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Roles of Histidine-103 and Tyrosine-235 in the Function of the Prolipoprotein Diacylglyceryl Transferase of *Escherichia coli*†

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Phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt) is the first enzyme in the posttranslational sequence of reactions resulting in the lipid modification of lipoproteins in bacteria. A previous comparison of the primary sequences of the Lgt enzymes from phylogenetically distant bacterial species revealed several highly conserved amino acid sequences throughout the molecule; the most extensive of these was the region 103HGGLIG108 in the *Escherichia coli* Lgt (H.-Y. Qi, K. Sankaran, K. Gan, and H. C. Wu, J. Bacteriol. 177:6820–6824, 1995). These studies also revealed that the kinetics of inactivation of *E. coli* Lgt with diethylpyrocarbonate were consistent with the modification of a single essential histidine or tyrosine residue. The current study was conducted in an attempt to identify this essential amino acid residue in order to further define structure-function relationships in Lgt. Accordingly, all of the histidine residues and seven of the tyrosine residues of *E. coli* Lgt were altered by site-directed mutagenesis, and the in vitro activities of the altered enzymes, as well the abilities of the respective mutant *lgt* alleles to complement the temperature-sensitive phenotype of *E. coli* SK634 defective in Lgt activity, were determined. The data obtained from these studies, in conjunction with additional chemical inactivation studies, support the conclusion that His-103 is essential for Lgt activity. These studies also indicated that Tyr-235 plays an important role in the function of this enzyme. Although other histidine and tyrosine residues were not found to be essential for Lgt activity, alterations of His-196 resulted in a significant reduction of in vitro activity.

More than 130 different lipoproteins have been discovered in a wide variety of both gram-positive and gram-negative bacteria (1). All bacterial lipoproteins possess an N-terminal lipid-modified cysteine residue, *N*-acyl-S-*sn*-1,2-diacylglycerylcysteine, which is now recognized as a signature component. The lipid modification of bacterial lipoproteins is the result of a posttranslational sequence of reactions (Fig. 1) (10). Thus, bacterial lipoproteins are synthesized as prolipoproteins, and the cysteine residue targeted for lipid modification is located in the C-terminal region of the signal sequence of the prolipoprotein in a modification and processing motif known as the lipobox. Analyses of lipobox sequences from many prolipoproteins have revealed the consensus sequence L(A/S)(G/A)C (1). The initial step in the modification pathway involves the transfer of *sn*-1,2-diacylglyceryl from phosphatidylglycerol to the sulfhydryl group of the cysteine catalyzed by the enzyme phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt) (10, 12). This is followed by cleavage of the signal peptide by a lipoprotein-specific signal peptidase, prolipoprotein signal peptidase (signal peptidase II), to yield apolipoprotein (11, 16). Finally, the amino group of the N-terminal 1,2-diacylglyceryl-modified cysteine of apoplipoprotein is acylated by the enzyme apolipoprotein:phospholipid *N*-acyl transferase (Lnt) (6, 12).

The *lgt* gene has been identified in *Escherichia coli* (5, 17), *Salmonella typhimurium* (4), *Staphylococcus aureus* (9), and *Haemophilus influenzae* (3). The deduced amino acid sequence of Lgt has revealed it to be a 28-kDa enzyme whose sequence has been fairly conserved regardless of the organism. The reaction catalyzed by Lgt presumably involves cleavage of the C-O bond at the *sn*-3 position of the 1,2-diacylglycerol moiety of phosphatidylglycerol (Fig. 1). The unique nature of this posttranslational modification reaction, as well as the importance of lipoprotein synthesis in prokaryotes, has prompted an interest in the relationship between the structure and function of Lgt. We previously reported comparisons of the Lgt sequences from *E. coli*, *S. typhimurium*, *S. aureus*, and *H. influenzae*, and several highly conserved regions that might be important for function were identified (9). Accordingly, point mutations that rendered the enzyme temperature sensitive were located in these conserved regions. Chemical modification studies using diethylpyrocarbonate (DEPC) also indicated that a histidine or a tyrosine was essential for enzyme function (9). The current work is a continuation of these studies, and it was undertaken in consideration of formulated strategies and approaches designed to identify amino acids that are essential for enzyme activity in an attempt to ultimately define their roles in enzyme catalysis (8). Accordingly, all of the histidine residues and seven of the tyrosine residues in *E. coli* Lgt that are conserved in both gram-negative and gram-positive bacteria were systematically altered by site-directed mutagenesis in order to identify the essential amino acid residue. The data obtained from these studies, as well as from additional chemical modification studies, support the conclusion that His-103 is essential for Lgt activity. In addition, Tyr-235 was also found to play an important role in the function of Lgt.

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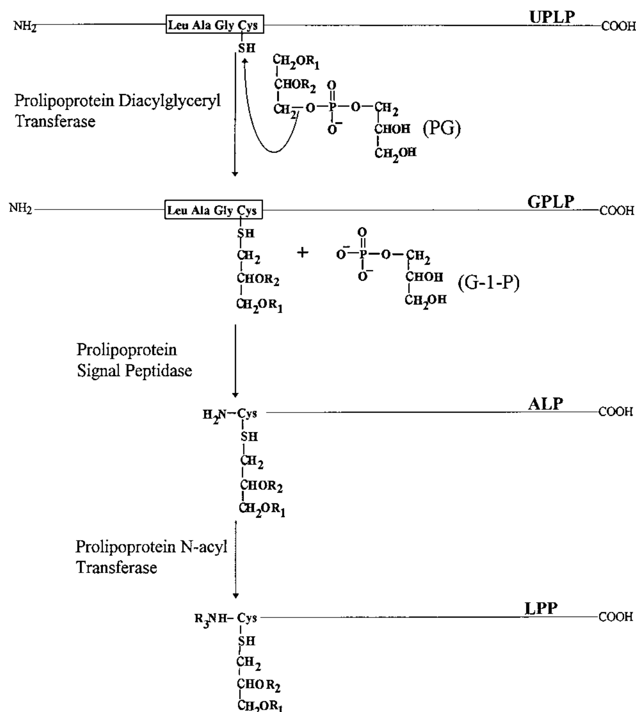

FIG. 1. Posttranslational steps in the assembly of bacterial lipoproteins. Lipid modification is initiated by the transfer of a *sn*-1,2-diacylglyceryl moiety from phosphatidylglycerol to the nucleophilic sulfhydryl group of cysteine present in the lipobox at the C-terminal end of the signal peptide of unmodified prolipoprotein. Subsequent steps involve cleavage of the signal peptide by prolipoprotein signal peptidase (signal peptidase II) followed by fatty acylation of the newly generated N-terminal amino group of the lipid-modified cysteine by prolipoprotein *N*-acyl transferase. Abbreviations: UPLP, unmodified prolipoprotein; GPLP, diacylglyceryl-modified prolipoprotein; ALP, apolipoprotein; LPP, lipoprotein; PG, phosphatidylglycerol; G-1-P, *sn*-glycerol-1-phosphate; R_1, R_2, and R_3, fatty acyl chains.

MATERIALS AND METHODS

Bacterial strains and culture media. The experiments described here were conducted with *E. coli* BL21(DE3) (Novagen, Madison, Wis.), *E. coli* SK634 [*argA103 lgt-634*(*umpA1*)], and *E. coli* SD9 (*pssA cls-1*). *E. coli* SK634 was the kind gift of S. R. Kushner, University of Georgia, Athens. Strain SK634 is temperature sensitive in growth due to a point mutation in *lgt*. *E. coli* SD9 was the kind gift of Isao Shibuya, Saitama University, Urawa, Japan. This strain was used for the preparation of [9,10-^{3}H]palmitoyl-labeled phospholipid as previously described (10). Under the conditions used, 75% of the radiolabeled phospholipid is phosphatidylglycerol and the remainder is phosphatidylethanolamine (10, 14). Bacteria were grown in Luria-Bertani (LB) broth or LB agar at either 37°C (all *lgt* clones and strain BL21) or 30°C (strain SK634). *E. coli* SD9 was grown at 30°C in LB broth or on LB agar plates; in either case, the media contained 50 mM magnesium chloride and 400 mM sucrose. When appropriate, ampicillin was added to culture media to give a final concentration of 50 μg/ml.

E. coli BL21(DE3) was the host strain used in experiments to overexpress the *lgt* gene. Although a variety of expression vectors were examined for this purpose, optimal expression of *lgt* resulted with use of plasmid pT7-7, in which *lgt* was placed under control of the T7 promoter (15). However, it is important to note that the levels of Lgt resulting from induction of T7 polymerase with isopropyl-β-D-thiogalactoside (IPTG) were lethal, and stable expression of *lgt* leading to levels of Lgt activity that were 20 to 25 times that observed in wild-type strains occurred in the absence of IPTG induction. This elevated level of expression was apparently due to leaky expression of the T7 polymerase.

Radiochemicals, chemicals, and reagents. [9,10-^{3}H(N)]palmitic acid (specific activity, 54 Ci/mmol) was purchased from DuPont NEN (Wilmington, Del.). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.). DNA sequencing was performed with a DNA Taq DyeDeoxy Terminator Cycle Sequencing kit obtained from Applied Biosystems, Inc. (Foster City, Calif.). TA cloning kits were obtained from Invitrogen Corp. (San Diego, Calif.). PCR reagents were purchased from Roche Molecular Systems, Inc. (Branchburg, N.J.). DEPC and phospholipids were purchased from Sigma Chemical Co. (St. Louis, Mo.). The detergent *n*-octyl-β-D-glucoside was purchased from Boehringer Mannheim.

In vitro assay of diacylglyceryl transferase modification of prolipoprotein. The following in vitro assay procedure was routinely used to compare the activities of various mutant Lgt enzymes. Cultures were grown to late log phase, and membranes were prepared from the cells in 1 ml of culture following lysozyme-EDTA lysis (18). The membranes were then resuspended in TED buffer (20 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 2 mM dithiothreitol) to give a final protein concentration of 5 mg/ml. The enzyme was subsequently solubilized at 0 to 2°C with 1% *n*-octyl-β-D-glucoside. Aliquots of the soluble fraction (5 μl) were assayed for diacylglyceryl transferase activity as previously described (10, 12). Briefly, the soluble fraction containing the enzyme was incubated in 50 μl of TED buffer containing 50 mM guanidine hydrochloride (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 phosphatidylglycerol per mmol), and 400 μM peptide acceptor for 20 min at 37°C. The peptide acceptor consisted of the N-terminal 24 amino acids (MKATKLVLGAVILGSTLLAGCSSN) of Braun's lipoprotein, which was synthesized as previously described (10). The reaction was terminated by the addition of 4 μl of a 20% saturated ammonium sulfate solution followed immediately by the addition of 400 μl of acetone. The mixture was allowed to stand at room temperature for 15 min, and the pellet was then collected by centrifugation and washed once with 90% acetone saturated with ammonium sulfate. The washed pellet was dissolved in 100 μl of 1% sodium dodecyl sulfate, and the amount of radioactivity incorporated into the peptide acceptor was determined by liquid scintillation counting. Specific activity values represent the averages of five determinations corrected for the background activity present in extracts obtained from strain BL21; each determination was made by using the cells obtained from a single colony of each isolate.

Kinetic analyses were performed on enzyme obtained from inverted membrane vesicles prepared by the French press method as previously described (10, 12). Briefly, cells were grown to late log phase in 2 liters of LB broth, collected by centrifugation, and washed once with 50 ml of 20 mM Tris-HCl (pH 8.0). The washed pellets were resuspended in a volume of TED equal to the weight of the pellets and then disrupted by two passages through a French press at 10,000 lb/in^2. The preparations were centrifuged at 18,000 × *g* for 30 min to remove debris, and membranes were isolated by centrifugation of the supernatant solutions at 200,000 × *g* for 2 h. The membrane fraction was washed once with 10 ml of TED and then resuspended in TED containing 1% *n*-octyl-β-D-glucoside to give a protein concentration of 5 mg/ml. Lgt activity was solubilized by subsequent incubation of the suspension for 1 h on ice. The soluble fraction was then obtained by centrifugation at 200,000 × *g* for 1 h. The amount of protein in membranes was determined by solubilizing an aliquot of the membrane preparation in 1% sodium dodecyl sulfate and measuring the optical density at 280 nm. In this assay, 1 optical density unit was taken to be equal to 1 mg of protein per ml.

Chemical modification of Lgt. Inactivation of Lgt with DEPC was carried out as previously described (7, 9).

Site-directed mutagenesis. PCR-based site-directed mutagenesis of cloned wild-type *lgt* was used to systematically alter each of the histidine and tyrosine residues in Lgt. Two strategies were used to create these mutations (Fig. 2). The strategy outlined in Fig. 2A was used to create substitutions for internal histidine residues (His-24, -103, and -196) and seven internal tyrosine residues (Tyr-26, -62, -76, -190, -201, -235, and -282). Accordingly, two separate PCRs (PCR 1 and PCR 2) were performed to construct each mutation. PCR 1 involved two separate amplifications, each using a unique set of primers. One of the primer sets (amplification 1) consisted of a reverse primer (primer 1) which encoded the desired mutation as well as a second forward primer (PT7) that was complementary to the nonsense strand in the region immediately upstream of the *lgt* gene. The second primer set (amplification 2) consisted of a forward primer (primer 2) that was complementary to primer 1 and a second reverse primer (PT7R) that was complementary to the sense strand in the region immediately downstream of the *lgt* gene. Following PCR, the partial *lgt* genes encoding the desired mutation were isolated by agarose gel electrophoresis and used as templates for the second PCR. Although the second PCR was carried out in the presence of primers PT7 and PT7R, the annealed overlapping strands of the partial *lgt* gene functioned as the initial primers for strand completion. Subsequent amplification of the complete mutant gene used primers PT7 and PT7R as the forward and reverse primers, respectively.

A second single-step PCR strategy was used for the mutational alteration of histidine residues 7 and 289 located at the N- and C-terminal ends of the protein, respectively (Fig. 2B). In each case, one of the primers was designed to include the appropriate mutation, whereas the other primer was complementary to a region immediately flanking the gene at the opposite end of the opposing strand. The implementation of both of these strategies was followed by the isolation of products, and the nucleotide sequence of each construct was determined to ensure that the gene was altered only by the desired point mutation.

Complementation of strain SK634 by mutant *lgt* alleles. *E. coli* SK634 possesses a temperature-sensitive Lgt which renders the organism unable to grow at 42°C. The defective enzyme is the result of a missense mutation in *lgt* in which Gly-104 has been replaced with serine (5). Strain SK634 was transformed with wild-type and mutant *lgt* alleles subcloned into plasmid pT7-7, and the ability of the mutant alleles to complement the temperature-sensitive defect in growth was evaluated. Although all of these alleles were under the control of the T7 promoter, expression of the wild-type allele, as well as certain mutant alleles, was

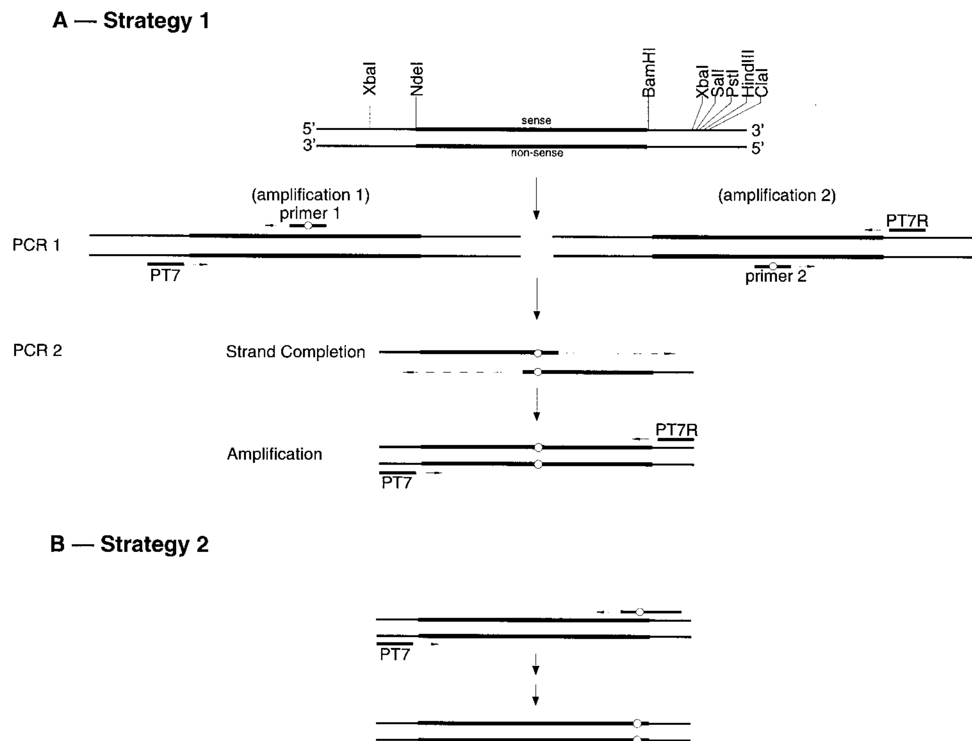

FIG. 2. Strategy for site-directed mutagenesis of Lgt by PCR. This strategy was used to alter specific histidine and tyrosine residues in the *lgt* gene product of *E. coli*. Specific details are provided in the text.

observed even though strain SK634 lacks the T7 RNA polymerase. This is apparently due to the ability of the *E. coli* RNA polymerase to recognize the T7 promoter to some extent. When wild-type *lgt* was expressed in this system, the in vitro diacylglyceryl transferase activity was comparable to that observed in wild-type strains.

RESULTS AND DISCUSSION

Effects of mutagenic alteration of Lgt histidine residues on in vitro and in vivo activities. *E. coli* Lgt contains five histidine residues: His-7, His-24, His-103, His-196, and His-289. The possible importance of His-103 and His-196 for catalytic activity is indicated by the observation that they are the only histidine residues that are conserved in the Lgt enzymes of *E. coli*, *S. typhimurium*, *S. aureus*, and *H. influenzae* (9). Accordingly, mutation of all five histidine residues of *E. coli* Lgt by site-directed mutagenesis revealed that only alteration of His-103 and His-196 had an effect on in vitro and in vivo Lgt activities (Table 1). An essential role for His-103 in Lgt activity was indicated by the abrogation of in vitro diacylglyceryl transferase activity following deletion of His-103 or substitution of His-103 with either asparagine or glutamine. Indeed, no activity was detected when Lgt altered at His-103 was assayed in the absence of detergent or under conditions where the pH and temperature of reaction mixtures were varied considerably (data not shown). Expression of mutant *lgt* alleles that resulted in the synthesis of enzymes with either an H103Q or an H103N substitution also failed to complement mutant strain SK634, which is temperature sensitive for growth due to a single amino acid substitution (G104S) located in the conserved 103HGG LIG108 region of Lgt (5). In contrast, substitution of either His-7, His-24, or His-289 with glutamine did not alter in vitro enzyme activity or the ability of the mutant enzymes to complement the temperature-sensitive phenotype of strain SK634. This was the case even with a mutant enzyme that contained a glutamine substitution at all three of these positions; however, in this instance, further alteration resulting in an additional H103Q substitution abolished both in vitro and in vivo activities. In this regard, it is important to note that substitution of either glutamine or asparagine for histidine constitutes a conservative change since these amino acids can also participate in hydrogen bonding in a manner similar to that of histidine. Therefore, it can be only tentatively concluded that His-7, His-24, and His-289 are nonessential until such time as mutant

TABLE 1. Effects of mutational alteration of the histidine residues of *E. coli* Lgt on in vitro and in vivo enzyme activities

lgt allele(s)	Relative sp act of Lgt[a]	Complementation of the temperature-sensitive phenotype of strain SK634
Wild type	1.0	+
H7Q	1.0	+
H24Q	1.0	+
H103Q	0.0	−
H196Q	0.5	+
H289Q	0.9	+
H7Q, H289Q	1.1	+
H7Q, H24Q, H289Q	0.9	+
H7Q, H24Q, H103Q, H289Q	0.0	−
H103N	0.0	−
ΔH103	0.0	−
H196N	1.1	+
H196R	0.5	+
H196L	1.0	+
H7Q, H24Q, H196Q, H289Q	0.4	+

[a] Specific activity of each mutant enzyme relative to that of the wild-type enzyme. Specific activities were determined in crude extracts.

TABLE 2. Effects of mutations and DEPC modification on the kinetics of *E. coli* Lgt activity[a]

lgt Allele	Apparent K_m (μM) for:		V_{max} (nmol/min/mg of protein)	K'_{app} for DEPC inactivation (M^{-1} s^{-1})
	Peptide	PG		
Wild type	30	10	25.5	18.6
H196Q	28	7	13.0	33.2
Y235S	67	30	18.1	36.0

[a] Kinetic parameters were obtained from Lineweaver-Burk plots following assay of the enzymes present in the soluble fraction resulting from *n*-octyl-β-D-glucoside extraction of inverted membrane vesicles. PG, phosphatidylglycerol.

Lgt enzymes possessing less conservative substitutions at these positions are analyzed.

Several lines of evidence support the conclusion that His-196 is not essential for Lgt activity. The conservative substitution of His-196 with asparagine had no apparent effect on in vitro Lgt activity, and the conservative substitution of His-196 with glutamine resulted in only a 50% decrease in vitro activity (Table 1). Once again, the activity of the altered enzyme was not affected by the absence of detergent from reaction mixtures or by changes in assay conditions involving pH and temperature (data not shown). Although substitution of His-196 with arginine allowed retention of a positive charge at this position, the bulkier arginine nevertheless resulted in a 50% decrease in in vitro activity. Quite surprisingly, the nonconservative substitution of His-196 with leucine did not result in an alteration of in vitro activity. Indeed, substitution of leucine for His-196 did not affect the apparent maximal velocity of the reaction catalyzed by Lgt. In addition, none of the above-specified substitutions at His-196 resulted in the inability of the respective mutant Lgt enzymes to complement the temperature-sensitive phenotype of strain SK634. Kinetic analyses of the His-196Q mutant Lgt revealed that the apparent K_m values of the enzyme for both the peptide acceptor and phosphatidylglycerol were not significantly altered by the substitution (Table 2). Although the H196Q and H196R substitutions resulted in an apparent 50% reduction in the maximal velocity, it is difficult to explain the significance of this apparent decrease without knowing the actual amount of enzyme in reaction mixtures. In this regard, attempts to estimate the quantity of Lgt have been unsuccessful since it appears to be a minor lipoprotein, and it is only barely visible in silver-stained gels even after 20- to 25-fold overexpression. In addition, attempts to purify the enzyme by using a variety of conventional procedures have been frustrated by the apparent instability of the protein during the purification process; enzyme activity was rapidly lost following during the initial steps of protein purification procedures. However, the available data, particularly the ability of leucine to effectively replace His-196, suggest that the role of His-196 is most likely quite different from the conventional role of histidine as a nucleophile, as a general acid-base catalyst, or as a hydrogen bond donor as is the case in other enzymes.

Effect of chemical modification with DEPC. DEPC modifies histidyl and tyrosyl residues in proteins to the corresponding *N*-carbethoxy and *O*-carbethoxy derivatives, respectively (7). As described previously, DEPC modification of wild-type Lgt indicated the presence of a single essential histidine or tyrosine residue in the enzyme (9), and data presented later in this report suggest that tyrosine-235 plays an important role in Lgt function. However, treatment of H196Q and Y235S mutant Lgt enzymes with DEPC revealed that both enzymes were inactivated, and the inactivation followed second-order kinetics with a rate constant comparable to that observed for the wild-type enzyme (Table 2). Indeed, this was also the case following DEPC treatment of mutant Lgt enzyme in which all of the histidines except His-103 were changed by site-directed mutagenesis to glutamine (data not shown). Thus, if DEPC treatment of Lgt results in the modification of a single histidine residue, it appears quite likely that His-103 is the target of modification by DEPC, supporting the conclusion that this residue is essential for Lgt activity. Nevertheless, the existing data do not preclude the possibility that a conserved tyrosine or even some other reactive residue is modified by this reagent. In this regard, DEPC inactivation of Y235S clearly suggests that this tyrosine is not the target of modification. Furthermore, Lgt was not inactivated by DEPC in the presence of phospholipid or peptide substrates at saturating concentrations (data not shown). Accordingly, His-103 may not be part of the binding site for these substrates; rather, its function may be restricted to a catalytic role in the transfer of a diacylglyceryl moiety from phosphatidylglycerol to the sulfhydryl group of cysteine during the assembly of prolipoproteins. Although free glycerol-1-phosphate has been isolated from in vitro reaction mixtures (unpublished results), it is not known whether this is the case in vivo.

It is interesting that although the sequence 103HGGLIG108 is conserved in the Lgt enzymes of several related and unrelated organisms, the putative Lgt of *Mycoplasma genitalium* contains glutamine in the position normally occupied by histidine (2). However, it is not known whether this enzyme is functional. Indeed, it is doubtful if lipoproteins occur in *M. genitalium* since the *lnt* gene appears to be absent from its genome (2).

Effects of mutagenic alteration of Lgt tyrosine residues on in vitro and in vivo activities. The Lgt of *E. coli* contains 13 tyrosine residues; however, only 7 of these residues are conserved in the Lgt enzymes of other gram-negative and gram-positive bacteria (9). The importance of one of these conserved tyrosine residues, Tyr-235, in Lgt function was established by site-directed mutagenesis experiments in which all seven of the conserved tyrosine residues of *E. coli* Lgt were individually substituted by phenylalanine, thus retaining the aromatic ring structure. The in vitro and in vivo activities of all the resulting mutant enzymes, with the exception of the Lgt possessing the Y235F substitution, were essentially unaltered compared to that of the wild-type enzyme (Table 3). However, substitution of Tyr-235 with phenylalanine resulted in a total loss of in vitro Lgt activity, and expression of the corresponding mutant allele

TABLE 3. Effects of the mutational alteration of tyrosine residues of *E. coli* Lgt on in vitro and in vivo enzyme activities

lgt allele	Relative sp act of Lgt[a]	Complementation of the temperature-sensitive phenotype of strain SK634
Wild type	1.0	+
Y26F	1.2	+
Y62F	1.2	+
Y76F	1.1	+
Y190F	1.1	+
Y201F	1.0	+
Y235F	0.0	−
Y282F	0.9	+
Y235S	0.6	+
Y235T	0.0	−

[a] Specific activity of each mutant enzyme relative to that of the wild-type enzyme. Specific activities were determined in crude extracts.

in *E. coli* SK634 failed to complement the temperature-sensitive phenotype of this strain. The importance of the hydroxyl group of Tyr-235 was examined by substitution of this residue by both serine and threonine. Substitution of Tyr-235 with serine resulted in a 37% loss of in vitro activity; however, the in vivo activity of the Y235S Lgt was nevertheless sufficient to complement the temperature-sensitive phenotype of strain SK634, and the apparent kinetic parameters of the Y235S Lgt were not remarkably different from those of the wild-type enzyme. In contrast to the Y235S Lgt, the Y235T enzyme had only 3% of the in vitro activity of the wild-type enzyme, and it was unable to complement the temperature-sensitive phenotype of SK634. Thus, although the role of Tyr-235 in Lgt function remains unknown, it appears that the phenolic moiety or the primary hydroxyl group of this amino acid residue may be necessary to stabilize either the structure of Lgt or its transition states during catalysis. A similar role for the hydroxyl group of tyrosine has been demonstrated in other enzymes such as phospholipase A_2 (13). Furthermore, since both His-103 and Tyr-235 are conserved among the Lgt enzymes of other bacteria, it seems probable that they also play a critical role in the function of these enzymes.

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