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Structure-Function Relationship of Bacterial Prolipoprotein Diacylglyceryl Transferase: Functionally Significant Conserved Regions

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The structure-function relationship of bacterial prolipoprotein diacylglyceryl transferase (LGT) has been investigated by a comparison of the primary structures of this enzyme in phylogenetically distant bacterial species, analysis of the sequences of mutant enzymes, and specific chemical modification of the *Escherichia coli* enzyme. A clone containing the gene for LGT, *lgt*, of the gram-positive species *Staphylococcus aureus* was isolated by complementation of the temperature-sensitive *lgt* mutant of *E. coli* (strain SK634) defective in LGT activity. In vivo and in vitro assays for prolipoprotein diacylglyceryl modification activity indicated that the complementing clone restored the prolipoprotein modification activity in the mutant strain. Sequence determination of the insert DNA revealed an open reading frame of 837 bp encoding a protein of 279 amino acids with a calculated molecular mass of 31.6 kDa. *S. aureus* LGT showed 24% identity and 47% similarity with *E. coli*, *Salmonella typhimurium*, and *Haemophilus influenzae* LGT. *S. aureus* LGT, while 12 amino acids shorter than the *E. coli* enzyme, had a hydropathic profile and a predicted pI (10.4) similar to those of the *E. coli* enzyme. Multiple sequence alignment among *E. coli*, *S. typhimurium*, *H. influenzae*, and *S. aureus* LGT proteins revealed regions of highly conserved amino acid sequences throughout the molecule. Three independent *lgt* mutant alleles from *E. coli* SK634, SK635, and SK636 and one *lgt* allele from *S. typhimurium* SE5221, all defective in LGT activity at the nonpermissive temperature, were cloned by PCR and sequenced. The mutant alleles were found to contain a single base alteration resulting in the substitution of a conserved amino acid. The longest set of identical amino acids without any gap was H-103-GGLIG-108 in LGT from these four microorganisms. In *E. coli lgt* mutant SK634, Gly-104 in this region was mutated to Ser, and the mutant organism was temperature sensitive in growth and exhibited low LGT activity in vitro. Diethylpyrocarbonate inactivated the *E. coli* LGT with a second-order rate constant of 18.6 M^{-1} s^{-1}, and the inactivation of LGT activity was reversed by hydroxylamine at pH 7. The inactivation kinetics were consistent with the modification of a single residue, His or Tyr, essential for LGT activity.

Posttranslational lipid modification of prolipoproteins in bacteria involves three sequential reactions catalyzed by cytoplasmic membrane enzymes, i.e., prolipoprotein:phosphatidylglycerol diacylglyceryl transferase (LGT), prolipoprotein signal peptidase, and apolipoprotein *N*-acyl transferase, resulting in the formation of *N*-acyl diacylglycerylcysteine as the N-terminal amino acid of these lipid-modified proteins (10, 14). More than 130 lipoproteins have been identified in the bacterial kingdom. In general, a putative lipoprotein is identified by the presence of a lipobox sequence (commonly, -Leu_{-3}-Ser/Ala_{-2}-Ala/Gly_{-1}-Cys_{+1}) present at the C-terminal portion of the signal sequence of the preprotein (2). This pathway appears to be essential since mutants defective in the activity of any of these three enzymes are temperature sensitive in growth, suggesting that one or more lipoproteins are required for normal growth, division, and viability of bacterial cells (7, 9, 23).

The LGT protein catalyzes a novel lipid modification reaction by transferring the diacylglyceryl group from phosphatidylglycerol (PG) to the sulfhydryl group of the invariant cysteine residue in the lipobox of prolipoproteins (14), and this modification is a prerequisite for the subsequent cleavage of the prolipoprotein signal peptide by prolipoprotein signal peptidase (4, 19). The *lgt* gene has been identified in *Escherichia coli* (8), *Salmonella typhimurium* (7), and *Haemophilus influenzae* (5). The novelty of the reaction catalyzed by this enzyme, its importance as the first committed step in this essential pathway, and its ubiquity in bacteria have prompted us to examine its structure-function relationship and to identify the catalytically important residues. Since a comparison of homologous protein sequences among distantly related microorganisms can reveal highly conserved regions important for the structure and function of an enzyme, we have cloned and sequenced the *lgt* gene from *Staphylococcus aureus* and compared the deduced LGT sequence with those of *E. coli*, *S. typhimurium*, and *H. influenzae*. We have also sequenced four independent *lgt* mutant alleles from both *E. coli* (22) and *S. typhimurium* (7) that are defective in this enzyme at the nonpermissive temperature. The LGT enzyme was found to be readily inactivated by diethylpyrocarbonate (DEPC), and the inactivation kinetics indicated the presence of a single modifiable residue essential for the activity.

MATERIALS AND METHODS

Materials. [^{35}S]methionine (specific activity, 1,150 Ci/mmol) and [9,10-^{3}H]palmitate (specific activity, 54 Ci/mmol) were purchased from ICN Biomedical, Inc. (Irvine, Calif.), and Du Pont NEN (Wilmington, Del.), respectively. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc. (Beverly, Mass.). DNA Taq DyeDeoxy Terminator cycle sequencing kit was purchased from Applied Biosystems, Inc. (Foster City, Calif.). TA cloning kit was obtained from Invitrogen Corp. (San Diego, Calif.). PCR reagents were from Roche Molecular System, Inc. (Branchburg, N.J.). DEPC and phospholip-

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ids were from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were of the finest commercial grade available.

Bacterial strains, plasmids, and culture media. Strains used in the present study included temperature-sensitive *E. coli lgt* mutants SK634, SK635, and SK636 obtained from S. R. Kushner (University of Georgia, Athens) (22), *S. typhimurium lgt* mutant SE5221 (7), and *E. coli* DH5α as the host in cloning experiments. pACYC184 was used as a vector for subcloning, and pT7-7 was used as a vector for expression of the cloned *lgt* gene. *E. coli* SD9 (*pssA cls-1*) (17), a gift from Isao Shibuya (Saitama University, Urawa, Japan), was used for the preparation of [^{3}H]palmitate-labeled PG. Culture media included L broth (LB) and M9 minimal medium supplemented with glucose (0.4%) and required amino acids (20 μg of each per ml) (7). Antibiotic was added to a final concentration of 50 μg of ampicillin per ml, 35 μg of chloramphenicol per ml, or 10 μg of tetracycline per ml. Bacteria were grown at 30, 37, or 42°C with aeration in LB or on LB-agar plates. M9 minimal medium was used for radioactive labeling experiments.

Isolation of a *Staphylococcus aureus* genomic clone that complemented the temperature-sensitive *lgt* mutation. A commercial *S. aureus* (FDA no. 485 A B D *ent*$^+$ strain) genomic DNA library containing average 2.32-kb inserts in pGEM1 (Clontech Laboratories, Inc., Palo Alto, Calif.) was a gift from Motoyuki Sugai (Hiroshima University, Hiroshima, Japan). The library was transformed into the temperature-sensitive *lgt* mutant strain SK634 by electroporation, and the transformants were selected on LB plates containing 50 μg of ampicillin per ml at 30°C, replica plated, and incubated at 42°C. Plasmids isolated from the pool of temperature-resistant colonies from each plate were retransformed into the mutant strain at 30°C, and the transformants were screened for the temperature-resistant phenotype at 42°C. The physical map of the plasmid which conferred the temperature-resistant phenotype was determined. The DNA insert was subcloned into pACYC184, and the ability of the subclones to complement the temperature-sensitive mutation was determined.

DNA sequencing. Plasmids for DNA sequencing were purified with a Wizard Minipreps DNA purification system from the Promega Corp. (Madison, Wis.). Double-stranded DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (13) according to the supplier's instructions. On the basis of the sequence data obtained, appropriate oligonucleotide primers (19-mers) were synthesized. The sequence of the DNA insert corresponding to the 1.47-kb region beginning at the *Hin*dIII site in pGM01 was determined in both directions, and the sequence data were analyzed by the University of Wisconsin Genetics Computers Group's sequence analysis software package.

Recombinant DNA techniques. Restriction endonuclease and DNA ligase were used according to the manufacturer's specifications. Plasmid preparations and transformation were performed by standard procedures (12).

In vivo assay of diacylglyceryl modification of prolipoprotein. The in vivo activity of diacylglyceryl modification of prolipoprotein was assessed by pulse-labeling experiments according to the method described previously (7). Briefly, cells grown in M9 glucose medium to an optical density at 600 nm of 0.5 at 30°C were shifted to 42°C for 1 h, and 1 ml of culture was then labeled with 10 μCi of [^{35}S]methionine for 2 min. Labeling was terminated with the addition of trichloroacetic acid (10% final concentration), and the radiolabeled lipoprotein species were immunoprecipitated, separated on a Tricine-sodium dodecyl sulfate (SDS) gel (16), and subjected to autoradiography.

In vitro assay of prolipoprotein diacylglyceryl transferase activity. The enzyme was assayed as described previously (14) with the following modifications. *E. coli* SD9 (*pssA cls-1*) grown at 42°C in LB containing 400 mM sucrose and 50 mM $MgCl_2$ was labeled with [9,10-^{3}H]palmitate, and the labeled phospholipid preparation containing 75% PG and 25% phosphatidylethanolamine was used as the source of labeled PG substrate. For the assay, the enzyme preparation was incubated in 50 μl of 20 mM Tris-HCl, pH 8.0, containing 4 mM dithiothreitol, 5 mM EDTA (pH 8.0), 50 mM guanidinium chloride (pH 8.0), 0.15% *n*-octyl-β-D-glucoside, 250,000 cpm of [9,10-^{3}H]palmitate-labeled phospholipid (final specific activity, 0.8 μCi of PG per nmol), and 100 μM peptide for 25 min at 37°C (14). The reaction was terminated by the addition of 400 μl of acetone following the addition of 4 μl of 20% saturated ammonium sulfate which facilitated the precipitation of the peptide. After 15 min at room temperature, the pellet was collected by centrifugation and washed once with 90% acetone saturated with ammonium sulfate. The washed pellet was dissolved in 100 μl of 1% SDS, and the radioactivity was measured by scintillation counting.

Inactivation of prolipoprotein diacylglyceryl transferase activity by DEPC (11). The crude enzyme preparation (0.05 mg of protein per ml of 1% octylglucoside-solubilized inverted vesicle preparation) was incubated at 25°C with varying concentrations of DEPC in 25 μl of 100 mM sodium phosphate buffer (pH 6.0). At stipulated time intervals, the reaction with DEPC was terminated by the addition of 10 μl of imidazole (pH 6.0) to a final concentration of 10 mM. The prolipoprotein diacylglyceryl transferase activity was then measured in 50 μl of 50 mM phosphate buffer, pH 6.0; pH 6.0 was chosen for inactivation by DEPC because of the stability and specificity of DEPC towards histidine at this pH (11). The actual concentration of DEPC and its half-life (15 min) under the experimental conditions were determined by the imidazole method (11). Restoration of the activity of DEPC-treated enzyme by hydroxylamine was examined by incubating the DEPC-inactivated enzyme (0.5 mg of protein per ml) with 0.5 M hydroxylamine (pH 7.0) at 25°C for various periods of time. Aliquots containing 1 μg of protein were then assayed for the LGT activity. The final hydroxylamine concentration in the assay was 25 mM, and there was no significant loss of LGT activity even during prolonged incubations in the presence of hydroxylamine.

Nucleotide sequence accession number. The sequence of the 1.47-kb insert has been submitted to the GenBank database (accession number U35773).

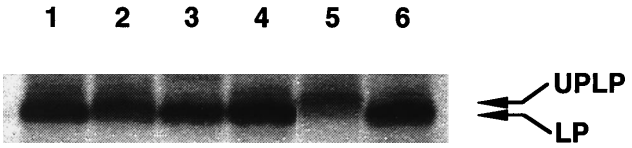

FIG. 1. In vivo assay of diacylglyceryl modification of prolipoprotein in *lgt* mutant strain SK634 with or without the complementing clone. [^{35}S]methionine-labeled lipoprotein in wild-type DH5α, *lgt* mutant SK634, and mutant SK634 carrying the complementing clone pGM01 was analyzed by Tricine-SDS-polyacrylamide gel electrophoresis. Lanes 1 and 4 represent DH5α grown at 30 and 42°C, respectively; lanes 2 and 5 represent mutant SK634 grown at 30 and 42°C, respectively; lanes 3 and 6 represent SK634 with pGM01 clone grown at 30 and 42°C, respectively. UPLP, unmodified prolipoprotein; LP, mature lipoprotein.

RESULTS

Isolation of DNA fragment encoding LGT in *S. aureus*. By selecting temperature-resistant transformants of the *E. coli lgt* mutant SK634 from a genomic library of *S. aureus* cloned in the plasmid vector pGME, we obtained a clone (pGM01) with a 3.8-kb insert that complemented both the temperature-sensitive phenotype and the defect in prolipoprotein diacylglyceryl modification (Fig. 1 and 2). In contrast to the mutant cells which accumulated the unmodified prolipoprotein at 42°C (Fig. 1, lane 5), the transformant with pGM01 contained mature lipoprotein at 42°C (Fig. 1, lane 6). In vitro assay for the LGT activity revealed that mutant cells harboring the complementing clone restored LGT activity (0.29 nmol/min/mg of protein compared with 0.04 nmol/min/mg of protein in the mutant cells). A restriction map of the DNA insert in pGM01 is shown in Fig. 2. The DNA sequence of a 1.5-kb region in pGM01 revealed a single open reading frame (ORF) of 837 bp encoding a protein of 279 amino acids with a calculated molecular mass of 31.6 kDa (Fig. 3). A putative promoter and a potential ribosome-binding site are present immediately upstream of this ORF. To ascertain that this ORF predicted from the DNA sequence encodes LGT activity, the entire ORF sequence was amplified by PCR and cloned in a T7 expression vector (18) to generate pT706. Plasmid pT706 complemented the temperature-sensitive phenotype of *lgt* mutant SK634 (Fig. 2). In addition, an elevated level of LGT activity was found in

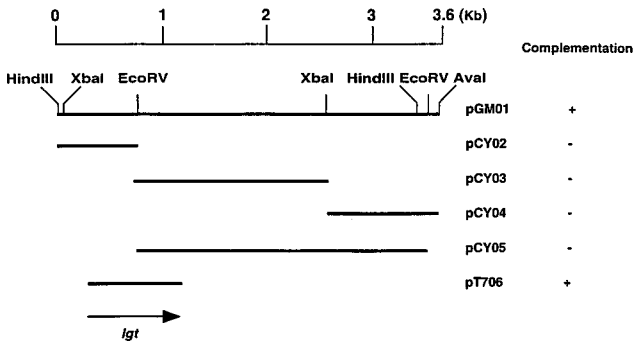

FIG. 2. The restriction enzyme map of pGM01 from *S. aureus* that complements the temperature-sensitive mutation in SK634 and restores the LGT activity. Results of complementation of the temperature-sensitive phenotype are expressed as + or −. The arrow denotes the direction of transcription of the *lgt* gene.

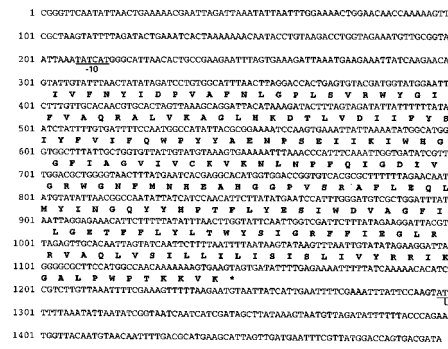

FIG. 3. The nucleotide sequence of the 1.5-kb fragment and the deduced amino acid sequence of the *S. aureus lgt* gene. Symbols: underline, putative promoter; double underline, putative ribosome-binding site; *, stop codon; ⌊→, start codon of an ORF 3′ to the *lgt* gene.

BL21 (F^- Δ*ompT* $r_B^-m_B^-$) cells harboring pT706 (specific activity of LGT in BL21, 0.16 nmol/min/mg of protein versus that of BL21 harboring pT706, 2.4 nmol/min/mg of protein). A partial ORF found downstream of *lgt* beginning at bp 1270 did not have significant homology to the *E. coli thyA* gene.

Comparison of the deduced amino acid sequence of *S. aureus* LGT with LGT proteins of *E. coli*, *S. typhimurium*, and *H. influenzae*. Comparison of the deduced amino acid sequence of *S. aureus* LGT with the sequences in the GenBank database revealed a high degree of homology with previously cloned LGT from *E. coli* (8), *S. typhimurium* (7), and *H. influenzae* (5). *S. aureus* LGT displayed 24% identity and 47% similarity with the *E. coli*, *S. typhimurium*, and *H. influenzae* LGT (Fig. 4). Like the LGT from *E. coli*, *S. typhimurium*, and *H. influenzae*, the *S. aureus* LGT contains significant stretches of hydrophobic segments interrupted by charged hydrophilic segments. It has a net charge of +9 at neutral pH (+5 for *E. coli* LGT, +6 for *S. typhimurium* LGT, and +3 for *H. influenzae* LGT) because of an abundance of basic amino acids, Arg and Lys. Whereas the *E. coli*, *S. typhimurium*, and *H. influenzae* enzymes have predominantly Arg residues (20, 21, and 16, respectively) and only a few Lys residues (three in *E. coli* and *S. typhimurium* and five in *H. influenzae*), the *S. aureus* LGT has about an equal number of Arg (11) and Lys (12) residues. The deduced pI is nearly the same for three enzymes (10.45 for *E. coli* LGT, 10.58 for *S. typhimurium* LGT, and 10.40 for *S. aureus* LGT) and is a little higher than that of *H. influenzae* LGT (9.77). Like the *E. coli*, *S. typhimurium*, and *H. influenzae* LGT, *S. aureus* LGT is also enriched in α-helix-breaking amino acids, glycine and proline, and has predominantly β-sheet structure and low α-helix content on the basis of the Chou-Fasman prediction (3). *S. aureus* LGT is smaller than *E. coli* and *S. typhimurium* LGT by 12 amino acids and is larger than *H. influenzae* LGT by 11 amino acids. In the sequence alignment, a major gap in *S. aureus* sequence is seen between the 166th and 180th amino acids with respect to the *E. coli* sequence, and a larger gap in this region (168 to 193) is found in the *H. influenzae* LGT; the sequences

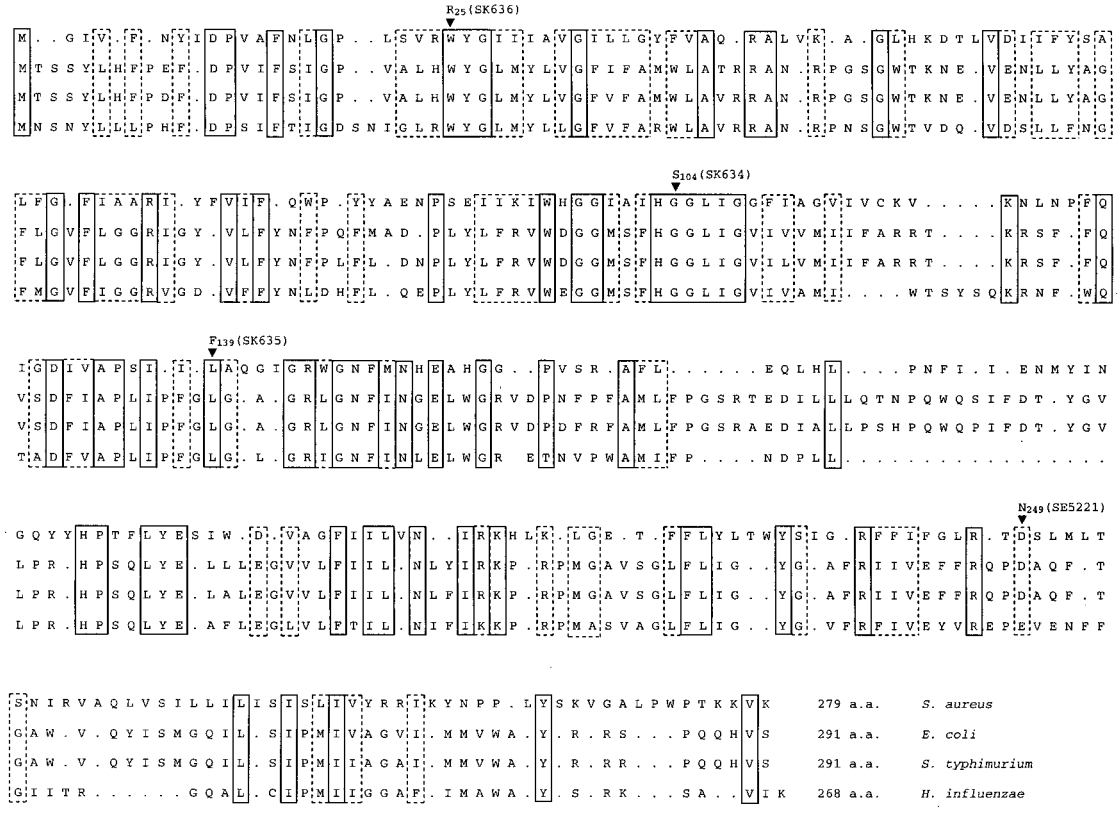

FIG. 4. Comparison of the deduced amino acid sequences of LGT from *E. coli*, *S. typhimurium*, *H. influenzae*, and *S. aureus*. Alignment was performed with the Pileup program in the Genetics Computer Group sequence analysis software package. These four LGT proteins have 24% identity and 47% similarity at the amino acid level. The conserved regions among LGT proteins are marked by solid (identity) or dashed (similarity) boxes. The symbol ..... represents the gap in the sequences. The amino acid alterations in each of the *lgt* alleles in the mutant strains of *E. coli* (SK634, SK635, and SK636) and *S. typhimurium* (SE5221) are shown above the wild-type sequences.

on both sides of the gap retain excellent homology. As a whole, the N-terminal and the middle part of LGT are more conserved than the C-terminal half.

The codon usage of the *S. aureus lgt* gene showed a bias indicative of the codon usage of this microorganism (21), e.g., Ile (AUA), Leu (UUA), Lys (AAG), Arg (AGG, AGA), and Thr (ACA), distinct from the codon usage of *E. coli*, *S. typhimurium*, and *H. influenzae lgt* genes. The *lgt* gene organization in *S. aureus* also differs from those in *E. coli*, *S. typhimurium*, and *H. influenzae*. In the latter organisms, the *thyA* gene is immediately 3′ to *lgt* and cotranscribed with the *lgt* gene, and the expression of these two genes are translationally coupled (5, 7, 8). In *S. aureus*, the *thyA* gene is not next to the *lgt* gene.

Cloning and sequencing of the mutant *lgt* alleles in *E. coli* and *S. typhimurium*. To gain further insight into the structure-function relationship of the LGT enzyme, the *lgt* alleles from the temperature-sensitive mutants of *E. coli* SK634, SK635, and SK636 (22) and *S. typhimurium* SE5221 (7) defective in diacylglyceryl modification of prolipoprotein were cloned by PCR and sequenced. Each of these four *lgt* alleles was found to contain a point mutation, resulting in single-amino-acid substitution [W-25 to R-25 in *lgt-636*, G-104 to S-104 in *lgt-634*, L-139 to F-139 in *lgt-635*, and D-249 to N-249 in *lgt-1* (SE5221)] in regions of the enzyme which are conserved among bacterial LGT proteins (Fig. 4). These results suggest that these regions could represent functionally important domains for the activity of this enzyme.

Inactivation of the LGT activity by DEPC. The most significant stretch of sequence (H-103-GGLIG-108) that was identical in all four LGT enzymes contains an invariant His (His-103) signifying its possible involvement in the catalytic activity of this enzyme. In addition, an alteration of the sequence H-103-GGLIG-108 in the *E. coli* LGT enzyme to HSGLIG in the *lgt-634*-encoded enzyme (Fig. 4) resulted in defective LGT activity in vivo and in vitro (8). In addition to His-103, His-196 is also conserved in all of the three LGT enzymes.

To test the possibility that the conserved histidine residues in the *E. coli* LGT might be important for its function, we investigated the effect of DEPC (11) treatment on the enzyme activity and the reversal of inactivation by hydroxylamine. We found that DEPC treatment reduced the LGT activity in a concentration- and time-dependent manner. The plot of the pseudo-first-order rate constant for inactivation versus DEPC concentration gave a straight line passing through the origin (Fig. 5), consistent with a second-order reaction. The second-order rate constant for DEPC inactivation of the enzyme was 18.6 M^{-1} s^{-1}. The slope of the plot of log k_{obs} versus log DEPC concentration was 1.04, suggesting that a single functional group essential for LGT activity is modified by DEPC (Fig. 5B, inset). The observations of (i) a nearly total inactivation (less than 1% residual activity) being achieved at a relatively low DEPC concentration at pH 6.0, (ii) the restoration of the enzyme activity by a subsequent treatment with hydroxylamine at pH 7.0 (Table 1), and (iii) the absence of Cys residue in the LGT protein suggest that the amino acid modified by DEPC is probably a conserved histidine. However, it is equally possible that any of the four conserved Tyr residues that can be modified by DEPC and reversed by neutral hydroxylamine could be critical for its activity. LGT activity in a 200-fold enriched enzyme preparation from a hyperexpressing *E. coli* clone was also found to be inactivated by DEPC (15). Like the *E. coli* enzyme, the LGT activity of *S. aureus* was also inactivated by DEPC with an apparent second-order rate constant of 7.9 M^{-1} s^{-1} (15). The second-order rate constant for DEPC inactivation of the mutant enzyme in SK634 (with the substitution Gly-104 to Ser-104) was 25.5 M^{-1} s^{-1} (15).

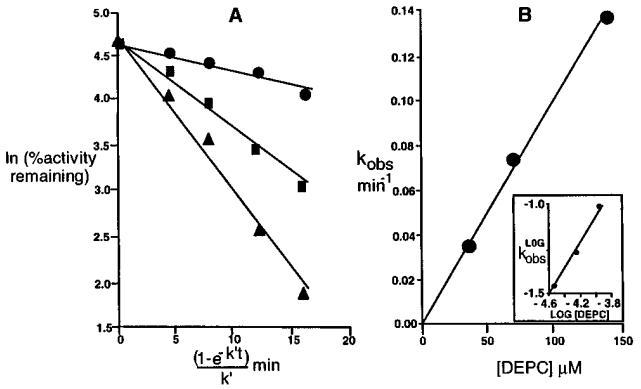

FIG. 5. Inactivation of prolipoprotein diacylglyceryl transferase with DEPC. (A) The crude enzyme preparation (1.25 μg of protein) was incubated with 35 (●), 70 (■), and 140 (▲) μM DEPC under conditions described in Materials and Methods. The parameter in the *x* axis has been corrected for the spontaneous decomposition of DEPC (k′ is the first-order rate constant for the decomposition of DEPC under the experimental conditions) and plotted against ln (percent activity remaining) to determine the k_{obs}, the pseudo-first-order rate constant for inactivation of the enzyme by DEPC at different DEPC concentrations. (B) Plot of k_{obs} versus DEPC concentration to obtain the second-order rate constant (k′) for DEPC inactivation. The straight line passing through the origin signifies a second-order reaction. (Inset) Plot of log k_{obs} versus log DEPC concentration has a slope of 1.04, indicating the presence of a single DEPC-modifiable residue affecting the activity.

Preliminary studies did not reveal any significant protection by either PG (the phospholipid substrate) or the peptide substrate against DEPC inactivation of LGT. The apparent K_m for PG and V_{max} of the SK634 mutant LGT in which the sequence H-103-GGLIG-108 is mutated to H-103-SGLIG-108 were 100 μM and 6.6 nmol/min/mg of protein, respectively; the differences between these values and those of the wild-type enzyme, 30 μM and 15.5 nmol/min/mg of protein, were not large enough to warrant a definitive conclusion on the role of the H-103-GGLIG-108 sequence in the binding of PG, the negatively charged phospholipid substrate of the enzyme.

DISCUSSION

To gain insight into the regions important for the structure and function of the LGT enzyme which catalyzes the transfer of diacylglyceryl moiety from PG to the sulfhydryl group in the lipobox of the prolipoprotein signal sequences, we have taken the approach of comparing the primary structure of this enzyme from a distantly related gram-positive bacterium with those from *E. coli*, *S. typhimurium*, and *H. influenzae* as well as

TABLE 1. Reversal of DEPC inactivation of prolipoprotein diacylglyceryl transferase with hydroxylamine[a]

Duration of hydroxylamine treatment (min)	% Activity	
	Expt 1	Expt 2
0	37	20
30	54	
60	67	
120	70	
240		70

[a] The crude enzyme preparation (0.5 mg of protein per ml) was partially inactivated with 70 μM DEPC in phosphate buffer (pH 6.0) at 25°C for 15 min. Subsequently, the enzyme was treated with 0.5 M hydroxylamine (pH 7.0) for various time intervals; the enzyme was diluted 10-fold and assayed at 37°C for LGT activity. The details are given in Materials and Methods.

comparing the nucleotide sequences of *E. coli* and *S. typhimurium lgt* mutant alleles with those of the wild-type genes. Comparison of the sequences of LGT enzymes from *E. coli*, *S. typhimurium*, *H. influenzae*, and *S. aureus* has revealed a number of highly conserved regions. The longest invariant stretch of amino acids, H-103-GGLIG-108, is likely to be important in the structure-function relationship of this enzyme inasmuch as a mutational alteration of Gly-104 to Ser-104 in the *E. coli* mutant LGT (strain SK634) results in a defective enzyme that is temperature sensitive in vivo and has a lower activity than the wild-type enzyme in vitro even at the permissive temperature. The conditions under which the DEPC inactivation is observed strongly favor the modification of a critical histidine or tyrosine residue or, less likely, an unusual serine residue (11). The lack of large quantities of homogeneous enzyme preparations has precluded further biochemical characterization of the modified enzyme to pinpoint the modified residue. The essential nature of the His and Tyr residues is being addressed by site-specific mutagenesis of the cloned *lgt* gene to systematically alter each of the histidine and tyrosine residues in the LGT protein.

To further ascertain the functional significance of the sequence H-103-GGLIG-108, we have searched for this motif in GenBank with the BLAST program (1). A similar sequence, H-1867-SGLIG-1872, is found in the A3 domain of the light chain of factor VIII, which has multiple binding sites for negatively charged phospholipids (6). This binding is essential for the cofactor activity of factor VIII and hence for the clotting function. A mutational alteration of His-1867 to Arg is known to cause moderate hemophilia (20). Whereas definitive evidence has been provided for the binding of phosphatidylserine to the C2 domain of the light chain (6), not much is known about the functional determinants in the other domains. The apparent lack of protection by PG or the peptide against DEPC inactivation and the lack of a large difference in the K_m and V_{max} values of wild-type and mutant SK634 LGT enzymes do not permit an assignment of a precise role of this conserved sequence in the function of this enzyme.

The temperature-sensitive mutations that affect the activity of this enzyme alter conserved amino acid residues generally in the well-conserved regions. Among the *lgt* mutations, the replacement of Trp-25 and Asp-249 with Arg-25 and Asn-249 in *E. coli* mutant SK636 and *S. typhimurium* mutant SE5221, respectively, would result in an increase in the N-terminal positive charge or a decrease in the C-terminal negative charge, respectively. Mutational alterations of Gly-104 and Leu-139 to Ser-104 and Phe-139 would change the polarity and/or size of the side chain in the LGT protein in *E. coli* mutants SK634 and SK635, respectively. Since these enzymes are active at the permissive temperature but defective at the nonpermissive temperature and exhibit low activity in vitro, these residues are not essential for catalysis but are important for the proper folding of the protein or represent important components in the substrate binding pockets. It might be worthwhile to probe the vicinity of these residues by site-directed mutagenesis and chemical modification techniques to functionally map the enzyme.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Braun, V., and H. C. Wu.** 1993. Lipoproteins, structure, function, biosynthesis and model for protein export, p. 319–342. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Comprehensive biochemistry, vol. 27. Bacterial cell wall. Elsevier Science Publishers, B.V., Amsterdam.
3. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47:**251–276.
4. **Dev, I. K., and P. H. Ray.** 1984. Rapid assay and purification of a unique signal peptidase that processes the prolipoprotein from *Escherichia coli* B. J. Biol. Chem. **259:**11114–11120.
5. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirknes, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. Mckenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Philips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
6. **Foster, P. A., C. A. Fulcher, R. A. Houghten, and T. S. Zimmerman.** 1990. Synthetic factor VIII peptides with amino acid sequences contained within the C2 domain of factor VIII inhibit factor VIII binding to phosphatidylserine. Blood **75:**1999–2004.
7. **Gan, K., S. D. Gupta, K. Sankaran, M. B. Schmid, and H. C. Wu.** 1993. Isolation and characterization of a temperature-sensitive mutant of *Salmonella typhimurium* defective in prolipoprotein modification. J. Biol. Chem. **268:**16544–16550.
8. **Gan, K. D., K. Sankaran, M. G. Williams, M. Aldea, K. E. Rudd, S. R. Kushner, and H. C. Wu.** 1995. The *umpA* gene of *Escherichia coli* encodes phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (*lgt*) and regulates thymidylate synthase levels through translational coupling. J. Bacteriol. **177:**1879–1882.
9. **Gupta, S. D., K. Gan, M. B. Schmid, and H. C. Wu.** 1993. Characterization of a temperature-sensitive mutant of *Salmonella typhimurium* defective in apolipoprotein N-acyltransferase. J. Biol. Chem. **268:**16551–16556.
10. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the mureinlipoprotein of the *Escherichia coli* outer membrane. Eur. J. Biochem. **34:** 284–296.
11. **Miles, E. W.** 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. Methods Enzymol. **47:**431–442.
12. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
14. **Sankaran, K., and H. C. Wu.** 1994. Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. J. Biol. Chem. **269:**19701–19706.
15. **Sankaran, K., and H. C. Wu.** Unpublished data.
16. **Schägger, H., and G. Von Jagow.** 1987. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
17. **Shibuya, I., and S. Hiraoka.** 1992. Cardiolipin synthase from *Escherichia coli*. Methods Enzymol. **209:**321–330.
18. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
19. **Tokunaga, M., H. Tokunaga, and H. C. Wu.** 1982. Post-translational modification and processing of *Escherichia coli* prolipoprotein *in vitro*. Proc. Natl. Acad. Sci. USA **79:**2255–2259.
20. **Tuddenham, E. G. D., D. N. Cooper, J. Gitschier, M. Higuchi, L. W. Hoyer, A. Yoshioka, I. R. Peake, R. Schwaab, K. Olek, H. H. Kazazian, J. M. Lavergne, F. Giannelli, and S. E. Antonarakis.** 1991. Haemophilia A: database of nucleotide substitutions, deletions, insertions and rearrangement of the factor VIII gene. Nucleic Acids Res. **19:**4821–4833.
21. **Wada, K., Y. Wada, H. Doi, F. Ishibashi, T. Gojobori, and T. Ikemura.** 1991. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. **19:**1981–1986.
22. **Williams, M. G., M. Fortson, C. C. Dykstra, P. Jensen, and S. R. Kushner.** 1989. Identification and genetic mapping of the structural gene for an essential *Escherichia coli* membrane protein. J. Bacteriol. **171:**565–568.
23. **Yamagata, H., C. Ippolite, M. Inukai, and M. Inouye.** 1982. Temperature-sensitive processing of outer membrane lipoprotein in an *Escherichia coli* mutant. J. Bacteriol. **152:**1163–1168.