or with antisera against Mizuno's lipoprotein (6). In addition, there is no extensive nucleotide sequence homology between Braun's lipoprotein gene and the genes for the new lipoproteins, as measured by hybridization of ^{32}P-labeled Braun's lipoprotein mRNA with restriction enzyme fragments of total *E. coli* DNA (21). Consequently, the enzyme(s) responsible for the post-translational modificaton reactions must recognize some common feature other than the primary structures of the mature proteins. It is likely that the amino acid sequence located in the vicinity of the site of modification and cleavage of these prolipoproteins constitutes such a common structural feature. This speculation is supported by the similarity in the NH_2-terminal sequences between Braun's lipoprotein and Mizuno's lipoprotein (Fig. 4) (6). It is further validated by the demonstration that *B. licheniformis* penicillinase synthesized in *E. coli* is modified and processed as a glycerolipoprotein (9).

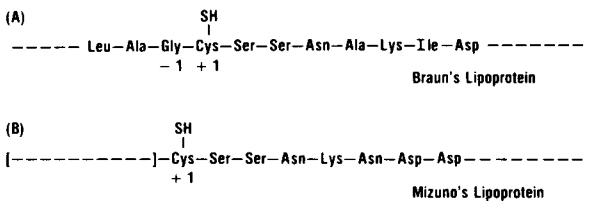

FIGURE 4 Amino-terminal sequence of Braun's lipoprotein and Mizuno's lipoprotein in *E. coli*.

B. licheniformis Penicillinase Synthesized in *E. coli* Contains Covalently Linked Glyceride-Cysteine.

Penicillinase from *B. licheniformis* exists in three forms: the exoenzymes, exo F and exo S, which are secreted into the culture medium, and the membrane-bound form which is associated with the cytoplasmic membrane, presumably via the hydrophobic segment at the NH_2-terminus (22). The structural gene for this enzyme has been cloned and its DNA sequence determined (23). The NH_2-terminal portion of the penicillinase contains a tetrapeptide, -Leu-Ala-Gly-Cys-, which is located at the end of the hydrophobic segment of the signal sequence of penicillinase (Fig. 5*B*). This is precisely the same tetrapeptide surrounding the site of posttranslational modification and processing of prolipoprotein in *E. coli* (Fig. 5*A*). Thus, the penicillinase gene from *B. licheniformis* cloned on a λ-transducing phage offers a unique opportunity to test our working hypothesis. As shown in Fig. 6, penicillinase of *B. licheniformis* synthesized in *E. coli* contains covalently linked glyceride and fatty acids. It is not labeled, however, with [^{32}P]phosphate (data not shown). Penicillinase doubly labeled with [2-^3H]glycerol and [^{35}S]sulfate was isolated from immunoprecipitate by preparative SDS/urea gel electrophoresis, oxidized with performic acid, and hydrolyzed with 6N HCl at 105°C *in vacuo* for 20 h. A peak doubly labeled with [2-^3H]glycerol and [^{35}S]sulfate with the same electrophoretic mobility of glycerylcysteine sulfone isolated from Braun's lipoprotein was detected in the total acid hydrolysate of oxidized penicillinase (data not shown). Free [2-^3H]glycerol was not found in the hydrolysate of penicillinase beyond the level expected from the destruction of glycerylcysteine sulfone during hydrolysis. This result indicates that the glyceride moiety in penicillinase is joined through a thioether linkage to the sulfhydryl group of cysteine, most likely at the NH_2-terminus of the membrane-bound penicillinase.

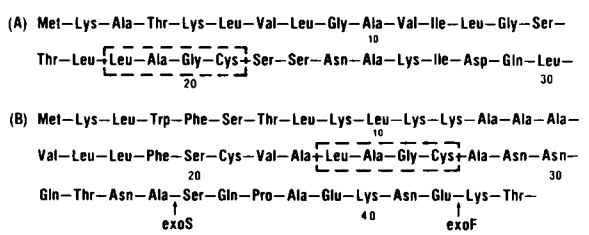

FIGURE 5 The amino acid sequence of the NH_2-terminal portion of the prolipoprotein in *E. coli* (*A*) and prepenicillinase in *B. licheniformis* (*B*). The complete peptide extension at the NH_2-terminus of the exopenicillinase is 42 amino acids long and the tetrapeptide marked borders the only hydrophobic section within this region. exo-S and exo-F denote slow and fast migrating forms of exopenicillinase.

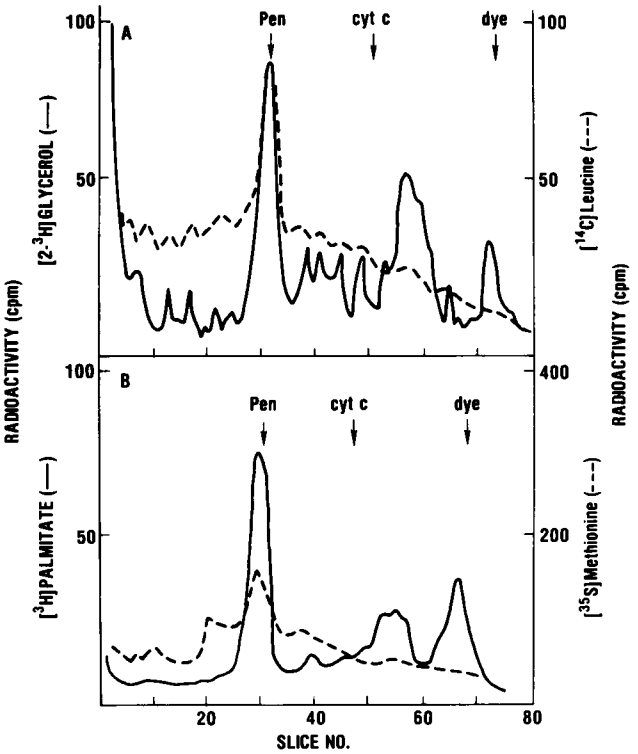

FIGURE 6 Incorporation of [2-^{3}H]glycerol and [^{3}H]palmitate into *B. licheniformis* penicillinase synthesized in *E. coli*. Cells were labeled with the radioactive precursors as indicated. Envelope fractions were isolated, extracted three times with chloroform/methanol (2:1), immunoprecipitated with anti-penicillinase antiserum, solubilized in 10 mM sodium phosphate buffer (pH 7.1) containing 1% SDS, 1% 2-mercaptoethanol and 10% glycerol at 70°C for 20 min and analyzed by SDS/urea polyacrylamide gel electrophoresis. *A*, Immunoprecipitated penicillinase from [2-^{3}H]glycerol- and [^{14}C]leucine-labeled *E. coli* strain WB956(λ pen). *B*, Immunoprecipitated penicillinase from [^{3}H]palmitate and [^{35}S]methionine-labeled *E. coli* strain WB956(λ pen). Pen, penicillinase; cyt *c*, cytochrome *c*; dye, pyronin Y.

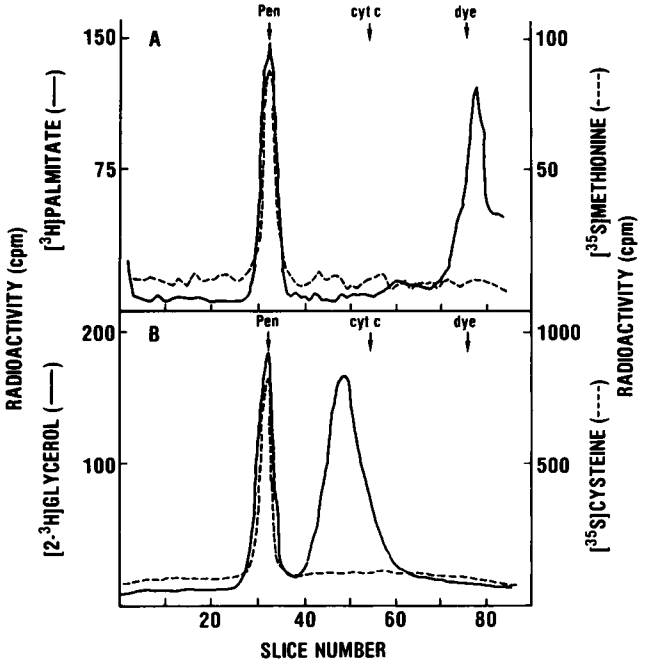

FIGURE 7 Incorporation of [^{3}H]palmitate and [2-^{3}H]glycerol into membrane-bound penicillinase in *B. licheniformis*. *B. licheniformis* cells were labeled with radioactive precursors as indicated. Envelope fractions were isolated, extracted three times with chloroform/methanol, solubilized, immunoprecipitated with anti-penicillinase anti-serum, and analyzed as described in the legend to Fig. 10. *A*, Immunoprecipitated penicillinase from [^{3}H]palmitate- and [^{35}S]methionine-labeled *B. licheniformis* strain IH4715. *B*, Immunoprecipitated penicillinase from [2-^{3}H]glycerol and [^{35}S]cysteine-labeled *B. licheniformis* strain IH4715. The [2-^{3}H]glycerol-labeled peak migrating slower than cytochrome *c* is most likely related to teichoic acid.

Membrane-bound Penicillinase of *B. licheniformis* Contains Glyceride-cysteine

While earlier reports have suggested the presence of covalently-linked phosphatidylserine at the NH_2-terminus of membrane-bound penicillinase in *B. licheniformis* (24), Simons et al. (22) found no phospholipid covalently attached to the purified membrane-bound penicillinase from the same species. Our observation that *B. licheniformis* penicillinase synthesized in *E. coli* is a glyceride-lipoprotein prompted us to reinvestigate the nature of this enzyme in *B. licheniformis*. As shown in Fig. 7, the membrane-bound penicillinase in *B. licheniformis* is a glyceride-lipoprotein since it can be readily labeled with [^{3}H]palmitate and [2-^{3}H]glycerol. There was no incorporation of [^{32}P]phosphate into this enzyme (data not shown). Glycerylcysteine sulfone doubly labeled with [2-^{3}H]glycerol and [^{35}]cysteine was found in the total acid hydrolysate of performic acid-oxidized membrane-bound penicillinase of *B. licheniformis* purified by SDS/urea preparative gel electrophoreis (data not shown). These results suggest that glyceride-lipoproteins containing modified cysteine at their NH_2-termini are not restricted to gram-negative bacteria, as previously assumed.

Effects of Globomycin on the Biosynthesis of *B. licheniformis* Penicillinase in *E. coli*

We have postulated that globomycin may not be a structural analogue of the signal sequence in prolipoproteins but instead interferes with the processing of prolipoproteins by binding noncovalently to the glyceride moiety in prolipoproteins. The modification of *B. licheniformis* penicillinase to a glyceride-lipoprotein in *E. coli* offers an opportunity to study the effect of globomycin on the processing of penicillinase. In contrast to the inhibition of processing of many different prolipoproteins by globomycin treatment, there appears to be a severe inhibition in the incorporation of palmitate or [2-^{3}H]glycerol into the membrane-bound penicillinase in both *E. coli* and in *B. licheniformis*. Similar results have been obtained by Lampen and his co-workers (25). They have also demon-

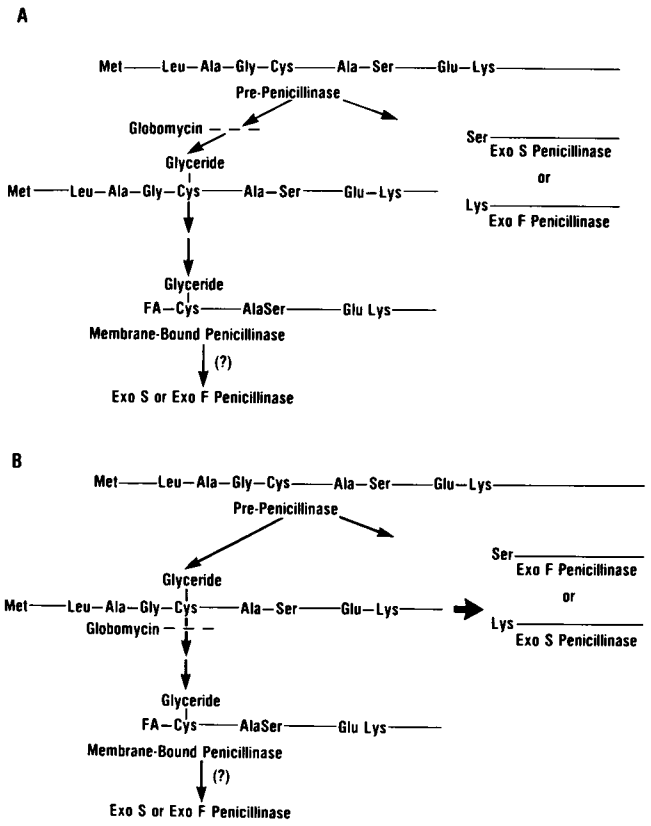

FIGURE 8 Postulated pathway for the biosynthesis, modification and processing of penicillinase from *B. licheniformis*.

strated an increased production of the exo-forms of penicillinase in globomycin treated cells (25). These results are consistent with the postulate that the membrane-bound penicillinase is not an obligatory intermediate in the formation and secretion of the exo-forms (Fig. 8). This is further supported by the work of Davis and his colleagues (26) demonstrating that there are two independent and cotranslational processings of prepenicillinase, leading to the formation of either the exo-forms by proteolytic cleavage of unmodified prepenicillinase, or the membrane-bound form which has been modified by the addition of the lipid moiety. Alternatively, globomycin may have inhibited the processing of modified prepenicillinase and our failure to detect an accumulation of glyceride-containing prepenicillinase in globomycin-treated cells may be due to enhanced processings of modified precursor to form exo-forms of penicillinase by the alternative pathway (Fig. 8).

DISCUSSION

In this paper, we have summarized some recent developments in the biogenesis of lipoproteins in bacteria. The outer membrane lipoprotein represents not only the most abundant protein in *E. coli*, but also one of the most extensively studied, largely due to the pioneering work of Braun, Inouye and their co-workers (27, 28). The identification of many lipoproteins with similar structures in both gram-negative and gram-positive bacteria (6, 9, 25, 26) has provided new impetus to the studies of Braun's lipoprotein as a prototype of lipoproteins in general. Moreover, the discovery of the novel antibiotic globomycin has provided an important tool in the studies of the biosynthesis and assembly of lipoproteins in bacteria.

It is clear from the work of Smith, et al. (26) that the modification of prepenicillinase is cotranslational. This finding is consistent with the suggestion that the modification of prolipoprotein by the successive transfer of glycerol and fatty acids precedes the proteolytic removal of the leader sequence, and the modified glyceride-cysteine may in fact constitute a "signal" for cleavage by a unique signal peptidase. The postulated sequence of events is shown in Fig. 9. Covalent modification of precursor proteins by the addition of glyceride prior to the removal of leader sequence may be especially important for the anchorage of otherwise hydrophilic proteins to the membrane such as *B. licheniformis* penicillinase. It is teleologically attractive to have proteins anchored to the membrane through the covalently attached lipid before they could leave the membrane to the aqueous milieu as a result of the removal of signal sequence. This anchorage may also be necessitated by the nature of other components of the modification reaction (phospholipids, membrane-bound enzymes). Our data show that the modification enzymes recognize a limited amino acid sequence near the end of the hydrophobic segment of the signal sequence of prolipoproteins. The overall structures of the mature lipoproteins appear to be less important. This is understandable in view of the fact that the modification reaction for penicillinase in *B. licheniformis* is cotranslational and presumably takes place prior to completion of the synthesis of polypeptide chains (26). The recognition of a restricted peptide

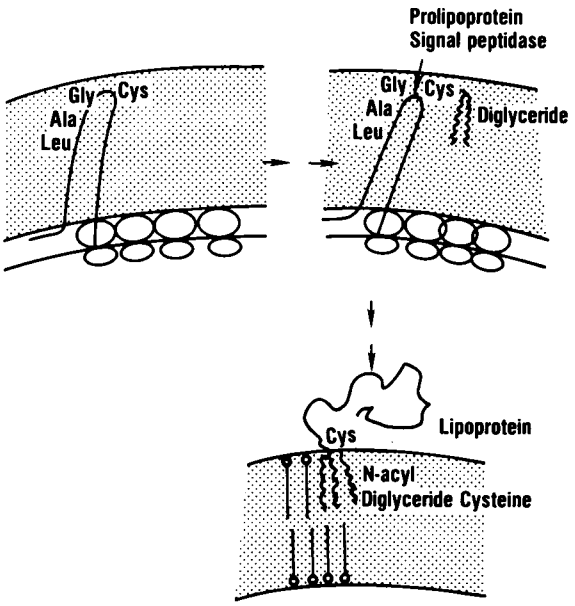

FIGURE 9 Cotranslational modification of prolipoproteins in *E. coli*.

sequence by the modification enzymes to form lipoproteins may be analogous to the substrate specificity for glycosylation of proteins to form glycoproteins (29).

The observation that mutant 6–23 and especially *lpm* mutant are resistant to globomycin raises an interesting question regarding the timing of various modification reactions and that of signal peptidase. Mutations within the mature lipoprotein allow either the proper cleavage of prolipoprotein in the presence of globomycin, or the translocation of altered prolipoprotein to the outer membrane thereby prevents the accumulation of fully modified and uncleaved prolipoprotein in the cytoplasmic membrane. Because the *lpm* mutation is known to alter the penultimate amino acid from the COOH-terminus of lipoprotein, a nearly completed prolipoprotein would have to be synthesized in order for this particular mutation to exert its effect against the action of globomycin. This would suggest that the cleavage of signal sequence in prolipoprotein occurs post-translationally. *Lpm* mutant lipoprotein also lacks one of the ester-linked fatty acids (17). Assuming that this defect in O-acylation of lipoprotein is caused by the substitution of COOH-subterminal arginine by cysteine, the transacylation of monoacylglyceryl-prolipoprotein must also be a post-translational event. In view of the small size of Braun's lipoprotein, it is probably not surprising that some or all of its modification and processing reactions take place post translationally.

The demonstration that *B. licheniformis* penicillinase is a glyceride-lipoprotein indicates that lipoproteins containing glyceride-cysteine at the NH_2-termini may be ubiquitous in prokaryotic cells. The localization of this hydrophobic moiety at the NH_2-termini of membrane-bound lipoproteins offers a simple way for the anchorage of otherwise hydrophilic polypeptides onto either side of the biological membrane (Fig. 10).

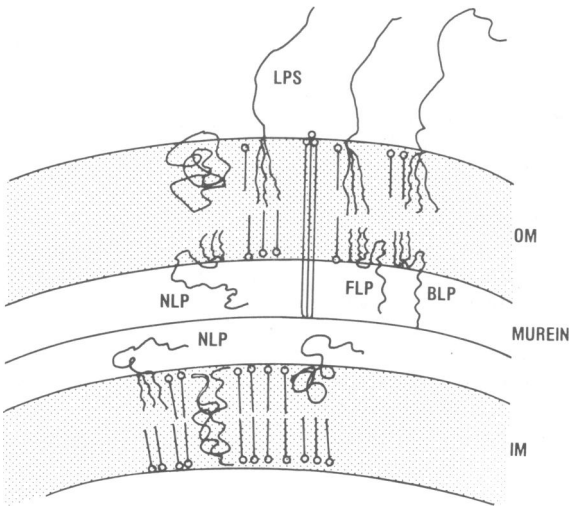

FIGURE 10 Schematic representation of the molecular organization of lipoproteins in the cell envelope of gram-negative bacteria. LPS: lipopolysaccharide; FLP: free-form Braun's lipoprotein; BLP: bound-form Braun's lipoprotein; NLP: new lipoprotein(s); OM: outer membrane; IM: inner membrane.

The function of Braun's lipoprotein has been a mystery. An *E. coli* mutant deleted for *lpp* gene shows very little change in phenotype (30). On the other hand, the primary structures of lipoprotein are extremely well conserved except for the signal sequence and the COOH-terminal sequence of lipoprotein, which is involved in covalent linkage to the peptidoglycan (31, 33). This highly conserved primary structure of lipoprotein and the extremely efficient expression of lipoprotein gene suggest there must be an important function of this protein. It is most likely that one major function of lipoprotein is mediated through its murein-bound form, especially in conjunction with other outer membrane proteins such as OmpA protein (7, 34). A physiological function of the free-form lipoprotein beyond its role as a precursor to the murein-bound form remains obscure. The recent discovery of minor lipoproteins with apparently similar lipid moieties attached to different inner and outer membrane proteins raises an intriguing question as to the functions of these lipoproteins and the role of the covalent lipid in the functioning of these lipoproteins. Regardless of what the physiological function of Braun's lipoprotein may be, it is conceivable that the functions of Braun's lipoprotein and the new lipoproteins may not be intimately dependent on the lipid moieties at their NH_2-termini. Rather, these lipoproteins may have diverse functions, structural or catalytic, while they share one feature in common, i.e., having their NH_2-termini anchored to the membrane via modified glyceride-cysteine residues. This common feature may have more to do with the biogenesis of these lipoproteins than their respective functions in the cell. This view is strengthened by the finding that a β-lactamase from *B. licheniformis* is synthesized as a lipoprotein. The lipid moiety at the NH_2-terminus of the membrane-bound penicillinase is not essential for its activity as a β-lactamase, since exo-forms of this enzyme containing no covalent lipid are secreted into the media and are fully active. Rather, the attachment of lipid to prepenicillinase allows the formation of membrane-bound form of this enzyme which is presumably anchored to the membrane via the hydrophobic lipid moiety at the NH_2-terminus.

Inasmuch as the modification enzymes are shared by these different lipoproteins, mutants deficient in these modification enzymes will be extremely valuable in the elucidation of the biosynthesis and function of these lipoproteins.

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DISCUSSION

Session Chairman: Franklyn G. Prendergast *Scribe*: Jennifer L. McKnight

PRENDERGAST: We begin with a question from a referee, John Cronan: "Is mutant 3 –2 deficient in the synthesis of lipoproteins other than Braun's?"

WU: No. We have been trying very hard to isolate a mutant which is defective in the modification of lipoproteins including the new lipoproteins, and so far we have not yet succeeded.

ENGELMAN: If the function of the linkage of lipid is to anchor the protein in the plasma membrane, how do you imagine the protein traversing the periplasmic space and arriving in the outer membrane? The most extreme case would be motion to the outer leaflet of the outer membrane bilayer.

WU: A very good question. The problem is that although we know there are many lipoproteins in *E. coli*, we don't have any information on their topology. So we actually don't know whether they go into the outer leaflet of the membrane or the inner leaflet. Braun's lipoprotein is the only one which is presumed to be facing inward because the C-terminal end of this protein is attached to the peptidoglycan. So it is reasonable to assume that the lipid is anchored in the inner leaflet of the outer membrane.

ENGELMAN: But if it is modified on the plasma membrane, how does it get across?

WU: That is a question that many of us would like to answer. That is, how does a protein go from the inner membrane to the outer membrane? The mature lipoprotein is translocated from the cytoplasmic membrane to the outer membrane extremely rapidly, with a transit time of seconds. If the lipid is not present but the leader sequence remains intact, for example a mutant prolipoprotein which cannot be modified, this lipid-deficient prolipoprotein can get to the outer membrane without much difficulty, and the transit time is also very rapid. In the globomycin treated cell, the prolipoprotein which contains lipid can not get out, and we don't know why. Presumably, the lipid moiety plus the hydrophobic leader sequence keep this protein from getting to the outer membrane. As far as the translocation of proteins to the outer membrane is concerned, several models have been proposed which involve Bayer's zone of adhesion (Osborn et al. 1980. *Annu. Rev. Microbiol.* 34:369 –422).

PRENDERGAST: Do you know anything about the protease responsible for the processing?

WU: All we know is that there is a leader peptidase for the bacteriophage M-13 procoat protein which has been purified by Wickner as a membrane bound enzyme. The leader peptidase for the prolipoprotein has been solubilized. We know it is a membrane bound enzyme, but there is no purification data. It is an endopeptidase which cleaves at the correct site.

PRENDERGAST: Have you any synthetic substrates for this enzyme?

WU: No. The substrate we have been using is the precursor protein.

STEVEN: There have been models around for a number of years which suggest that the lipoprotein forms an oligomer. There was Inouye's coiled-coil model (M. Inouye. 1974. *Proc. Natl. Acad. Sci. U.S.A.* 71:2396 –2400), in which lipoproteins were still being thought of as a potential porin. More recently, McLachlan (A. D. McLachlan. 1978. *J. Mol. Biol.* 121:493 –506) has formulated a model proposing a double-α-helical, coiled coil. Have there been any developments, experimental or otherwise, on the possible oligomeric state of lipoprotein in the membrane?

WU: Both schemes (Inouye and McLachlan) are based on model building. Inouye has built a model trying to use lipoprotein as a hydrophilic pore across the outer membrane. The porin has now been identified as being another protein.

There are no good physicochemical data to tell us how far the lipoprotein is embedded in the outer membrane, so in that sense we have no information about either the oligomeric structure, or even how deep it goes into the outer membrane.

TROY: Implicit in Fig. 9 is that the peptidase acts by "dipping" into the bilayer. Is there evidence as to where that peptide cleavage occurs relative to the face of the bilayer?

WU: Modification and processing are assumed to take place at the outside of the inner membrane, but there is no direct evidence to support this. It is part of the inner membrane.

TROY: So Fig. 9 then, where it shows the peptidase penetrating into the bilayer is speculative?

WU: It is hypothetical.

TROY: The model should be modified then to put the cleavage site outside of the phospholipid bilayer.

WU: We are postulating that the lipoprotein-specific signal peptidase may require modified protein as a substrate.