Lipid Modification of Bacterial Prolipoprotein
TRANSFER OF DIACYLGLYCERYL MOIETY FROM PHOSPHATIDYLGLYCEROL.*

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The peptide, MKATKIVGAVLGGSTLLAGCSSN, corresponding to the N-terminal 24 amino acids of Braun’s prolipoprotein, was used to study the lipid modification of prolipoprotein in Escherichia coli by measuring the rate of incorporation of either [2-3H]glycerol or [9,10-3H]palmitate from the corresponding labeled phosphatidylglycerol into the peptide. Using E. coli strains containing varying levels of prolipoprotein diacylglycerol modification activities due to mutations in or overexpression of the gene involved in diacylglycerol modification (lng), we have shown that the activities based on the peptide assay correlated well with the prolipoprotein-based assay. Further, we have followed the fate of the lipid substrate, phosphatidylglycerol, during the modification reaction and found that lipid modification of prolipoprotein involves the transfer of diacylglycerol moiety from phosphatidylglycerol to the sulfhydryl group of the cysteine residue with the concomitant formation of sn-glycerol 1-phosphate. This mechanism is contrary to the previously proposed two-step mechanism of an initial glyceryl transferase followed by O-acyltransferase (Chattopadhyay, P. K., and Wu, H. C. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5318-5322). Accordingly, the enzyme that catalyzes this activity has been named phosphatidylglycerol-prolipoprotein diacylglycerol transferase. The revised pathway for the lipoprotein biogenesis in bacteria consists of three successive reactions catalyzed by prolipoprotein diacylglycerol transferase, signal peptidase II, and apolipoprotein N-acyltransferase.

More than 130 lipoproteins of diverse structure, function, and bacterial origin have been identified to date (1), and they all contain N-acyldiacylglycerol-cysteine as their N-terminal amino acid (2). A common biosynthetic pathway for lipoproteins was proposed in 1982 by Tokunaga et al. (3) based on in vivo and in vitro studies using Braun’s lipoprotein of Escherichia coli (referred to as lipoprotein henceforth) as the prototype for all lipoproteins (3-6). The first step in the proposed pathway is the transfer of the non-acylated glycerol moiety of phosphatidylglycerol (PG) to the sulfhydryl group of the prospective N-terminal cysteine residue in the unmodified prolipoprotein by prolipoprotein-phosphatidylglycerol glycerol transferase (glycerol transferase) (4). This is followed by one or more O-acyltransferases acylating the sn-3 and sn-2 hydroxyls of the glycerol moiety of glycerolcysteine to form the diacylglycerol-modified prolipoprotein. In contrast to the specific requirement of PG for glycerol modification (5), liposomes containing [9,10-3H]palmitate-labeled phosphatidylethanolamine (PE), PG, or cardiolipin (CL) were found to serve as donors of the ester-linked fatty acyl chain in lipoproteins with comparable efficiencies (6). Diacylglycerol modification is a prerequisite for the cleavage of the signal peptide by a lipoprotein-specific signal peptidase called signal peptidase II (7-9). The amino-terminal of the product, apolipoprotein, is further N-acylated by N-acyltransferase which has no specificity with respect to phospholipids as acyl donors (10-12).

Two in vitro assays have been used in previous studies to measure glycerol transferase activity. The first measured the incorporation of [2-3H]glycerol from [2-3H]glycerol-labeled PG into unmodified prolipoprotein following immunoprecipitation and SDS-PAGE (7); however, the incorporation of [2-3H]glycerol was poor due to the low amount of prolipoprotein used as the acceptor. More recently, [35S]methionine-labeled prolipoprotein freshly prepared by in vitro transcription and translation was used in an assay system that relied on the shift in mobility of unmodified prolipoprotein in Tricine-SDS-PAGE (13) upon digestion with signal peptidase II. This assay required fresh substrate for efficient modification; however, heating the substrate with 1 M guanidinium chloride at 100 °C for 5 min increased the efficiency of conversion severalfold and allowed the substrate to be used following storage at -20 °C (15).

Both assays referred to above are laborious and time consuming (up to 24 h). Owing to the low amount (picomolar) of the protein substrate used in these assays, it is not possible to verify the proposed reaction mechanism for this enzyme; i.e. the release of phosphatic acid (PA) concomitant with the transfer of non-acylated glycerol moiety from PG to the prolipoprotein. A simpler, more rapid and quantitative assay was needed for the purification of the modification enzymes and the study of their reaction mechanisms. Since the structural determinants for the lipid modification reside in the signal peptide and adjacent few residues of the mature protein (1), we were able to develop a simpler, quicker, and more quantitative assay using a synthetic peptide containing N-terminal 24 amino acids of Braun’s prolipoprotein (16) as the substrate. More importantly, the results obtained with the new assay have led us to modify the proposed biosynthetic pathway. We report in this study that diacylglycerol modification of prolipoprotein proceeds by the transfer of diacylglycerol moiety from PG to the cysteine residue to form diacylglycerol-prolipoprotein with the concomitant formation of sn-glycerol 1-phosphate. Accordingly, the enzyme that catalyzes this reaction is named prolipoprotein-phosphatidylglycerol diacylglycerol transferase.

EXPERIMENTAL PROCEDURES

Synthesis of the Peptide—The peptide containing the N-terminal 24 amino acids (MKATKIVGAVLGGSTLLAGCSSN) of Braun’s prolipo-

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The abbreviations used are: PG, phosphatidylglycerol; PA, phosphatic acid; PE, phosphatidylethanolamine; CL, cardiolipin; GDPG, cytidine 5'-diphosphodiacylglycerol; Triene, N-[2-hydroxy-1,1,3-dioxymethyl]ethylglycine; PAGE, polyacrylamide gel electrophoresis; LB, Luria broth.
were washed once with 10 ml of TED, resuspended in TED containing with the rest being phosphatidic acid. The reaction mixture was fractionated by TLC, and the radioactive spot corresponding to PG was eluted and stored as described above.

The soluble fraction was obtained after centrifugation at 6,000 g for 1 h. The soluble fraction was then allowed to reach the late log phase. The soluble fraction was then added; the culture was then allowed to reach the late log phase.

For the remaining radioactivity.

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Phospholipids were isolated as described above.


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Acid Hydrolysis and Detection of Glycerolysteine—The modified peptide was desalted using a Sephadex G-50 mini-column and then subjected to hydrolysis at 110 °C for 16 h with constant boiling HCl containing phenylmethylsulfonyl fluoride (HMF). The standard, glycerolysteine, was prepared from acetaminophen and thioglycerol (Koch-Light Laboratories Ltd., England) according to the method described by Hantke and Braun (2).

The hydrolysate was analyzed by silica gel TLC using chloroform:methanol:water, 4:4:1 (v/v) systems separately. The standard glycerolysteine spot was revealed by ninhydrin spray (0.5 g of Ninhydrin in 100 ml of 1-butanol containing 3 ml of acetic acid), and the corresponding spot in the peptide hydrolysate lane and the zones above and below were scraped and counted.

Identification of Reaction Products Derived from Labeled PG—After an extended incubation of the peptide with [2-3H]glycerol-labeled PG and the enzyme preparation from the lgt clone, T7GT, the reaction mixture was extracted with chloroform:methanol:water, 4:4:1 (v/v), and butanol:acetic acid:water, 4:1:1 (v/v) systems separately. The standard glycerolysteine spot was revealed by ninhydrin spray (0.5 g of Ninhydrin in 100 ml of 1-butanol containing 3 ml of acetic acid), and the corresponding spot in the peptide hydrolysate lane and the zones above and below were scraped and counted.

PG Specifically Labeled at Non-acetylated Glycerol—This was synthesized enzymatically from CDP-diaclylglycerol (CDP-DG, Sigma) and sn-[2-3H]glycerol 3-phosphate according to the procedure of Shibuuya and Hirooka (18). sn-[2-3H]Glycerol 3-phosphate was prepared from 1 ml of 10 μCi of [2-3H]glycerine (specific activity 575,000 cpm) by glycerokinase and ATP (Sigma).

The yield of PG was 10%. Exhaustive digestion of the labeled PG with phospholipase D (Sigma) released 73% of the radioactivity into the aqueous phase of chloroform:methanol extract. TLC analysis of the chloroform phase revealed no PA, with the undigested PG accounting for the remaining radioactivity.

PG Specifically Labeled at the Diacylglycerol—Trans-phosphatidyl-

PG analysis of the reaction product showed a 69% conversion of PE to PG specifically labeled at the diacylglycerol moiety was developed. Under the assay conditions, the modified peptide was hydrolyzed with 0.1 N NaOH in methanol.

Preparation of Crude Membrane Fraction—Bacteria (E. coli wild-type strain [BL-21], lgt mutants [strain SK634, SK635] [15, 20] defec-
tative substrate. Based on this observation, a peptide-based assay that measures the incorporation of [2-3H]glycerol or [9,10-3H]palmitate into the peptide from the specifically labeled-PG as the lipid donor was developed. Under the assay conditions described, the modification was linear with respect to time and enzyme concentration (data not shown). The Kₘ for the peptide was 30 μM (Fig. 1B). Iodoacetate-treated peptide did not undergo modification (data not shown) as would be expected from the requirement of the SH group for modification. The levels of the prolipoprotein diacylglycerol modification activity measured by the peptide assay in lgt mutants (strain SK634, SK635), wild type (BL-21), and the clone (BL-

RESULTS

Synthetic Peptide Mimics the Protein Substrate for Lipid Modification—The synthetic peptide completely inhibited prolipoprotein modification at a concentration of 200 μM (0.5 mg/ml) in the diacylglycerol modification assay using unmodified prolipoprotein (Fig. 1A, compare T7GT with T7GT + PEP). The peptide inhibited modification presumably by virtue of being a competitive substrate. Based on this observation, a peptide-based assay that measures the incorporation of [2-3H]glycerol or [9,10-3H]palmitate into the peptide from the specifically labeled-PG as the lipid donor was developed. Under the assay conditions described, the modification was linear with respect to time and enzyme concentration (data not shown). The Kₘ for the peptide was 30 μM (Fig. 1B). Iodoacetate-treated peptide did not undergo modification (data not shown) as would be expected from the requirement of the SH group for modification. The levels of the prolipoprotein diacylglycerol modification activity measured by the peptide assay in lgt mutants (strain SK634, SK635), wild type (BL-21), and the clone (BL-21 [T7GT]) that hyperexpresses the lgt gene correlated very well with the results from the prolipoprotein assay (Fig. 1A).

Both [2-3H]glycerol- and [9,10-3H]palmitoyl-modified peptides migrated equally, but they both migrated slower than the native peptide in Tricine-SDS-PAGE (Fig. 2). This observation suggests that the peptide is modified with a diacylglycerol moiety, and the conclusion is consistent with previous observations of the electrophoretic mobility of diacylglycerol-modified prolipoprotein as compared to that of unmodified prolipoprotein (Figs. 1A and 3) (15). After alkali hydrolysis (0.1 N NaOH in 6 M guanidinium chloride, 20 mM dithiothreitol, and 1% n-octyl β-D-glucoside at a protein concentration of 5 mg/ml, and left under a stream of nitrogen and suspended in TED (20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA and 2 mM dithiothreitol) using glycerokinase and ATP (Sigma).

The yield of PG was 10%. Exhaustive digestion of the labeled PG with phospholipase D (Sigma) released 73% of the radioactivity into the aqueous phase of chloroform:methanol extract. TLC analysis of the chloroform phase revealed no PA, with the undigested PG accounting for the remaining radioactivity.

PG were added; the culture was then allowed to reach the late log phase. Phospholipids were isolated as described above.


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alkal-labile O-acyl linkages in the modified peptide. TLC analysis of the acid hydrolysate of glycerol-labeled peptide revealed a radioactive spot that had the same \( R_f \) values (0.56 and 0.17, respectively) as synthetic glycerolysteine in both chloroform: methanol:ammonia, 4:4:1 (v/v), and the 1-butanol:acetic acid: water, 4:1:1 (v/v), systems confirming that the modification is identical to that seen in lipoproteins. These data prove that the synthetic peptide (the first 24 amino acids of the N-terminal part of Braun’s prolipoprotein) behaved like its parent polypeptide with respect to diacylglycerol modification and that the assay provides a true measure of the modification activity.

**Phospholipid Specificity in Diacylglycerol Modification**—Only PG but not PE or CL was found to support the diacylglycerol modification activity using either the prolipoprotein (Fig. 3) or the peptide as the substrate (Table I). Transfer of the palmitoyl group from [9,10-\( ^3 \)H]palmitoyl-labeled PE or CL to the peptide was not observed during co-incubations with non-radioactive PG, nor was a significant reduction in the incorporation of [9,10-\( ^3 \)H]palmitate into the peptide observed after incubation of the peptide with palmitoyl-labeled PG and unlabeled PE or CL (data not shown). A reduction in [9,10-\( ^3 \)H]palmitate incorporation from PG into the peptide in presence of unlabeled PE or CL would be expected based on the two-step mechanism of glycerol transfer (with PG as the substrate) followed by O-acyl transfer (with PE, PG, or CL as substrates). Surprisingly, unmodified prolipoprotein was also found to be modified, albeit less efficiently, with PA or CDPDG, neither of which contains non-acylated glycercol (Fig. 3).

**Diacylglycerol Transfer to the Peptide and Release of sn-Glycerol 1-Phosphate from PG**—The fate of PG in the reaction was studied directly by analyzing the products of the reaction using PG labeled in either or both of the diacylglycerol and non-acylated glycerol portions of PG after chloroform:methanol extraction of the assay mixture. When PG labeled at both the acylated and the non-acylated portions was used as the substrate, equal amounts of counts were found in the aqueous phase of chloroform:methanol extracts and the acetone pellet (Table I, row 1). When PG labeled specifically at the non-acylated glycerol was used, almost all of the radioactivity was found in the aqueous phase (Table I, row 2); the small amount of radioactivity associated with the pellet was found to be eliminated by further washings. In contrast, using PG labeled specifically in the diacylglycerol portion as the substrate, the radioactivity was found exclusively in the peptide (Table I, row 3), strongly supporting the idea that the diacylglycerol moiety of PG is transferred to the peptide/prolipoprotein. According to this mechanism, sn-glycerol 1-phosphate will be produced concomitant with diacylglycerol transfer. In contrast to sn-glycerol 3-phosphate, the radioactive product in the aqueous phase whose migration was the same as sn-glycerol 3-phosphate in paper electrophoresis (Fig. 4A) and TLC (data not shown), was not oxidized by glycerol-3-phosphate dehydrogenase as measured spectrophotometrically (data not shown). In addition, analysis of the chloroform phase by two-dimensional TLC did not reveal the presence of PA (Fig. 4B), the formation of which would be expected according to the previously proposed pathway. The tiny radioactive spots seen near the origin (mature lipopéptide) of the chromatogram (Fig. 4B) and at the position of Lysy-PG (phospholipid product from N-acylation reaction) may be due to signal peptidase II processing and N-acylation of the diacylglycerol-modified peptide during a prolonged incubation in the absence of globomycin, a specific inhibitor of signal peptidase II.

**DISCUSSION**

Earlier investigations of lipoprotein biosynthesis using in vivo pulse-chase experiments with [2-\( ^3 \)H]glycerol in the pres-
ence of cerulenin showed incorporation of radioactivity preferentially into the non-acylated glyceryl moiety of PG and also lipoprotein (4). Comparison of the \(^{3}H/^{14}C\) ratio of delipitated lipoprotein doubly labeled with \(^3H\) or \(^{14}C\) arginine and sn-{1,3-\(^{14}C\)}, sn-{1,3-\(^{14}C\)}, or \([^{2-3}H\)labeled glycerol before and after periodate treatment led to the conclusion that the non-acylated glyceryl moiety of PG is transferred to the sulphydryl group of the cysteine in the unmodified prolipoprotein (4). \textit{In vivo} vesicle fusion studies containing \([^{2-3}H\)glycerol- or \([^{9,10-3}H\)palmitoyl-labeled phospholipid lent support to the above idea and also suggested a lack of specificity with respect to the utilization of the major phospholipids as acyl donors of ester-linked fatty acids in lipoproteins (5, 6). The finding that an \(lpp\) mutant lipoprotein (strain JE5511, \(lpp-1\ Arg^76 \rightarrow Cys^76\)) is deficient in ester-linked fatty acids (24, 25) is consistent with the two-step pathway. On the other hand, the lipoprotein precursor that accumulated in globomycin-treated cells of strain JE5511 was found to be labeled with \([^{9,10-3}H\)palmitate and showed a mobility in Tricine-SDS-PAGE expected for diacylglycerol-modified prolipoprotein.\(^5\)

Our attempts to identify the \(O\)-acyltransferase(s) by \textit{in vitro} conversion of glycerol prolipoprotein (prepared \textit{in vitro} by the treatment of diacylglycerol-modified prolipoprotein with either pancreatic lipase or ethanolic NaOH) to diacylglycerol-modified prolipoprotein with inverted vesicle preparations was not successful. Under the same conditions, diacylglycerol modification of unmodified prolipoprotein was very efficient. When we attempted to hyperexpress the \(lgt\) gene for the putative prolipoprotein glyceryl transferase we found that the rate of overall diacylglycerol modification increased. Since the insert in the clone contains only a single open reading frame corresponding to \(lgt\) (15), this finding is surprising unless the putative \(O\)-acyltransferases are present in large excess of glyceryl transferase in wild-type \textit{E. coli}. Finally, partial purification of diacylglycerol-modifying activity from crude extracts of the clones did not result in the separation of these two activities.

The results presented here based on the peptide assay clearly show a direct transfer of the diacylglycerol portion of PG to the cysteine —SH group in the peptide/prolipoprotein. Such a mechanism provides an explanation for the similarity in the fatty acid composition of the ester-linked fatty acids in lipoprotein with that of bulk phospholipid including the presence of cyclopropane fatty acid. Interestingly, the diacylglycerol transferase does not recognize the diacylglycerol group of PE or CL which is made from two molecules of PG. In contrast, CDPDG and PA with CDP and phosphate as the polar head-group, respectively, are substrates for this enzyme, albeit less efficient. The revised pathway predicts that sn-glycerol 1-phosphate is released during lipoprotein maturation. With outer membrane (Braun’s) lipoprotein as the major substrate of this pathway \textit{in vivo}, the metabolic fate of sn-glycerol 1-phosphate deserves further study.

The peptide-based assay for prolipoprotein diacylglycerol transferase is not only more quantitative but also simpler and more rapid than the prolipoprotein-based assay using SDS-PAGE. It is anticipated that this assay will greatly facilitate purification of this enzyme. Though a variety of labeled PG substrates have been used here, the use of \([^{9,10-3}H\)palmitate-labeled PG (from SD9 (\(pssA-1\) \(cis-1\)) grown at 42 °C in LB containing 400 mM sucrose and 20 mM MgCl\(_2\)) (18) seems advantageous as the substrate for routine assays because of the ease of obtaining higher specific activity preparations. We find that the concentration of \(n\)-octyl \(\beta\)-glucoside and guanidinium chloride in the assay should be below 0.25% and 100 mM, respectively, and many commonly used detergents are not compatible with the enzyme activity (7). Alternatively, diacylglycerol modification activity can be assayed indirectly but more rapidly by counting the aqueous phase of the reaction mixture for labeled sn-glycerol 1-phosphate, after chloroform-methanol extraction.

### Table 1

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>input counts</th>
<th>aqueous phase counts</th>
<th>acetone pellet counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^{2-3}H)glycerol PE</td>
<td>443,794</td>
<td>896</td>
<td>960</td>
</tr>
<tr>
<td>([^{2-3}H)glycerol CL</td>
<td>386,432</td>
<td>699</td>
<td>1,468</td>
</tr>
<tr>
<td>([^{9,10-3}H)palmitoyl PG</td>
<td>224,105</td>
<td>752</td>
<td>27,580</td>
</tr>
<tr>
<td>([^{9,10-3}H)palmitoyl PE</td>
<td>424,546</td>
<td>1,150</td>
<td>1,221</td>
</tr>
<tr>
<td>([^{9,10-3}H)palmitoyl CL</td>
<td>410,464</td>
<td>873</td>
<td>1,497</td>
</tr>
</tbody>
</table>
Bacterial Lipoprotein Biosynthesis

A

![Graph showing cpm vs. distance in cm for Glycerol Phosphate and Glycerol](image)

B

![TLC analysis of chloroform:methanol extract with PA, PG, and LPG](image)

FIG. 4. A, paper electrophoresis of the radioactive product in the aqueous phase of the chloroform:methanol extract of the diacylglycerol modification reaction mixture containing [2-3H]glycerol-labeled PG as the substrate. B, two-dimensional TLC analysis of the chloroform layer of the chloroform:methanol extract of the diacylglycerol modification reaction mixture after an extended incubation of the peptide and [2-3H]glycerol-labeled PG with the enzyme preparations from the E. coli clone T7GT (bottom) and E. coli lgt mutant, SK634 (middle). The top panel shows schematically the position of the phospholipids. First direction (1), chloroform:methanol:ammonia, 65:35:4 (v/v); second direction (2), chloroform:methanol:acetic acid:water, 85:25:5:4 (v/v).

FIG. 5. The proposed pathway for lipoprotein maturation.

It is known from lpp mutants and lipoprotein-β-lactamase fusions that most of the sequence of the mature portion of the prelipoprotein is not required for the lipid modification (6, 27). Using bacteriocin release protein (BRP) fused to β-lactamase, it was shown that the signal peptide plus the Cys of BRP was sufficient for the lipid modification and processing (28). Our in vitro study using the signal peptide plus four N-terminal amino acids of mature lipoprotein supports this conclusion. It appears likely that this assay will also facilitate the use of the N-terminal sequence of any lipoprotein to study its lipid modification. It may also be useful for studies on the kinetics of modification and the structure-function relationship in modification.

The kinetics of pulse-chase experiments with [2-3H]glycerol as the precursor for lipoprotein (4, 29) and the lethality of the pgsA3 mutant (30) in the presence of major outer membrane lipoprotein (31) strongly argue that PG is the physiological substrate in lipoprotein maturation. The ability of PA and CDPDG to function as diacylglycerol donors in vitro suggests that lipid modification of certain essential lipoprotein(s) could go on in the pgsA3 mutant lacking or containing unmodifiable Braun’s lipoprotein even in the absence of PG, because of a lack of competition for the scarce supply of the lipid donor from the major outer membrane lipoprotein.

The revised pathway for the biosynthesis of bacterial lipoproteins (Fig. 5) involves the following three steps: diacylglycerol modification, cleavage of signal peptide, and N-acylation. This pathway predicts the existence of three genes encoding prelipoprotein modification and processing enzymes. All three genes have been identified; they are, lgt (prelipoprotein diacylglycerol transferase) (15), lsp (prelipoprotein signal peptidase) (32, 33), and int (apolipoprotein N-acyltransferase) (34, 35). All three genes appear to be essential, suggesting that lipid modification of certain minor lipoprotein(s) in E. coli is essential for its viability, growth or division.
Acknowledgments—We thank Dr. Isao Shibuya for E. coli strains SD9 and AD10(pMAL), Dr. Sydney R. Kushner for the mutant strains SK634 and SK635, Mike Flora for synthesis of the peptide, Keda Gan for the lgt clone (T7GT), and Dr. Paul Rick for many helpful suggestions.

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