



HAL
open science

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

Nienke Buddelmeijer, Ry Young

► **To cite this version:**

Nienke Buddelmeijer, Ry Young. The Essential Escherichia coli Apolipoprotein N -Acyltransferase (Lnt) Exists as an Extracytoplasmic Thioester Acyl-Enzyme Intermediate. *Biochemistry*, 2010, 49 (2), pp.341 - 346. 10.1021/bi9020346 . pasteur-01407685

HAL Id: pasteur-01407685

<https://pasteur.hal.science/pasteur-01407685v1>

Submitted on 2 Dec 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Thioester acyl-Lnt intermediate

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

**THE ESSENTIAL *E. COLI* APOLIPOPROTEIN N-ACYLTRANSFERASE (LNT) EXISTS AS
AN EXTRACYTOPLASMIC THIOESTER ACYL-ENZYME INTERMEDIATE[‡]**

Nienke Buddelmeijer* and Ry Young[§]

Institut Pasteur, Molecular Genetics Unit and CNRS URA 2172, 25 rue du docteur Roux, 75724 Paris
cedex 15, France

*Address correspondence : N. Buddelmeijer, 25 rue du docteur Roux, 75724 Paris cedex 15, France,

Phone +331403683, Fax +33145688960, E-mail : niebud@pasteur.fr

Running title: Thioester acyl-Lnt intermediate

[‡]R. Young was supported by NIH grant GM27099 and by the Molecular Genetics Unit of the Institut Pasteur. The work was supported by the Institut Pasteur.

Thioester acyl-Lnt intermediate

23

24 **FOOTNOTES**

25 [§]Permanent address: Ryland F. Young III, Sadie Hatfield Professor of Agriculture, Center for Phage
26 Technology, Dept. of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College
27 Station TX USA 77843-2128, Phone: 979-845-2087, Fax: 979-862-4718, E-mail: ryland@tamu.edu

28

29 Abbreviations used in the text: methoxypoly(ethylene glycol)-maleimide (malPEG), hydroxyl amine
30 (HA).

31

32

33 **ABSTRACT**

34

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

52

53

Thioester acyl-Lnt intermediate

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

73

74 **EXPERIMENTAL PROCEDURES**

75

76 *Strains, plasmids and growth media.* Strains PAP105, MC4100 and MG1655 have been described (5,
77 6). Strains carrying plasmids were grown in LB with 100 μ g/ml ampicillin.

Thioester acyl-Lnt intermediate

78

79 *Induction of lnt genes.* Strains were grown at 37°C with shaking in LB to OD₆₀₀ = 0.2. Expression of
80 *lnt* from the p_{lac} promoter in pUC18 plasmids and from the p_{ara} promoter of the pBAD18 constructs was
81 induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG), and 0.2% L-arabinose, respectively.
82 The final OD₆₀₀ was measured after 1 h of induction at 37°C, at which time the cultures were placed on
83 ice.

84

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

91

92 *Hydroxylamine treatment.* Bacteria (1.6 ml) were centrifuged at 16,000 g for 5 min and resuspended in
93 1 ml Phosphate Buffer (PB) (50 mM KPi pH 7.0). Samples were split into two tubes and centrifuged at
94 16,000 g for 5 min. One pellet was resuspended in 1M neutral hydroxylamine (HA; made immediately
95 before use by dissolving 1.4 g NH₂OH in 20 ml H₂O and adjusting to pH 7.0 by gradual addition of
96 32% NaOH) containing 1% SDS. The second pellet was treated identically except 1M Tris-HCl pH 7.0
97 containing 1% SDS was used instead of HA. After incubation for 30 min at room temperature, proteins
98 were precipitated by incubation with 10% TCA for 30 min on ice, collected by centrifugation at 16,000
99 g for 15 min at 4°C, washed twice with acetone, and resuspended either in PEG buffer (see below) for
100 alkylation with malPEG, or in SDS sample buffer for analysis by gel electrophoresis (see below).

101

Thioester acyl-Lnt intermediate

102 *Alkylation with MalPEG.* Pellets were air-dried and resuspended in 100 μ l PEG buffer (1M Tris-HCl
103 pH 7.0, 10 M Urea, 1% SDS and 1mM EDTA). MalPEG was added to all samples to 0.2 mM and they
104 were then incubated for 30 min at room temperature. Cold 100% ethanol (1.5 ml) was added and
105 proteins were precipitated at -20°C. Protein samples were centrifuged at 16,000 g for 15 min at 4°C.
106 Supernatants were carefully removed and pellets were dried at room temperature and then resuspended
107 in SDS sample buffer and heated at 100°C for 5 min prior to analysis by gel electrophoresis and
108 immunoblotting.

109

110 *³H-palmitate labeling of whole cells and Fluorography.* Overnight cell cultures were diluted 100 fold
111 in 5 ml fresh medium and grown at 37°C to OD₆₀₀ = 0.2. ³H-palmitate was added to 100 μ Ci per ml
112 and *lnt* expression was induced simultaneously with 0.2%-L-arabinose. The cultures were incubated for
113 a further 60 min at 37°C. Harvested cells were treated with HA as described above. Proteins were
114 separated on 10% polyacrylamide-SDS gels. Gels were fixed in 25% isopropanol + 10% acetic acid for
115 30 min, washed twice in H₂O for 15 min and incubated in Amplify (GE Healthcare) for 20 min. The
116 dried gels were exposed to Kodak BioMax XAR film at -80°C.

117

118 *SDS-PAGE, immunoblotting.* For the preparation of whole cell extracts, 1 ml of cells were centrifuged
119 for 5 min at 16,000 g. Cell pellets were resuspended in SDS sample buffer containing 4 mM DTT and
120 heated for 5 min at 100°C. Protein samples were loaded at an equivalent of 0.1 OD₆₀₀ units and
121 separated in 10% polyacrylamide SDS-PAGE gels. After transfer onto nitrocellulose membranes,
122 proteins were detected by incubating the membrane with antibodies against c-Myc (Sigma) followed by
123 horseradish peroxidase conjugated secondary antibodies to rabbit immunoglobulin G (Amersham).
124 Secondary antibodies were detected by chemiluminescence (Thermo Scientific) (8).

125

Thioester acyl-Lnt intermediate

126 **RESULTS**

127

128 *The periplasmic thiol of Lnt is blocked in vivo.*

129 The proposed mechanism of *N*-acylation of lipoproteins involves a nucleophilic attack by the
130 activated thiol of Lnt on the *sn*-1-glycerolphospholipid carbonyl group to generate a lysophospholipid
131 byproduct and an acyl enzyme intermediate, which is then resolved by the apolipoprotein α -amino
132 group. Besides the catalytic cysteine, C387, in the large periplasmic domain, there are two other Cys
133 residues, neither conserved: C23 in the first transmembrane segment (TMS1) and C62 in TMS3 (Fig.
134 1b). To facilitate biochemical analysis of Lnt enzymatic function we altered the non-conserved Cys
135 residues to Ala, both individually and in combination, in the context of an *lnt* allele also encoding a
136 double c-Myc tag at the cytoplasmic C-terminus. As expected, both C23A and C62A alleles and the
137 double substitution complemented an *E. coli lnt* conditional null mutant. Surprisingly, when the high-
138 molecular-weight alkylating agent, methoxypoly(ethylene glycol)-maleimide (malPEG; 5 kDa) was
139 used to assess the state of the thiols in denatured protein samples prepared from whole cells, both of the
140 single substitutions exhibited only a single modifiable thiol, and the double substitution had none (Fig.
141 2). This conclusion was confirmed by showing that modifying the Cys387 to Ser in the parental or
142 either single substitution had no effect on the number of malPEG-reactive thiols, although as expected
143 the ability to complement the *lnt* defect was lost. These results indicated that the essential C387 residue
144 exists in covalently blocked state *in vivo*.

145

146 *Lnt exists as a periplasmic thioester acyl enzyme intermediate.*

147 The most likely covalent modification of Lnt would be an acyl-enzyme intermediate. To test this
148 idea, we examined the thiol state of Lnt after treatment with neutral hydroxylamine (HA), which
149 specifically cleaves thioesters but not oxygen esters or amides (9). HA treatment of cells producing the
150 Lnt(C23,C62A) double mutant quantitatively liberated the C387 thiol, as shown by malPEG

Thioester acyl-Lnt intermediate

151 modification (Fig. 3). Lnt lacking all cysteines (**Lnt(C23,C62AC387S)**) was not modified by malPEG
152 after HA treatment . These data indicate that the extra-cytoplasmic catalytic thiol of Lnt exists in a
153 thioester acyl enzyme intermediate *in vivo*.

154
155 *Lnt(C23,C62AC387S) forms an oxygen-ester acyl enzyme intermediate.*

156 To demonstrate directly that Lnt was acylated on C387, cells induced for synthesis of Lnt variants
157 with different substitutions at C387 were labeled with ³H-palmitate and analyzed by fluorography. ³H-
158 palmitate was incorporated into both Lnt(C23,C62A) and **Lnt(C23,C62AC387S)**, but not
159 Lnt(C23,C62,C387A) (Fig. 4). The thioester formed at C387 with the labeled palmitate proved to be
160 sensitive to neutral HA, both in terms of losing the label and becoming sensitive to derivitization with
161 malPEG, whereas the C387S label did not. These results indicate that C387 indeed forms a thioester
162 bond with palmitate and also that, if the conserved Cys is replaced by Ser, Lnt is able to form an
163 oxygen-ester that is resistant to HA treatment. Furthermore, only residue 387 is involved in the
164 formation of acyl enzyme intermediates, since **Lnt(C23,C62,C387A)** does not incorporate fatty acids .

165
166 *Residues affecting thioester acyl enzyme formation and N-acylation of apolipoproteins.*

167 Previously, several essential residues in Lnt were identified by site-directed mutagenesis and an *in*
168 *vivo* complementation assay (5). To examine whether the defects in these mutants could be correlated
169 with thio-acylation of Lnt, each mutation was created in the Lnt(C23,C62A) background and the effect
170 on the state of the C387 thiol determined by malPEG alkylation. Lnt variants were also analyzed for
171 maleimide modification after HA treatment as a control for the accessibility of the thiol group of C387.
172 A double band was detected in **Lnt(C23,C62AC387S)**, probably representing non-acylated Lnt (lower
173 band) and oxygen-ester acyl-Lnt (upper band) (Fig. 5). This Lnt variant was not modified by malPEG
174 because it lacks C387. Lnt(C23,C62A) was modified by malPEG only after treatment with HA as seen
175 before (Figs. 3, 4 and 5). Lnt variants with substitutions at two glutamate residues E267 and E343

Thioester acyl-Lnt intermediate

176 exhibited malPEG modification independent of HA treatment, indicating that both mutations cause a
177 defect in thio-acylation of C387 (Fig. 5). These residues therefore play a role in the first step of the *N*-
178 acyltransferase reaction. Lnt containing the K335A substitution is partially alkylated by malPEG in the
179 absence of HA, suggesting that it plays a role in the stabilization of the tetrahedral intermediates that
180 are formed as part of both steps (10). The W237, Y388 and E389 substitutions showed no malPEG
181 alkylation (or slight modification in case of W237) in the absence of HA, indicating that C387 is
182 acylated in these allele products. These substitutions affect the maturation of major lipoprotein Lpp (5)
183 and are thus required for the *N*-acylation of substrate apolipoproteins.

184

185 **DISCUSSION**

186

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

192 To our knowledge, Lnt is one of the few enzymes that exists *in vivo* in its reaction intermediate form
193 and is the only protein found to have a persistent extracytoplasmic thioester of any kind. This suggests
194 that the thioester linkage is sequestered from the aqueous milieu of the periplasm, presumably by
195 having the C387 at the membrane interface, which might also facilitate the thermodynamically
196 unfavorable loading reaction where the *sn*-1 acyl group of linked in oxygen-ester to
197 phosphatidylethanolamine is transferred to the C387 thiol. Since each of the $\sim 10^6$ lipoproteins
198 produced in the bacterial cell per generation must be processed by Lnt, the total flux of fatty acid
199 through the Lnt thio-ester must be upwards of 2×10^4 molecules/min. Thus the persistent thio-ester
200 form of Lnt is likely to be in a state of rapid synthesis and discharge into nascent lipoproteins.

Thioester acyl-Lnt intermediate

201 Nevertheless, the persistent intermediate state of Lnt stands in distinct contrast to Sortase, which
202 catalyzes the covalent attachment of secreted proteins carrying the LPXTG motif to the peptidoglycan
203 of Gram-positive bacteria, and which has also been shown to use a covalent thiol intermediate (12).
204 However, probably due to its transient character, the thioester between sortase and LPXTG-containing
205 surface proteins has not been directly demonstrated *in vivo*. Suree *et al.* recently reported the solution
206 structure of sortase with a covalently bound peptide analog of the LPXTG sorting signal (13). The
207 attack of the carbonyl group of threonine by the active site cysteine leads to the formation of a thioester
208 and results in structural changes in the overall sortase structure. This movement creates an entry point
209 for the peptide portion of lipid II.

210 The first step of the *N*-acyltransferase reaction requires the active site residues C387 and E267 as
211 well as E343. In the predicted structure of the nitrilase domain of Lnt, E343 is located on a flexible
212 loop facing away from the active site (5). The data presented here suggest a role for E343, together
213 with E267, in the activation of C387 for attack on the carbonyl group of the phospholipid.
214 Alternatively, this residue might be involved in positioning the phospholipid in close proximity of the
215 active site pocket to facilitate nucleophilic attack by C387. The acyl moiety at the 1-position of PtdEtn
216 is the acyl donor for Lnt (11). Electrostatic interactions between the positively charged head group of
217 PtdEtn and the negatively charged E343 could therefore stabilize the interaction between phospholipid
218 and Lnt. Phosphatidylglycerol (PtdGro) and cardiolipin (CL) can also act as acyl donors for Lnt in
219 strains lacking the phosphatidylserine synthase gene (*pss*) (14). The intermediate state of Lnt has not
220 been examined under these conditions.

221 In the second step of the reaction, the acyl group is transferred to the α -amino group of
222 apolipoprotein. The active site residue C387 is required, as are W237, Y388 and E389. Like E343,
223 W237 faces away from the active site pocket. We hypothesize that W237 and Y388 recognize and
224 interact with the diacylglyceryl moiety of C₊₁ of apolipoprotein. Five of the seven essential residues are
225 conserved in Lnt of *M. smegmatis*, but W237 and Y388 are altered (2). Mycobacterial lipoproteins are

Thioester acyl-Lnt intermediate

226 modified with a diacylglyceryl containing mycobacterial specific fatty acids. The fact that Lnt of *M.*
227 *smegmatis* cannot complement a conditional *E. coli lnt* mutant suggests that W237 and Y388 play a
228 role in substrate specificity. In *Mycobacteria*, *lnt* is found in an operon (*M. smegmatis*) or fused (*M.*
229 *tuberculosis*) with a gene (*ppm-1*) encoding polyprenol monophosphomannose synthase (15). The *N*-
230 acyltransferase domain enhances Ppm-1 activity but the effect of mannosyltransferase on Lnt function
231 is currently unknown (15). K335 probably stabilizes the oxyanion of the tetrahedral intermediates that
232 are formed as part of the Lnt reaction similar to the reaction catalyzed by members of the nitrilase
233 family. Apolipoprotein fulfills hereby the role of a water molecule in the second step of the Lnt
234 reaction (16).

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

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

246

247 **ACKNOWLEDGMENT**

248 We thank all members of the Molecular Genetics Unit for support and helpful discussions.

249

250 **REFERENCES**

- 251 1. Sankaran, K., and Wu, H. C. (1994) Lipid modification of bacterial prolipoprotein. Transfer of
252 diacylglyceryl moiety from phosphatidylglycerol, *J. Biol. Chem.* 269, 19701-19706.
- 253 2. Tschumi, A., Nai, C., Auchli, Y., Hunziker, P., Gehrig, P., Keller, P., Grau, T., and Sander, P.
254 (2009) Identification of apolipoprotein N-acyltransferase (LNT) in Mycobacteria, *J. Biol.*
255 *Chem.* 284, 27146-27156.
- 256 3. Fukuda, A., Matsuyama, S.-I., Hara, T., Nakayama, J., Nagasawa, H., and Tokuda, H. (2002)
257 Aminoacylation of the N-terminal cysteine is essential for Lol-dependent release of lipoproteins
258 from membranes but does not depend on lipoprotein sorting signals., *J. Biol. Chem.* 277, 43512-
259 43518.
- 260 4. Pace, H. C., and Brenner, C. (2001) The nitrilase superfamily: classification, structure and
261 function, *Genome Biol.* 2, 1-9.
- 262 5. Vidal-Ingigliardi, D., Lewenza, S., and Buddelmeijer, N. (2007) Identification of essential
263 residues in apolipoprotein N-acyltransferase, a member of the CN hydrolase family, *J.*
264 *Bacteriol.* 189, 4456-4464.
- 265 6. Berry, J., Summer, E. J., Struck, D. K., and Young, R. (2008) The final step in the phage
266 infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes, *Mol.*
267 *Microbiol.* 70, 341-351.
- 268 7. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation,
269 and high-level expression by vectors containing the arabinose PBAD promoter, *J. Bacteriol.*
270 177, 4121-4130.
- 271 8. Buddelmeijer, N., Krehenbrink, M., Pecorari, F., and Pugsley, A. P. (2009) Type II secretion
272 system secretin PulD localizes in clusters in the *Escherichia coli* outer membrane, *J. Bacteriol.*
273 191, 161-168.

Thioester acyl-Lnt intermediate

- 274 9. Olson, E. N., Towler, D. A., and Glaser, L. (1985) Specificity of fatty acid acylation of cellular
275 proteins, *J. Biol. Chem.* 260, 3784-3790.
- 276 10. Nakai, T., Hasegawa, T., Yamashita, E., Yamamoto, M., Kumasaka, T., Ueki, T., Nanba, H.,
277 Ikenaka, Y., Takahashi, S., Sato, M., and Tsukihara, T. (2000) Crystal structure of N-carbamyl-
278 D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases,
279 *Structure* 8, 729-737.
- 280 11. Jackowski, S., and Rock, C. O. (1986) Transfer of fatty acids from the 1-position of
281 phosphatidylethanolamine to the major outer membrane lipoprotein of *Escherichia coli*, *J. Biol.*
282 *Chem.* 261, 11328-11333.
- 283 12. Ton-That, H., Liu, G., Mazmanian, S. K., Faull, K. F., and Schneewind, O. (1999) Purification
284 and characterization of sortase, the transpeptidase that cleaves surface proteins of
285 *Staphylococcus aureus* at the LPXTG motif, *Proc. Natl. Acad. Sci. USA* 96, 12424-12429.
- 286 13. Suree, N., Liew, C. K., Villareal, V. A., Thieu, W., Fadeev, E. A., Clemens, J. J., Jung, M. E.,
287 and Clubb, R. T. (2009) The structure of the *Staphylococcus aureus* sortase-substrate complex
288 reveals how the universally conserved LPXTG sorting signal is recognized, *J. Biol. Chem.* 284,
289 24465-24477.
- 290 14. Gupta, S. D., Dowham, W., and Wu, H. C. (1991) Phosphatidylethanolamine is not essential for
291 the N-acylation of apolipoprotein in *Escherichia coli*, *J. Biol. Chem.* 266, 9983-9986.
- 292 15. Gurcha, S. S., Baulard, A. R., Kremer, L., Locht, C., Moody, D. B., Muhlecker, W., Costello,
293 C. E., Crick, D. C., Brennan, P. J., and Besra, G. S. (2002) Ppm1, a novel polyprenol
294 monophosphomannose synthase from *Mycobacterium tuberculosis*, *Biochem. J.* 365, 441-450.
- 295 16. Wang, W. C., Hsu, W. H., Chien, F. T., and Chen, C. Y. (2001) Crystal structure and site-
296 directed mutagenesis studies of N-carbamoyl-D-amino-acid amidohydrolase from
297 *Agrobacterium radiobacter* reveals a homotetramer and insight into a catalytic cleft, *J. Mol.*
298 *Biol.* 306, 251-261.

Thioester acyl-Lnt intermediate

- 299 17. Robichon, C., Vidal-Ingigliardi, D., and Pugsley, A. P. (2005) Depletion of apolipoprotein N-
300 acyltransferase causes mislocalization of outer membrane lipoproteins in *Escherichia coli*, *J.*
301 *Biol. Chem.* 280, 974-983.

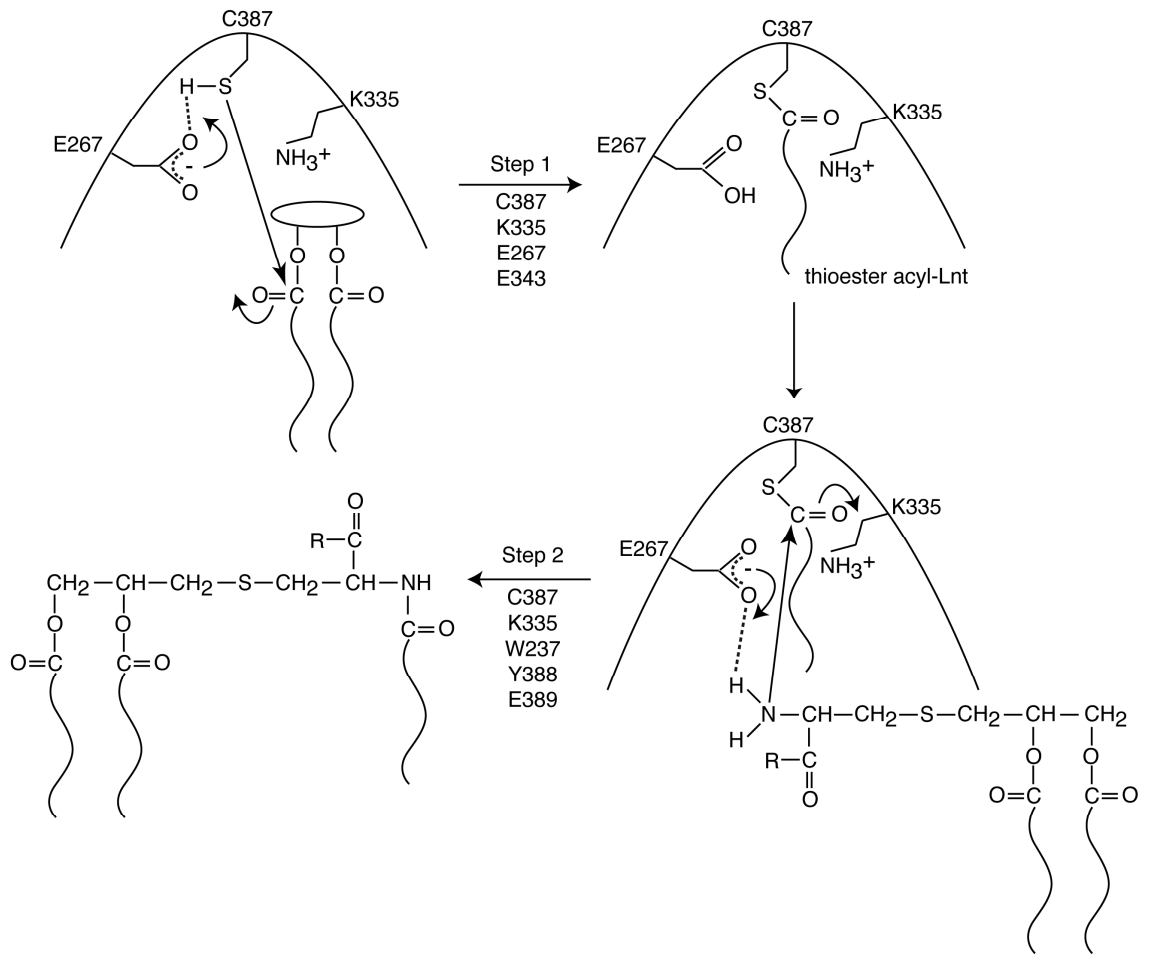
302

303

304

Thioester acyl-Lnt intermediate

Scheme 1



305

306

Thioester acyl-Lnt intermediate

307 **FIGURE LEGENDS**

308

309 **Figure 1 : Lipoprotein modification in Gram-negative bacteria**

310 a. Maturation of tri-acylated lipoproteins is subsequently catalyzed by
311 phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt), prolipoprotein signal peptidase
312 (LspA) and apolipoprotein *N*-acyltransferase (Lnt). b. Membrane topology of Lnt in the cytoplasmic
313 membrane of *E. coli* (17). Native cysteine residues are indicated, C387 is located in the nitrilase
314 domain (residues 220-468) facing the periplasm. The catalytic triad residues E267-K335-C387 are
315 illustrated as rectangles, other essential residues W237, E343, Y388 and E389 are indicated as circles.
316 Charged residues surrounding the transmembrane segments are shown with – and + symbols.

317

318 **Figure 2 : Lnt is blocked from alkylation by malPEG**

319 Cells producing variants of Lnt were treated with alkylating reagent malPEG after sample denaturation
320 with CHCl₃/MeOH. Gene expression was induced with 1 mM IPTG using pUC18-*lnt* derivatives.
321 Equal amounts of protein were analyzed on an immunoblot that was developed with antibodies against
322 c-Myc. Non-modified Lnt (Lnt), alkylated Lnt (Lnt-malPEG) and Lnt dimer (*) are indicated. The
323 dimeric form of Lnt is dependent on C23 and can be reduced in the presence of reducing agents (not
324 shown).

325

326 **Figure 3 : Lnt is alkylated by malPEG after treatment with neutral hydroxylamine**

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

331

Thioester acyl-Lnt intermediate

332 **Figure 4 : Lnt forms thioester acyl enzyme intermediate**

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

341
342 **Figure 5 : Effect of essential residues on the acylation state of Lnt**

343 Seven substitutions that abolished function were combined in Lnt(C23,C62A) and analyzed for Lnt
344 acylation by malPEG alkylation. **Lnt(C23,C62AC387S)** lacks all cysteines and is included as a control.
345 Whole cells were treated (+) or not (-) with 1M neutral hydroxylamine and subsequently with malPEG.
346 Samples were separated by SDS-10%PAGE and analyzed by immunoblotting with antibodies against
347 c-Myc. Alkylated (Lnt-malPEG) and non-alkylated forms of Lnt are indicated.

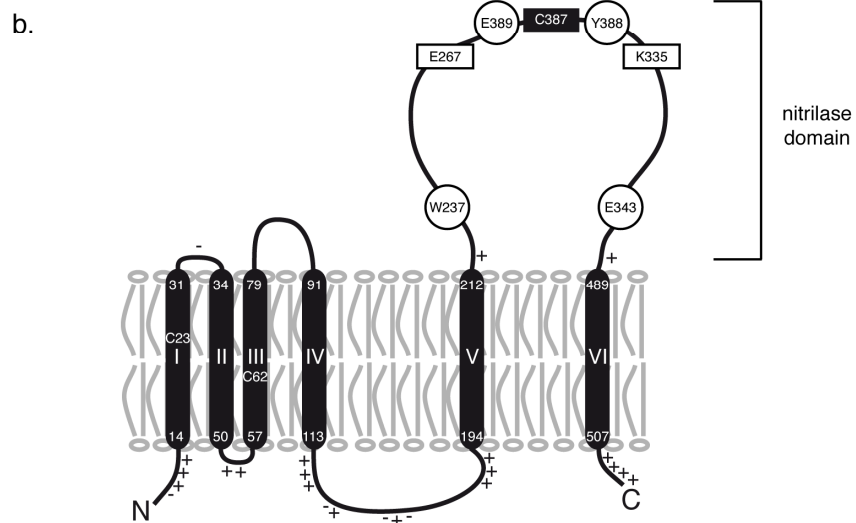
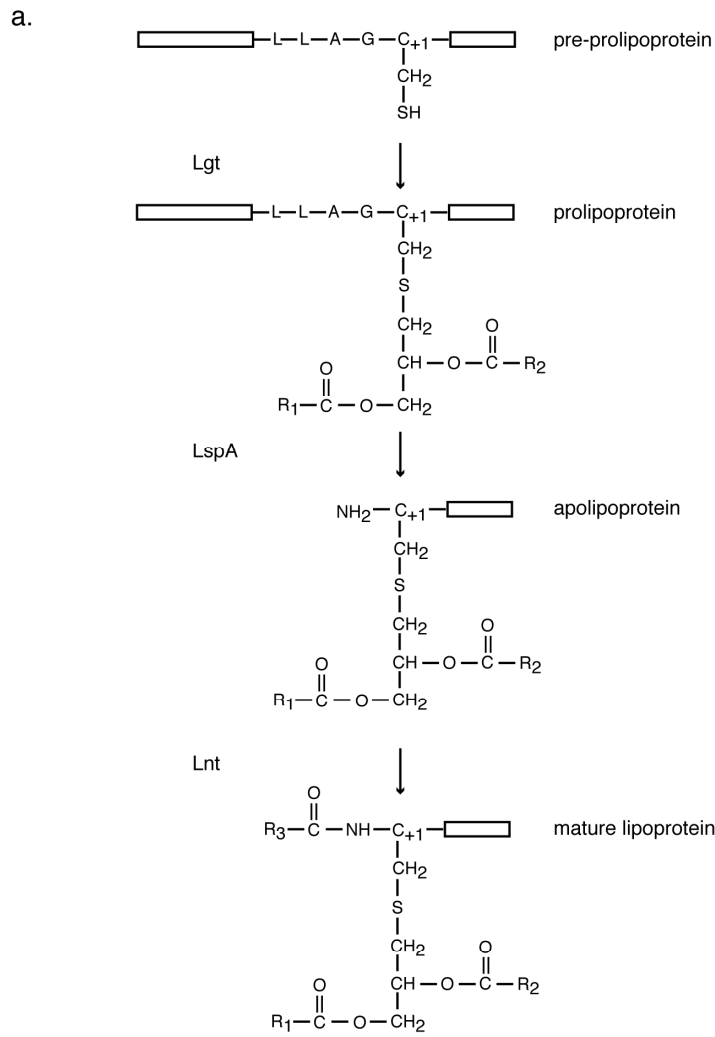
348
349 **Scheme 1: Model for N-acyltransferase reaction and the role of essential residues**

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

356

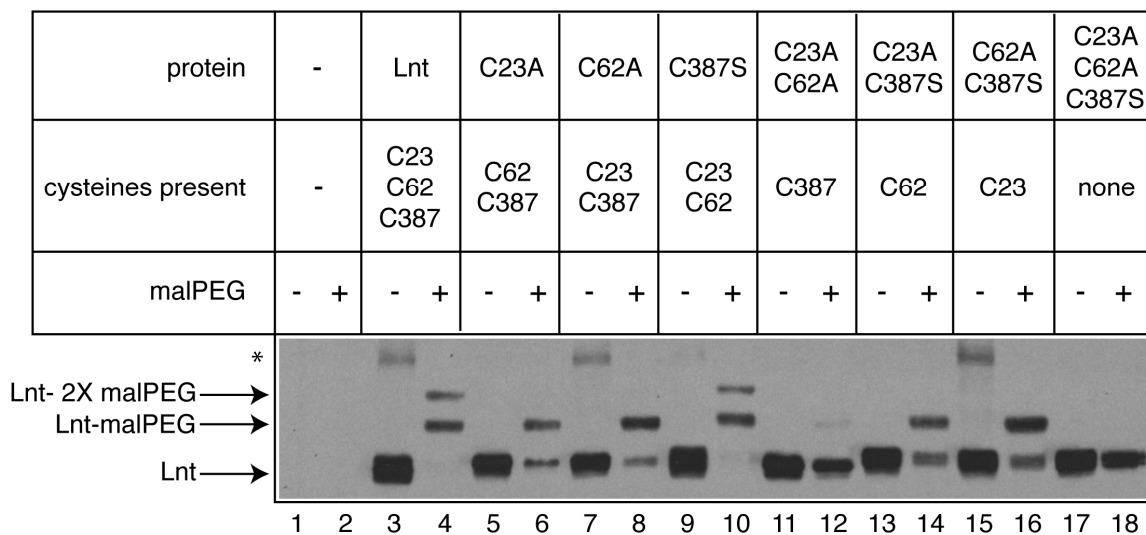
Thioester acyl-Lnt intermediate

Figure 1



Thioester acyl-Lnt intermediate

Figure 2

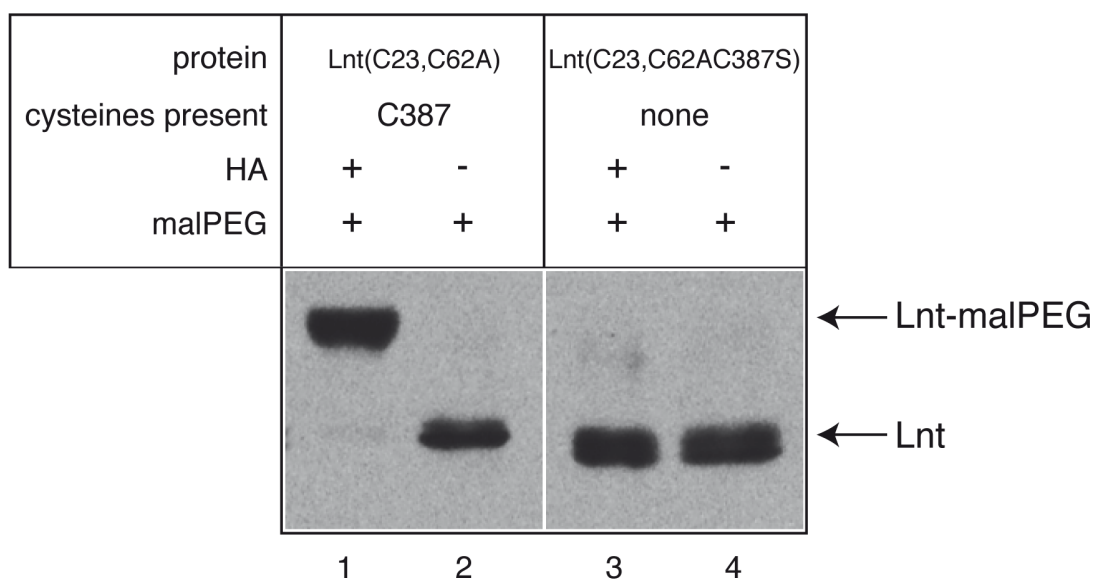


358

359

Thioester acyl-Lnt intermediate

Figure 3



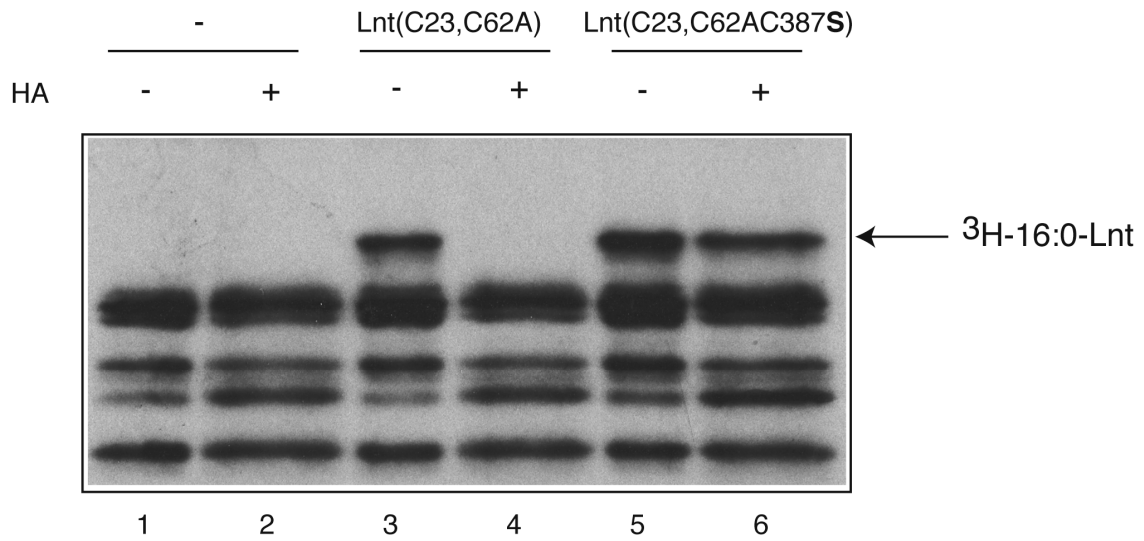
360

361

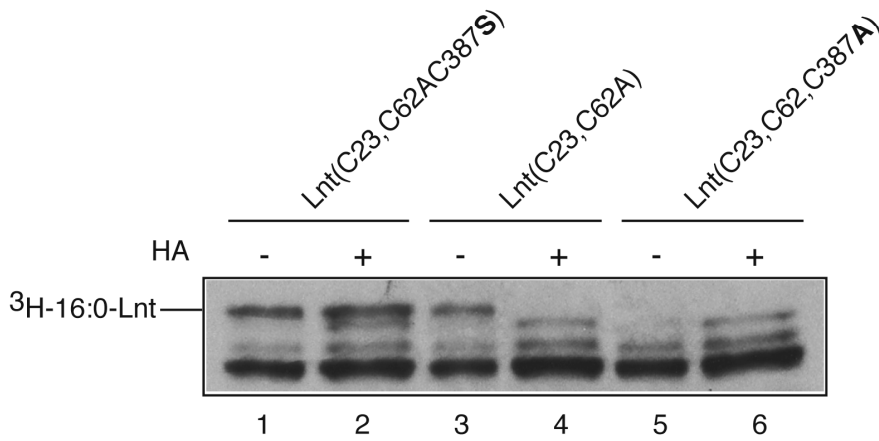
Thioester acyl-Lnt intermediate

Figure 4

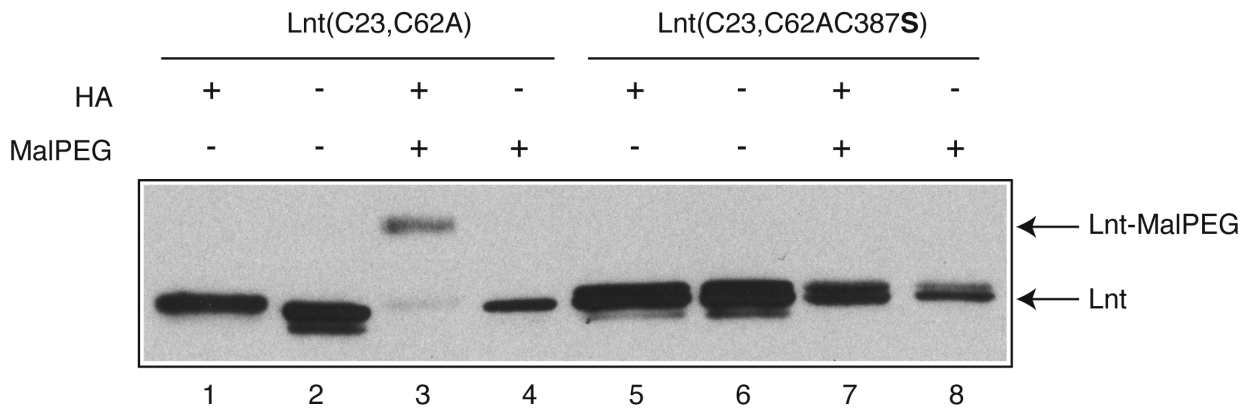
a.



b.



c.

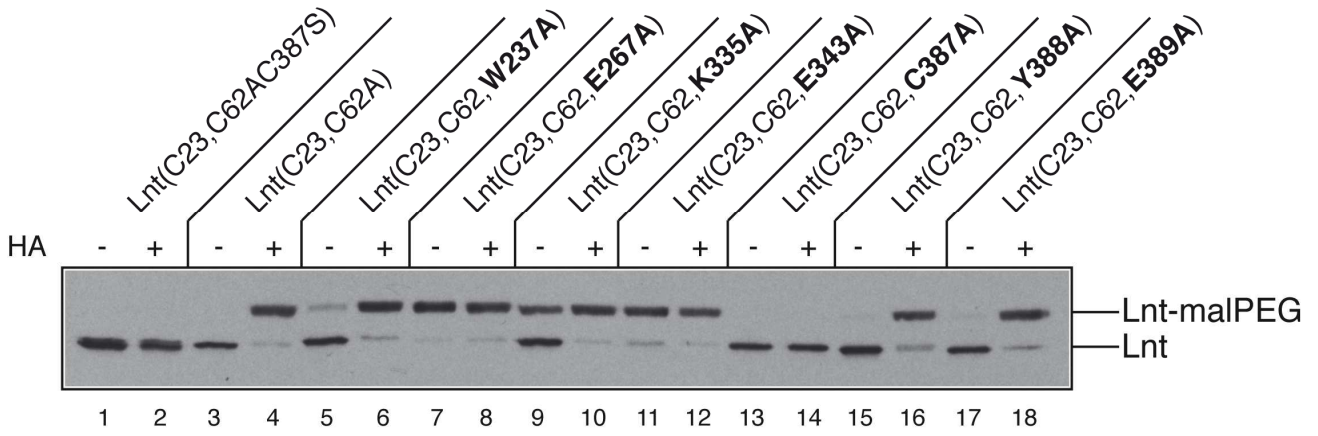


362

363

Thioester acyl-Lnt intermediate

Figure 5



364

365

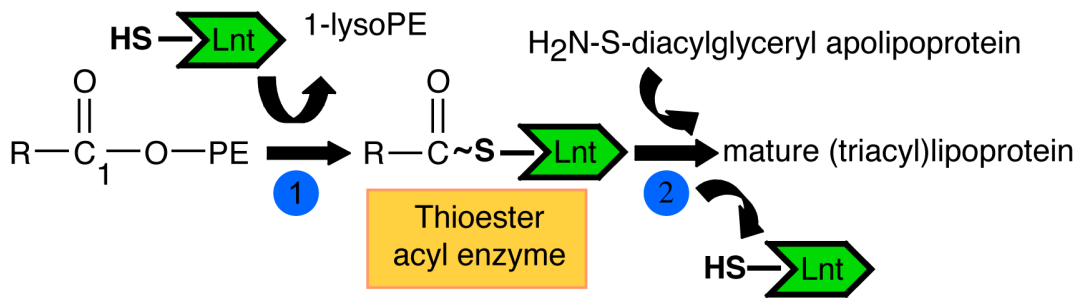
Thioester acyl-Lnt intermediate

366 THE ESSENTIAL *E. COLI* APOLIPOPROTEIN N-ACYLTRANSFERASE (LNT) EXISTS AS AN
367 EXTRACYTOPLASMIC THIOESTER ACYL-ENZYME INTERMEDIATE

368 Nienke Buddelmeijer and Ry Young

369

For Table Of Contents only



370