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The Essential *Escherichia coli* Apolipoprotein *N*-Acyltransferase (Lnt) Exists as an Extracytoplasmic Thioester Acyl-Enzyme Intermediate†

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ABSTRACT: *Escherichia coli* apolipoprotein *N*-acyltransferase (Lnt) transfers an acyl group from *sn*-1-glycerophospholipid to the free α-amino group of the N-terminal cysteine of apolipoproteins, resulting in mature triacylated lipoprotein. Here we report that the Lnt reaction proceeds through an acyl-enzyme intermediate in which a palmitoyl group forms a thioester bond with the thiol of the active site residue C387 that was cleaved by neutral hydroxylamine. Lnt(C387S) also formed a fatty acyl intermediate that was resistant to neutral hydroxylamine treatment, consistent with formation of an oxygen−ester linkage. Lnt(C387A) did not form an acyl-enzyme intermediate and, like Lnt(C387S), did not have any detectable Lnt activity, indicating that acylation cannot occur at other positions in the catalytic domain. The existence of this thioacyl-enzyme intermediate allowed us to determine whether essential residues in the catalytic domain of Lnt affect the first step of the reaction, the formation of the acyl-enzyme intermediate, or the second step in which the acyl chain is transferred to the apolipoprotein substrate. In the catalytic triad, E267 is required for the formation of the acyl-enzyme intermediate, indicating its role in enhancing the nucleophilicity of C387. E343 is also involved in the first step but is not in close proximity to the active site. W237, Y388, and E389 play a role in the second step of the reaction since acyl-Lnt is formed but N-acylation does not occur. The data presented allow discrimination between the functions of essential Lnt residues in catalytic activity and substrate recognition.

Lipoproteins are major components of the bacterial cell envelope; in *Escherichia coli*, Braun's lipoprotein (Lpp) at $\sim 10^6$ copies, is easily the most numerous protein. Lipoprotein signal sequences terminate in a lipobox motif, L(A/V)$_{-4}$-L$_{-3}$-A(S)$_{-2}$-G(A)$_{-1}$-C$_{+1}$, identifying a Cys residue as the site of posttranslational modification processing (Figure 1a) (*1*). First, phosphatidylglycerol:apolipoprotein diacylglyceryl transferase (Lgt) adds a phosphatidylglycerol- (PG-) derived *sn*-1,2-diacylglyceryl group via a thioether bond. Next, the signal sequence is removed by prolipoprotein signal peptidase (LspA, or signal peptidase II), liberating the α-amino group of C$_{+1}$. In Gram-negative bacteria and in Mycobacteria (*2*), a third step, N-acylation of diacylglyceride C$_{+1}$ by apolipoprotein *N*-acyltransferase (Lnt), follows, generating the mature triacylated lipoprotein. N-Acylation is required for engaging the Lol machinery (*3*), which either transfers the lipoprotein to the inner leaflet of the outer membrane or leaves it tethered in the outer leaflet of the inner membrane. All three processing enzymes, as well as the Lol system, are essential. Despite its central role in the generation of the bacterial envelope, the catalytic mechanism of Lnt is largely unknown. The reaction, in which the carbonyl group of *sn*-1-glycerophospholipid is linked to the free α-amino group of apolipoprotein, involves the formation of an acyl-enzyme intermediate, analogous to reactions described for members of the nitrilase superfamily, to which Lnt belongs by virtue of the similarity of its largest periplasmic domain (Figure 1b) (*4*). We previously identified the catalytic triad, E267-K335-C387, and residues W237, E343, Y388, and E389 in the periplasmic domain as essential for Lnt activity in *E. coli* (*5*). Here we describe genetic, physiological, and biochemical experiments aimed at further defining Lnt catalysis.

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EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth Media. Strains PAP105, MC4100, and MG1655 have been described (*5, 6*). Strains carrying plasmids were grown in LB with 100 μg/mL ampicillin.

Induction of lnt Genes. Strains were grown at 37 °C with shaking in LB to OD_{600} = 0.2. Expression of *lnt* from the p_{lac} promoter in pUC18 plasmids and from the p_{ara} promoter of the pBAD18 constructs was induced with 1 mM isopropyl β-thiogalactopyranoside (IPTG) and 0.2% L-arabinose, respectively. The final OD_{600} was measured after 1 h of induction at 37 °C, at which time the cultures were placed on ice.

Standard DNA Manipulation. Plasmid pCHAP7530, encoding Lnt with a double c-Myc tag at the C-terminus, has been described previously (*5*). Site-directed mutagenesis was performed with pairs of synthetic oligonucleotides (data not shown) using a two-step polymerase chain reaction based on the QuickChange site-directed mutagenesis protocol (Stratagene) with pCHAP7530 as template DNA. All constructs were verified by sequencing. Fragments containing *lnt* in pUC18 derivatives were digested with *Eco*RI and *Hin*dIII and inserted into pBAD18 for p_{ara}-controlled expression (*7*).

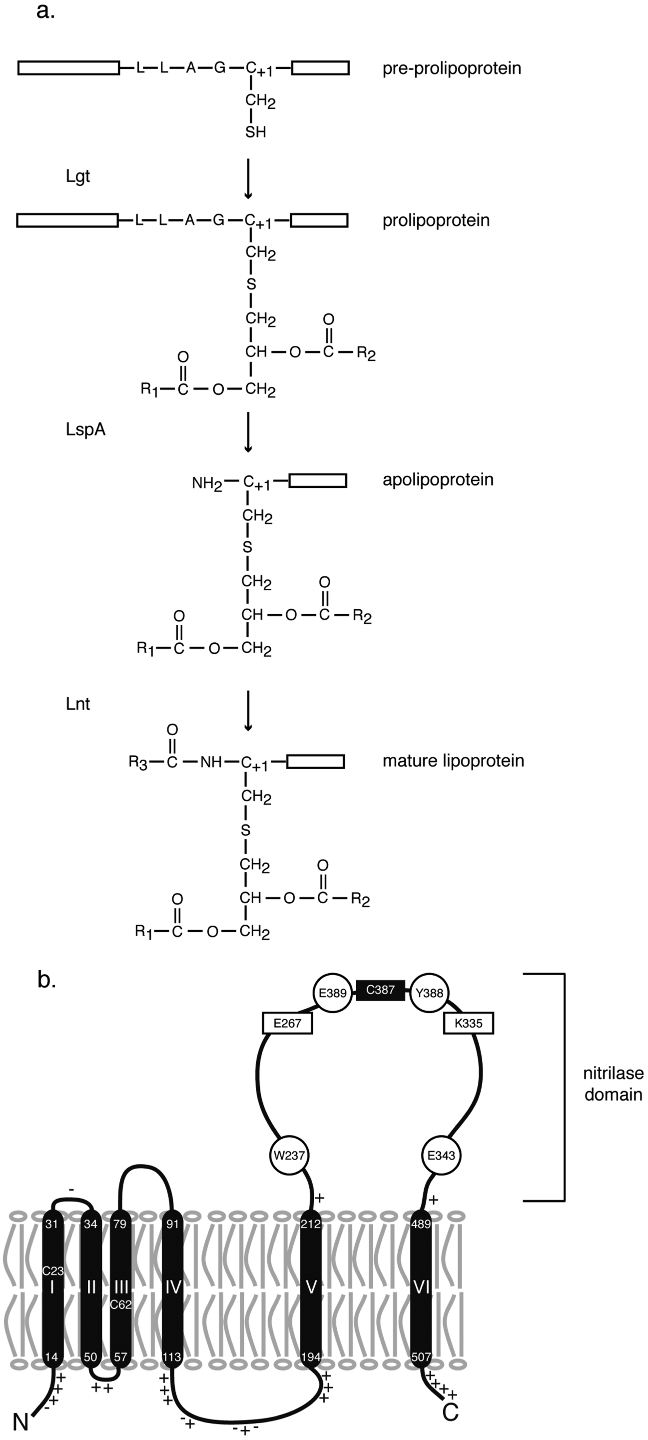


FIGURE 1: Lipoprotein modification in Gram-negative bacteria. (a) Maturation of triacylated lipoproteins is subsequently catalyzed by phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt), prolipoprotein signal peptidase (LspA), and apolipoprotein *N*-acyltransferase (Lnt). (b) Membrane topology of Lnt in the cytoplasmic membrane of *E. coli* (*17*). Native cysteine residues are indicated; C387 is located in the nitrilase domain (residues 220−468) facing the periplasm. The catalytic triad residues E267-K335-C387 are illustrated as rectangles; other essential residues, W237, E343, Y388, and E389, are indicated as circles. Charged residues surrounding the transmembrane segments are shown with (−) and (+) symbols.

Hydroxylamine Treatment. Bacteria (1.6 mL) were centrifuged at 16000*g* for 5 min and resuspended in 1 mL of phosphate buffer (PB) (50 mM KP_i, pH 7.0). Samples were split into two tubes and centrifuged at 16000*g* for 5 min. One pellet was resuspended in 1 M neutral hydroxylamine (HA;[1] made immediately before use by dissolving 1.4 g of NH_2OH in 20 mL of H_2O and adjusting to pH 7.0 by gradual addition of 32% NaOH) containing 1% SDS. The second pellet was treated identically except 1 M Tris-HCl, pH 7.0, containing 1% SDS was used instead of HA. After incubation for 30 min at room temperature, proteins were precipitated by incubation with 10% TCA for 30 min on ice, collected by centrifugation at 16000*g* for 15 min at 4 °C, washed twice with acetone, and resuspended either in PEG buffer (see below) for alkylation with malPEG or in SDS sample buffer for analysis by gel electrophoresis (see below).

Alkylation with MalPEG. Pellets were air-dried and resuspended in 100 μL of PEG buffer (1 M Tris-HCl, pH 7.0, 10 M urea, 1% SDS, and 1 mM EDTA). MalPEG was added to all samples to 0.2 mM, and they were then incubated for 30 min at room temperature. Cold 100% ethanol (1.5 mL) was added, and proteins were precipitated at −20 °C. Protein samples were centrifuged at 16000*g* for 15 min at 4 °C. Supernatants were carefully removed, and pellets were dried at room temperature and then resuspended in SDS sample buffer and heated at 100 °C for 5 min prior to analysis by gel electrophoresis and immunoblotting.

[^{3}H]Palmitate Labeling of Whole Cells and Fluorography. Overnight cell cultures were diluted 100-fold in 5 mL of fresh medium and grown at 37 °C to OD_{600}=0.2. [^{3}H]Palmitate was added to 100 μCi/mL, and *lnt* expression was induced simultaneously with 0.2% L-arabinose. The cultures were incubated for a further 60 min at 37 °C. Harvested cells were treated with HA as described above. Proteins were separated on 10% polyacrylamide−SDS gels. Gels were fixed in 25% 2-propanol plus 10% acetic acid for 30 min, washed twice in H_2O for 15 min, and incubated in Amplify (GE Healthcare) for 20 min. The dried gels were exposed to Kodak BioMax XAR film at −80 °C.

SDS−PAGE and Immunoblotting. For the preparation of whole cell extracts, 1 mL of cells was centrifuged for 5 min at 16000*g*. Cell pellets were resuspended in SDS sample buffer containing 4 mM DTT and heated for 5 min at 100 °C. Protein samples were loaded at an equivalent of 0.1 OD_{600} unit and separated in 10% polyacrylamide SDS−PAGE gels. After transfer onto nitrocellulose membranes, proteins were detected by incubating the membrane with antibodies against c-Myc (Sigma) followed by horseradish peroxidase conjugated secondary antibodies to rabbit immunoglobin G (Amersham). Secondary antibodies were detected by chemiluminescence (Thermo Scientific) (*8*).

RESULTS

The Periplasmic Thiol of Lnt Is Blocked in Vivo. The proposed mechanism of N-acylation of lipopoteins involves a nucleophilic attack by the activated thiol of Lnt on the *sn*-1-glycerophospholipid carbonyl group to generate a lysophospholipid byproduct and an acyl-enzyme intermediate, which is then resolved by the apolipoprotein α-amino group. Besides the catalytic cysteine, C387, in the large periplasmic domain, there are two other Cys residues, neither conserved: C23 in the first transmembrane segment (TMS1) and C62 in TMS3 (Figure 1b). To facilitate biochemical analysis of Lnt enzymatic function, we altered the nonconserved Cys residues to Ala, both individually and in combination, in the context of an *lnt* allele also encoding a double c-Myc tag at the cytoplasmic C-terminus. As expected,

[1]Abbreviations: malPEG, methoxypoly(ethylene glycol)maleimide; HA, hydroxylamine.

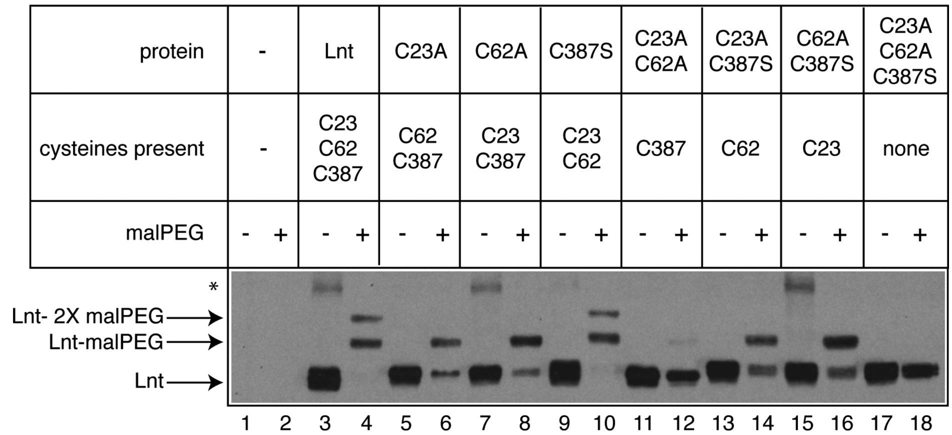


FIGURE 2: Lnt is blocked from alkylation by malPEG. Cells producing variants of Lnt were treated with alkylating reagent malPEG after sample denaturation with $CHCl_3$/MeOH. Gene expression was induced with 1 mM IPTG using pUC18-*lnt* derivatives. Equal amounts of protein were analyzed on an immunoblot that was developed with antibodies against c-Myc. Nonmodified Lnt (Lnt), alkylated Lnt (Lnt-malPEG), and Lnt dimer (*) are indicated. The dimeric form of Lnt is dependent on C23 and can be reduced in the presence of reducing agents (not shown).

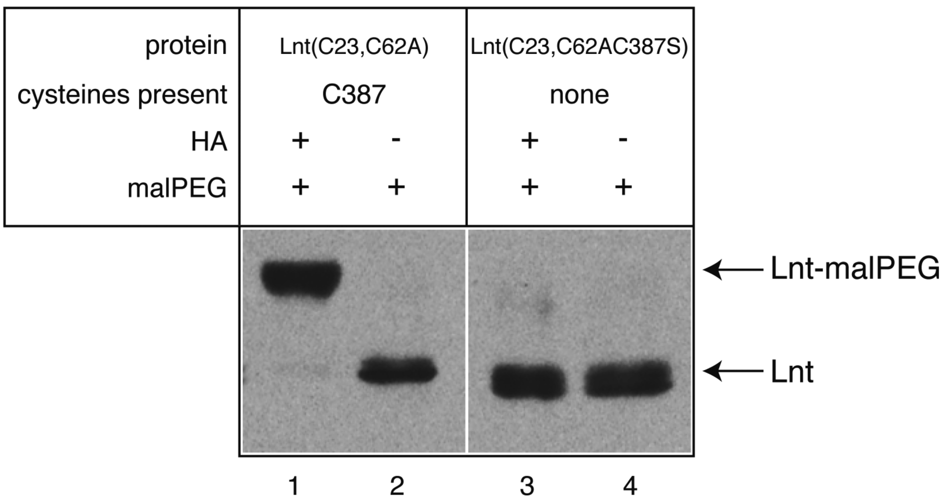


FIGURE 3: Lnt is alkylated by malPEG after treatment with neutral hydroxylamine. Total extracts of cells producing Lnt with C387 as the only cysteine residue, Lnt(C23,C62A), or Lnt lacking all three cysteines, Lnt(C23,C62AC387S), were alkylated with malPEG after treatment with neutral hydroxylamine. Samples were separated by SDS−10% PAGE and analyzed by immunoblotting with antibodies against c-Myc.

both C23A and C62A alleles and the double substitution complemented an *E. coli lnt* conditional null mutant. Surprisingly, when the high molecular weight alkylating agent methoxypoly(ethylene glycol)maleimide (malPEG; 5 kDa) was used to assess the state of the thiols in denatured protein samples prepared from whole cells, both of the single substitutions exhibited only a single modifiable thiol, and the double substitution had none (Figure 2). This conclusion was confirmed by showing that modifying the Cys387 to Ser in the parental or either single substitution had no effect on the number of malPEG-reactive thiols, although as expected the ability to complement the *lnt* defect was lost. These results indicated that the essential C387 residue exists in the covalently blocked state *in vivo*.

Lnt Exists as a Periplasmic Thioester Acyl-Enzyme Intermediate. The most likely covalent modification of Lnt would be an acyl-enzyme intermediate. To test this idea, we examined the thiol state of Lnt after treatment with neutral hydroxylamine (HA), which specifically cleaves thioesters but not oxygen esters or amides (*9*). HA treatment of cells producing the Lnt(C23,C62A) double mutant quantitatively liberated the C387 thiol, as shown by malPEG modification (Figure 3). Lnt lacking all cysteines (Lnt(C23,C62AC387S)) was not modified by malPEG after HA treatment . These data indicate that the extracytoplasmic catalytic thiol of Lnt exists in a thioester acyl-enzyme intermediate *in vivo*.

Lnt(C23,C62AC387S) Forms an Oxygen−Ester Acyl-Enzyme Intermediate. To demonstrate directly that Lnt was acylated on C387, cells induced for synthesis of Lnt variants with different substitutions at C387 were labeled with [^{3}H]palmitate and analyzed by fluorography. [^{3}H]Palmitate was incorporated into both Lnt(C23,C62A) and Lnt(C23,C62AC387S) but not Lnt(C23,C62,C387A) (Figure 4). The thioester formed at C387 with the labeled palmitate proved to be sensitive to neutral HA, in terms of both losing the label and becoming sensitive to derivitization with malPEG, whereas the C387S label did not. These results indicate that C387 indeed forms a thioester bond with palmitate and also that, if the conserved Cys is replaced by Ser, Lnt is able to form an oxygen−ester that is resistant to HA treatment. Furthermore, only residue 387 is involved in the formation of acyl-enzyme intermediates, since Lnt(C23,C62, C387A) does not incorporate fatty acids.

Residues Affecting Thioester Acyl-Enzyme Formation and N-Acylation of Apolipoproteins. Previously, several

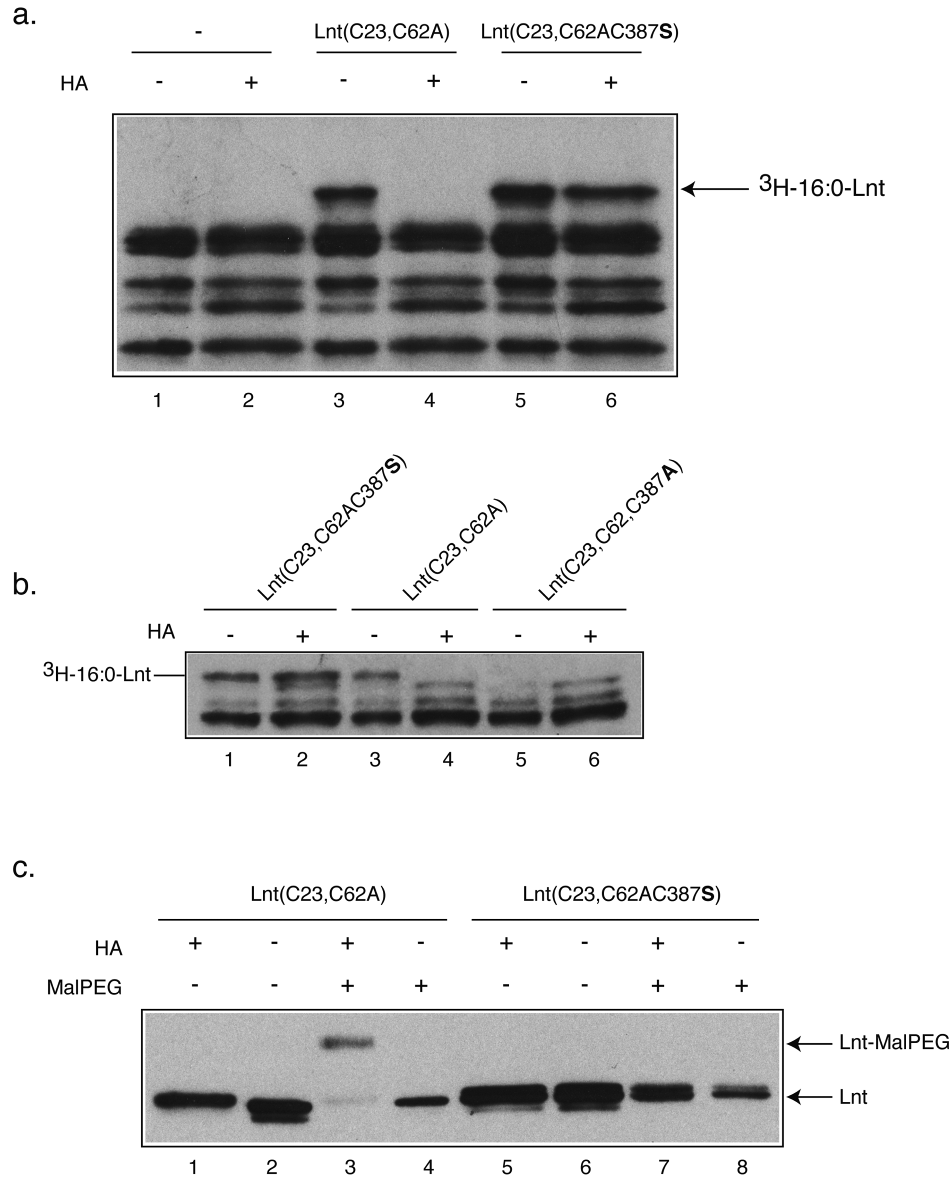


FIGURE 4: Lnt forms a thioester acyl-enzyme intermediate. (a) Labeling of Lnt(C23,C62A) and Lnt(C23,C62AC387S) with [^{3}H]palmitate. (−) vector only sample. Gene expression was induced from the p_{ara} promoter of pBAD18-*lnt* derivatives with 0.2% L-arabinose. Whole cells were treated with neutral hydroxylamine to release [^{3}H]palmitate from the thioester acyl-enzyme linkage (lanes 2, 4, and 6). (b) Lnt(C23,C62C387A) does not incorporate [^{3}H]palmitate (lane 5). Lnt(C23,C62A) and Lnt(C23,C62AC387S) were labeled in parallel and served as controls. (c) Release of [^{3}H]palmitate was verified by alkylation with malPEG and analysis on immunoblot using antibodies against c-Myc. Lnt(C23,C62A) was alkylated by malPEG after treatment with HA (lane 3), but Lnt(C23,C62AC387S) was not (lane 7).

essential residues in Lnt were identified by site-directed mutagenesis and an *in vivo* complementation assay (*5*). To examine whether the defects in these mutants could be correlated with thioacylation of Lnt, each mutation was created in the Lnt(C23, C62A) background and the effect on the state of the C387 thiol determined by malPEG alkylation. Lnt variants were also analyzed for maleimide modification after HA treatment as a control for the accessibility of the thiol group of C387. A double band was detected in Lnt(C23,C62AC387S), probably representing nonacylated Lnt (lower band) and oxygen−ester acyl-Lnt (upper band) (Figure 5). This Lnt variant was not modified by malPEG because it lacks C387. Lnt(C23,C62A) was modified by malPEG only after treatment with HA as seen before (Figures 3, 4, and 5). Lnt variants with substitutions at two glutamate residues, E267 and E343, exhibited malPEG modification independent of HA treatment, indicating that both mutations cause a defect in thioacylation of C387 (Figure 5). These residues

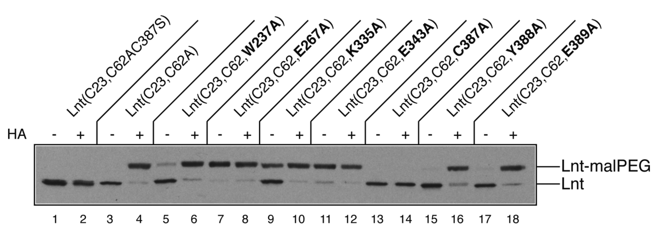
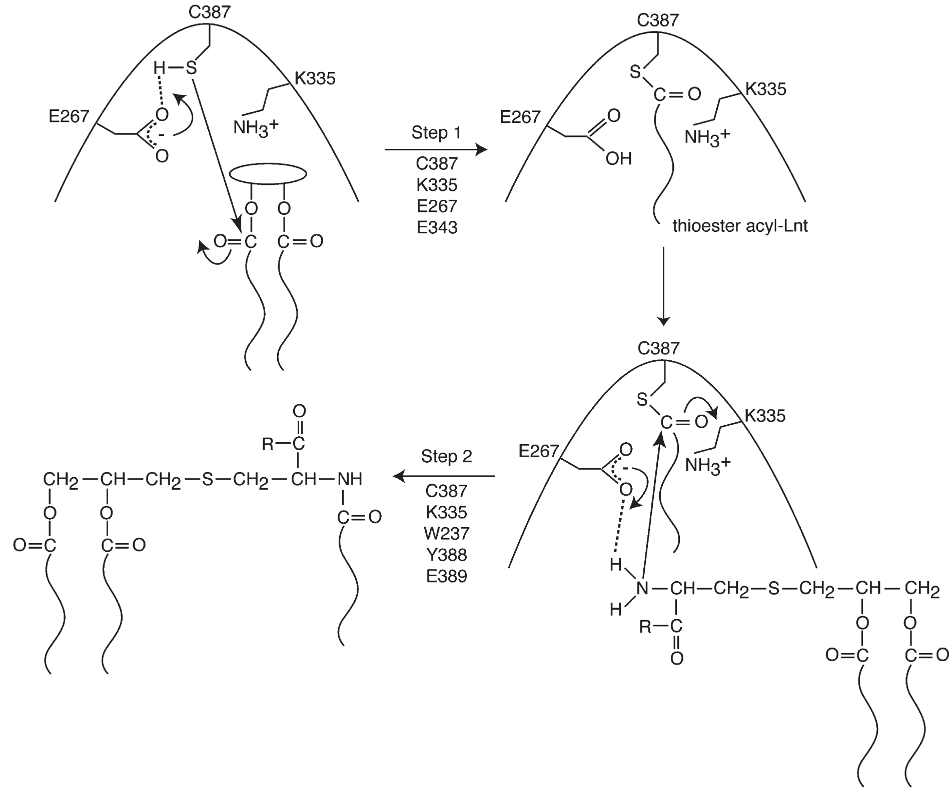


FIGURE 5: Effect of essential residues on the acylation state of Lnt. Seven substitutions that abolished function were combined in Lnt(C23, C62A) and analyzed for Lnt acylation by malPEG alkylation. Lnt(C23, C62AC387S) lacks all cysteines and is included as a control. Whole cells were treated (+) or not (−) with 1 M neutral hydroxylamine and subsequently with malPEG. Samples were separated by SDS−10% PAGE and analyzed by immunoblotting with antibodies against c-Myc. Alkylated (Lnt-malPEG) and nonalkylated forms of Lnt are indicated.

therefore play a role in the first step of the *N*-acyltransferase reaction. Lnt containing the K335A substitution is partially

Scheme 1: Model for the *N*-Acyltransferase Reaction and the Role of Essential Residues[a]



[a]Model of the Lnt reaction. Step 1: formation of the thioester acyl-enzyme intermediate. E267 activates the sulfydryl group of C387 which can then attack the 1-carbonyl group of phospholipids. Besides the active site residues C387 and E267, residues E343 and K335 are also required. Step 2: formation of *N*-acyl-*S*-diacylglyceryl (mature) lipoprotein. W237, Y388, E389, and the active site residues C387 and K335 are necessary for this step. Tetrahedral intermediates are formed as part of the reaction: in step 1 between Lnt and the 1-acyl group of phospholipids; in step 2 between Lnt and apolipoprotein (not shown).

alkylated by malPEG in the absence of HA, suggesting that it plays a role in the stabilization of the tetrahedral intermediates that are formed as part of both steps (*10*). The W237, Y388, and E389 substitutions showed no malPEG alkylation (or slight modification in the case of W237) in the absence of HA, indicating that C387 is acylated in these allele products. These substitutions affect the maturation of major lipoprotein Lpp (*5*) and are thus required for the N-acylation of substrate apolipoproteins.

DISCUSSION

Based on similarities of the Lnt periplasmic domain with the nitrilase superfamily, it has been proposed that the apolipoprotein N-acylation involves a thioester acyl-enzyme intermediate between an acyl group derived from 1-*sn*-glycerophospholipid, usually phosphatidylethanolamine (PtdEtn) *in vivo* (*11*), and the thiol of C387 (see Scheme 1). Here we have presented evidence supporting this model, showing that *in vivo* the bulk of Lnt molecules exists as the C387 acyl-enzyme intermediate.

To our knowledge, Lnt is one of the few enzymes that exists *in vivo* in its reaction intermediate form and is the only protein found to have a persistent extracytoplasmic thioester of any kind. This suggests that the thioester linkage is sequestered from the aqueous milieu of the periplasm, presumably by having the C387 at the membrane interface, which might also facilitate the thermodynamically unfavorable loading reaction where the *sn*-1 acyl group linked in the oxygen−ester to phosphatidylethanolamine is transferred to the C387 thiol. Since each of the $\sim 10^6$ lipoproteins produced in the bacterial cell per generation must be processed by Lnt, the total flux of fatty acid through the Lnt thioester must be upward of 2×10^4 molecules/min. Thus the persistent thioester form of Lnt is likely to be in a state of rapid synthesis and discharge into nascent lipoproteins. Nevertheless, the persistent intermediate state of Lnt stands in distinct contrast to sortase, which catalyzes the covalent attachment of secreted proteins carrying the LPXTG motif to the peptidoglycan of Gram-positive bacteria and which has also been shown to use a covalent thiol intermediate (*12*). However, probably due to its transient character, the thioester between sortase and LPXTG-containing surface proteins has not been directly demonstrated *in vivo*. Suree et al. recently reported the solution structure of sortase with a covalently bound peptide analogue of the LPXTG sorting signal (*13*). The attack of the carbonyl group of threonine by the active site cysteine leads to the formation of a thioester and results in structural changes in the overall sortase structure. This movement creates an entry point for the peptide portion of lipid II.

The first step of the *N*-acyltransferase reaction requires the active site residues C387 and E267 as well as E343. In the predicted structure of the nitrilase domain of Lnt, E343 is located on a flexible loop facing away from the active site (*5*). The data presented here suggest a role for E343, together with E267, in the activation of C387 for attack on the carbonyl group of the phospholipid. Alternatively, this residue might be involved in positioning the phospholipid in close proximity of the active site pocket to facilitate nucleophilic attack by C387. The acyl moiety at the 1-position of PtdEtn is the acyl donor for Lnt (*11*).

Electrostatic interactions between the positively charged head-group of PtdEtn and the negatively charged E343 could therefore stabilize the interaction between phospholipid and Lnt. Phosphatidylglycerol (PtdGro) and cardiolipin (CL) can also act as acyl donors for Lnt in strains lacking the phosphatidylserine synthase gene (*pss*) (*14*). The intermediate state of Lnt has not been examined under these conditions.

In the second step of the reaction, the acyl group is transferred to the α-amino group of apolipoprotein. The active site residue C387 is required, as are W237, Y388, and E389. Like E343, W237 faces away from the active site pocket. We hypothesize that W237 and Y388 recognize and interact with the diacylglyceryl moiety of C_{+1} of the apolipoprotein. Five of the seven essential residues are conserved in Lnt of *Mycobacterium smegmatis*, but W237 and Y388 are altered (*2*). Mycobacterial lipoproteins are modified with a diacylglyceryl containing mycobacterial specific fatty acids. The fact that Lnt of *M. smegmatis* cannot complement a conditional *E. coli lnt* mutant suggests that W237 and Y388 play a role in substrate specificity. In Mycobacteria, *lnt* is found in an operon (*M. smegmatis*) or fused (*Mycobacterium tuberculosis*) with a gene (*ppm*-1) encoding polyprenol monophosphomannose synthase (*15*). The *N*-acyltransferase domain enhances Ppm-1 activity, but the effect of mannosyltransferase on Lnt function is currently unknown (*15*). K335 probably stabilizes the oxyanion of the tetrahedral intermediates that are formed as part of the Lnt reaction, similar to the reaction catalyzed by members of the nitrilase family. In this perspective, the apolipoprotein fulfills hereby the role of a water molecule in the second step of the Lnt reaction (*16*).

Lnt forms an oxygen−ester acyl-enzyme intermediate when the thiol group of cysteine is replaced with the hydroxyl group of serine. This enzyme is not functional because it is unable to donate the acyl group to apolipoprotein. Whether E267 is required for the acylation of serine is unknown.

In the structural model, E343 and W237 are located on loops facing away from the active site pocket (*5*) that are predicted to be flexible and to open and close upon phospholipid and substrate binding, respectively. The formation of the thioester enzyme probably involves structural rearrangements of the active site allowing entry of the apolipoprotein substrate, similar to sortase. Alternatively, the loops may be closely located to the outer leaflet of the inner membrane in order to cap the active site pocket on top of the phospholipid bilayer, thereby allowing easy access to the 1-carbonyl group of PtdEtn in the first step (E343) and positioning of the diacylglyceryl cysteine for acylation in the second step (W237).

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