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Residues located on membrane-associated flexible loops are essential for the second step of the apolipoprotein N-acyltransferase (Lnt) reaction

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Running title: Functional analysis of Apolipoprotein N-acyltransferase (Lnt) of E. coli

Key words: lipoprotein modification, Apolipoprotein N-acyltransferase, membrane topology, essential residues, error-prone PCR mutagenesis.

Abbreviations:
Isopropyl β-D-1-thiogalactopyranoside (IPTG), hydroxylamine (HA), (2-
sulfoethyl)-methane thiosulfonate (MTSES), maleimide polyethylene glycol 5000 (malPEG).
Summary

Apolipoprotein N-acyltransferase (Lnt) is an essential membrane-bound enzyme that catalyzes the third and last step in the post-translational modification of bacterial lipoproteins. In order to identify essential residues implicated in substrate recognition and/or binding we screened for non-functional variants of Lnt obtained by error-prone PCR in a complementation assay using a lnt depletion strain. Mutations included amino acid substitutions in the active site and of residues located on flexible loops in the catalytic periplasmic domain. All, but one mutation, led to the formation of the thioester acyl-enzyme intermediate and to the accumulation of apo-Lpp, suggesting that these residues are involved in the second step of the reaction. A large cytoplasmic loop contains a highly conserved region and two hydrophobic segments. Accessibility analysis to alkylating reagents of substituted cysteine residues introduced in this region demonstrated that the hydrophobic segments do not completely span the membrane. Two residues in the highly conserved cytoplasmic region were shown to be essential for Lnt function. Together, our data suggest that amino acids located on cytoplasmic and periplasmic membrane-associated loops are required for efficient N-acylation of lipoproteins.
Introduction

Bacterial lipoproteins fulfill a variety of functions that include nutrient uptake, maintenance of cell wall integrity, cell wall growth, adhesion to surfaces and host cells, modulation of inflammatory processes and translocation of virulence factors (Kovacs-Simon et al., 2010, Sutcliffe & Russell, 1995). Although their primary sequences and three-dimensional structures differ to a large extend, they all possess fatty acids at their amino terminus the source of which are membrane phospholipids (Lai et al., 1980). Modification of lipoproteins occurs in a post-translational fashion by the sequential action of three membrane-bound enzymes: two acyltransferases and one signal peptidase. Lipoproteins are synthesized in the cytoplasm and inserted into the cytoplasmic membrane via the Sec or Tat secretion machinery (Zuckert, 2014, Sugai & Wu, 1992, Thompson et al., 2010). A specific signature sequence, composed of a signal peptide sequence containing a so-called lipobox (consensus sequence Leu(Ala/Val)-Leu-Ala(Ser)-Gly(Ala)-Cys for lipoproteins in Proteobacteria), allows membrane insertion and recognition by the modification machinery (Wu et al., 1983). The invariant cysteine in the lipobox becomes fatty-acid acylated. After complete maturation this cysteine is the first amino acid of mature lipoprotein. The first step in the modification process is catalyzed by prolipoprotein-diacylglycerol:phosphatidylglycerol transferase (Lgt), which attaches a diacylglycerol from phosphatidylglycerol to the thiol group of cysteine resulting in a thioether linked S-diacylglycerol group of prolipoprotein (Sankaran & Wu, 1994). The second step in lipoprotein maturation is the cleavage of the signal peptide from S-diacylglycerol prolipoprotein by signal peptidase II (Lsp) resulting in a free α-amino group of S-diacylglycerol-cysteine (Hussain et al., 1982). The third and last step is catalyzed by
apolipoprotein N-acyltransferase (Lnt) that attaches a fatty acid from phospholipids to this α-amino group resulting in mature triacylated lipoprotein (Hillmann et al., 2011, Jackowski & Rock, 1986). The mechanism of the Lnt-catalyzed reaction involves a two-step ping-pong mechanism in which, upon hydrolysis of phospholipids, the enzyme is first auto-acylated on the active site cysteine residue resulting in the formation of a thioester acyl-enzyme intermediate and lyso-phospholipid (Buddelmeijer & Young, 2010, Hillmann et al., 2011). In the second step, the α-amino group of diacylglycerol-cysteine of apolipoprotein is acylated. Phospholipids with small polar head groups, preferentially phosphatidylethanolamine (PE), and saturated- and unsaturated fatty acid on sn-1 and sn-2 position, respectively, are the preferred substrates for Lnt of E. coli (Hillmann et al., 2011, Jackowski & Rock, 1986). Incomplete modification of lipoproteins in γ-proteobacteria, for example through inhibition of Lsp activity by globomycin (Inukai et al., 1978) or myxovirescin (Xiao et al., 2012), or depletion of genes encoding the modification enzymes (Pailler et al., 2012, Robichon et al., 2005, Xiao & Wall, 2014), leads to arrest of cell growth and cell lysis. Accumulation of the abundant outer membrane lipoprotein Lpp (Braun’s lipoprotein) in an unmodified form and covalently linked to the cell wall in the cytoplasmic membrane, is the main cause of cell death (Hussain et al., 1980). However, modification and correct sorting of other essential outer membrane lipoproteins, such as BamD (Malinverni et al., 2006) LpoA and LpoB (Paradis-Bleau et al., 2010, Typas et al., 2010), is important since deletion of lpp still leads to cell lysis in the absence of Lgt (Pailler et al., 2012). Retention of these outer membrane lipoproteins in the cytoplasmic membrane likely leads to cell death. The lipoprotein modification machinery is conserved in all bacterial species, and although N-acylation occurs in low GC Gram-positive bacteria such as firmicutes and mollicutes, the gene
encoding Lnt has not yet been identified in these species (Nakayama et al., 2012). In these organisms not only does the composition of the N-acyl group vary, but also the length and degree of saturation of the S-diacylglycerol entity of lipoprotein is variable (Asanuma et al., 2010, Kurokawa, 2009, Kurokawa et al., 2012b, Serebryakova et al., 2011). Recent findings suggest that the extent of N-acylation varies depending on growth conditions probably by regulation of lnt expression (Kurokawa et al., 2012a).

The role of Lnt in lipoprotein modification has also been studied in high GC Gram-positive bacteria (Actinomycetales). In Mycobacterium smegmatis (Tschumi et al., 2009) and Corynebacterium glutamicum (Mohiman et al., 2012) a palmitoyl group is transferred by Lnt onto apolipoproteins. It was shown recently that lipoproteins in M. bovis also contain N-acyl tuberculostearic acid (C19) (Brülle et al., 2013). Thus, both the S-diacylglycerol and the N-acyl moieties of lipoproteins in Mycobacteria contain tuberculostearic acid (Tschumi et al., 2009). Furthermore, several lipoproteins in actinomycetes are modified by sugars, mainly hexose, via O-glycosylation (Brülle et al., 2010, Mohiman et al., 2012, Sartain & Belisle, 2009, Garbe et al., 1993). In C. glutamicum apolipoprotein N-acyltransferase (Lnt or Cg-ppm2) and O-glycosyltransferase (Cg-ppm1) operate in a common biosynthetic pathway resulting in glycosylated lipoproteins in this organism (Mohiman et al., 2012), but N-acylation is not a prerequisite for glycosylation in M. bovis (Brülle et al., 2013). Two lnt genes were identified in Streptomyces scabies. Lnt1 is the enzyme responsible for N-acylation of lipoproteins since lipoproteins are diacylated in lnt1 and lnt1lnt2 mutants. Lnt2 is required for efficient fatty acid acylation of lipoproteins since in its absence lipoproteins are both di- and triacylated (Widdick et al., 2011).

Although the mechanism of apolipoprotein N-acyltransferase is likely to be conserved among bacteria, differences in substrate specificity result in different types of
lipidation. This led us to ask the question of how Lnt recognizes and interacts with its substrates, phospholipid and apolipoprotein, and what residues are involved in the two steps of the reaction. Here we identified various essential residues in Lnt of *E. coli* that are located on flexible loops and are predicted to be membrane-associated. Our results suggest that amino acids located on the cytoplasmic- and on the periplasmic side of the cytoplasmic membrane are involved in the transfer of the acyl group onto the apolipoprotein substrate.
Results

Identification of essential residues in full-length Lnt

Topology studies with Lnt from *E. coli* using reporter fusions with cytoplasmic β-galactosidase and periplasmic alkaline phosphatase showed that this enzyme spans the cytoplasmic membrane via six transmembrane segments (TMS) with both its amino-terminus and carboxy-terminus facing the cytoplasm (Robichon *et al.*, 2005). Lnt contains a large cytoplasmic loop (R_{114}-R_{193}) (CL-2) between TMS-IV and TMS-V, with two hydrophobic sequences (TMS-IVa and TMS-IVb) and a highly conserved sequence (T_{144}GFPWL_{149}) (Fig. 1).

In order to identify residues in Lnt involved in substrate recognition and/or binding we performed random PCR mutagenesis on full-length *lnt* using a two-step PCR protocol where in the first step long complementary oligos were synthesized using error-prone DNA polymerase and in the second step the entire plasmid was synthesized using the mutagenized oligos. The PCR conditions were chosen such that on average one to three mutations would occur per kilobase (kb) of target DNA. The *Int* gene is 1.5 kb in length and between 1 and 5 mutations were expected.

Functionality of the mutated Lnt-s was addressed in a complementation assay using the *lnt* depletion strain (Robichon *et al.*, 2005). In this strain endogenous *lnt* is under control of an arabinose-inducible promoter. In the presence of L-arabinose *lnt* is expressed, in the absence of L-arabinose *lnt* is not expressed and cells are depleted for Lnt. The newly created plasmids were, either transformed directly into the *lnt* depletion strain and selected for growth under permissive conditions (with L-arabinose), or *lnt* was first re-cloned into a new vector backbone and then transformed
into the *lnt* depletion strain. Gene expression was not induced by IPTG and complementation was observed due to basal expression levels of *lnt* mutants. Colonies that did not grow in the absence of L-arabinose, and thus express non-functional variants of *lnt*, were analyzed further. We screened 450 colonies from two independent rounds of mutagenesis and 34 of those did not complement the *lnt* depletion strain (Table S1). The *int* gene from mutants obtained after direct screening for non-complementation of the *lnt* depletion strain was also re-cloned into a new vector backbone to exclude plasmid-linked mutations. Several mutants contained substitutions on C387 of the Lnt active site (E267-K335-C387), and some had stop codons. More than two substitutions were frequently observed and in several cases multiple mutations resulted in non-functional variants (Table S1). Among those, several mutants contained substitutions located in the first transmembrane segment of Lnt (Fig. 1) (Robichon et al., 2005) and five mutations in NB24 map in TMS-VI. This suggests that membrane insertion of Lnt was probably affected in these cases. Most mutants possess substitutions of amino acids located in both CL-2 and the periplasmic catalytic domain. We have shown that Lnt is a low abundant enzyme, about 100-200 molecules exist per cell (Hillmann et al., 2011). Single mutations that only slightly affect Lnt function may not be found because higher protein levels due to expression from a high-copy plasmid may overcome a functional defect. This might explain why several mutations are required to observe a strong phenotype in our screen.

Mutants that contained one or two substitutions and produced Lnt protein were analyzed in more detail. Re-cloning of *lnt* from NB8 (L183R, T404S), NB10 (R123C, I126N), NB14 (W192R, I211T) led to restoration of growth of the *lnt* depletion strain in the absence of L-arabinose suggesting that additional mutations had occurred either on the plasmid or on the chromosome. Reconstruction of Q155R from NB34 (Q155R,
T315N) was not successful, perhaps due to a dominant negative phenotype of this mutation, but T315N alone did not affect Lnt function.

The substitutions in mutants NB12 (F358V, T481R), NB22 (N244I, Y406C) and NB31 (P353S, L392H) were constructed individually by site-directed mutagenesis and analyzed for functionality in the lnt depletion strain. A spot dilution assay was used as a complementation assay that allowed comparison of the various mutants. A single colony from a fresh plate containing L-arabinose was inoculated in liquid medium without L-arabinose and cells were grown for two hours to deplete Lnt. Cells were serial diluted and spotted on plates containing or not L-arabinose and/or IPTG. The lnt depletion strain shows a slight growth defect on L-arabinose when a non-functional copy of Lnt is present (Fig. 2). Depletion of Lnt in these strains probably resulted in loss of viability prior to plating. The results show that both F358V and T481R result in non-functional Lnt. L392H is functional. When combining P353S and L392H into a single protein, a non-complementing phenotype is again observed. Complementation was also observed with G447S but the strain has a mucoid morphology. Both N244I and Y406C (from NB22) resulted in functional enzyme (data not shown).

Previous studies identified W237 and E343 as essential for Lnt function and were shown to be located on flexible loops in the catalytic domain (Vidal-Ingigliardi et al., 2007). Two substitutions, P361A (NB30) and M362I (NB28), are found close to F358 and P353. Construction of M362I resulted in non-functional Lnt (Fig. 2). NB30 contains several substitutions including P147L that results in non-functional Lnt (see below). We have previously shown that when highly conserved residues P340 or P346, located closely to E343, resulted in functional Lnt (Vidal-Ingigliardi et al., 2007). P361A was therefore not analyzed separately.
Addition of IPTG resulted in restoration of growth in the absence of arabinose in non-functional Lnt variants T481R and P353S/L392H and to a lesser extend in F358V and M362I, suggesting that the enzymes are less efficient and that increase in enzyme concentration compensates for their reduced activity. All variants produced protein, although less in the case of T481R, as judged by Western blotting (Fig. S1).

In conclusion, the essential residues identified here are located in the catalytic periplasmic domain of Lnt.

Conserved residues R139 and P147 in cytoplasmic loop 2 are required for Lnt function

In the screen for essential residues using full-length int described above, we did not identify amino acids located in the cytoplasmic loop 2 (CL-2) region of Lnt. Due to their hydrophobic character, TMS-IVa and TMS-IVb might position the CL-2 with the conserved T_{144}GFPWL_{149} sequence in close proximity to or associated with the membrane (Fig. 1). Upstream of this sequence a charge distribution is conserved in TMS-IVa (E136 and R139 in Lnt of E. coli) (Fig. 1). To gain additional insights into the role of this loop in Lnt function we performed random PCR mutagenesis targeting the part of int encoding CL-2. Restriction sites not affecting the amino acids were created in codons that allowed for exchange of CL-2 encoding fragments between wild type int and mutated fragments. Induction of the plasmids was not required to observe full complementation with plasmids expressing wild type Lnt. Deletion of CL-2 resulted in non-functional Lnt, demonstrating that this region is required for Lnt function (data not shown). A total of 200 mutated clones were screened for loss of Lnt activity. All clones restored growth of the int depletion strain under restrictive
conditions. Efficient mutagenesis was confirmed by sequencing of several clones. Several mutants contained multiple substitutions as described above (Table S2).

Conserved residues E136 and R139 were replaced separately by alanine using site-directed mutagenesis. Lnt with R139A partially complements the lnt depletion mutant, but Lnt containing E136A was functional (Fig. 2). We previously showed that Lnt in which conserved residue P147 was substituted by alanine was still functional (Vidal-Ingigliardi et al., 2007). NB30 contained P147L among others substitutions (Table S1) and was reconstructed and analyzed separately. Lnt with P147L was not functional, but addition of IPTG restored growth in the absence of L-arabinose (Fig. 2). Both R139A and P147L result in a mixed-colony phenotype. Substitution of P147 by leucine increases the overall hydrophobicity of the region containing TMS-IVa and T_{144}GFPWL_{149} and probably results in an orientation of CL-2 relative to the membrane that leads to inactive Lnt. Thus, R139 is essential and P147 is important for Lnt function.

### TMS-IVa and TMS-IVb do not completely span the cytoplasmic membrane

CL-2, with 79 amino acids in length, is a relative long cytoplasmic loop for a bacterial membrane protein. Furthermore, although not always predicted, the two hydrophobic regions in CL-2 are putative transmembrane segments, where TMS-IVa is less hydrophobic than TMS-IVb. We used the substituted cysteine accessibility method (SCAM) to determine whether TMS-IVa and TMS-IVb are real transmembrane segments such that CL-2 is exposed to the periplasm (Bogdanov et al., 2005, Pailler et al., 2012). Single cysteine substitutions were constructed in previously identified cytoplasmic and periplasmic loops and between the putative membrane-spanning
segments TMS-IVa and TMS-IVb (Fig. 1). The substitutions were introduced by site-directed PCR mutagenesis in \textit{Int} encoding both an active variant and an inactive variant that has active site cysteine 387 replaced by serine (C387S). To facilitate determination of membrane topology by SCAM, all constructs have cysteine 23 and cysteine 62 replaced by alanine. These substitutions do not affect Lnt function (Buddelmeijer & Young, 2010). The cysteine substitutions in active Lnt were used to test the biological activity in a complementation assay with the \textit{Int} depletion mutant (Robichon \textit{et al.}, 2005). All variants restored growth of the \textit{Int} depletion mutant under restrictive growth conditions, in the absence of L-arabinose, indicating that all Lnt cysteine mutants are functional enzymes (data not shown).

The second series of cysteine substitutions in inactive Lnt was used to determine membrane topology. Whole cells were first treated with EDTA to permeabilize the outer membrane. Cells were then treated with or without membrane-impermeable alkylating reagent MTSES and subsequently labeled with a second alkylating reagent maleimide polyethylene glycol (malPEG). The PEG buffer contains urea, SDS and EDTA that denatures all proteins and renders all unprotected cysteine residues accessible to malPEG. When cysteine residues are accessible to MTSES, and thus exposed to the periplasm, their free thiol groups covalently react with MTSES and as a consequence are blocked for alkylation with malPEG. In the case of cytoplasmic or membrane-embedded cysteine residues, MTSES does not react with thiol groups and proteins are alkylated by malPEG resulting in an increase in molecular weight of 5 kDa. Active Lnt and inactive Lnt were not alkylated by malPEG independent of treatment with MTSES since C387 is acylated in both proteins, as a thioester in Lnt and as an oxygen-ester in Lnt$^{C387S}$ (Fig. 3). Substitutions Y31C, G87C and T221C were protected by MTSES and not alkylated by malPEG. S5C, S56C, T120C, S154C,
N191C and S513C were not protected by MTSES and therefore alkylated with malPEG [Fig. 3]. Alkylation by malPEG of N191C and S513C was less efficient compared to the other cysteines. These results suggest that residues Y31, G87 and T221 are accessible to MTSES and are facing the periplasm and that S5, S56, T120, S154, N191 and S513 are not accessible to MTSES and are located either in the cytoplasm or embedded in the membrane. Similar results were obtained with an inactive variant of Lnt in which C387 was replaced by alanine and thus unable to form an acyl-enzyme intermediate (data not shown) (Buddelmeijer & Young, 2010). This indicates that the overall membrane topology of Lnt does not change upon auto-acylation.

Our membrane topology analyses show that S154C is not accessible to MTSES in whole cells and therefore not exposed to the periplasm. Thus the hydrophobic segments TMS-IVa and TMS-IVb do not completely span the membrane (Fig. 1). This indicates that the conserved sequence T144GFPWL149 is located in the cytoplasm and may be in close proximity to the membrane due to the hydrophobic character of TMS-IVa and TMS-IVb.

Conservation of essential residues in Lnt of other bacterial species

The Lnt sequence of E. coli was used to retrieve all Lnt orthologous sequences from over 2700 complete bacterial genomes. The workflow included comparison of the Lnt E. coli sequence to itself using BLASTP to determine the maximal bit score then matching the Lnt sequence against sequenced genomes using TBLASTN in a single representative for each genus. Lnt orthologs with a score of 15% or higher were analyzed further using Clustal Omega alignment and the conserved residues were...
identified (Fig. S2). Known Lnt sequences from high GC gram-positive bacteria fell below this cut-off and were analyzed separately (Fig. S3).

Residue G447 is found in Lnt of all bacterial species and P147 and T481 are conserved in γ-proteobacteria (Fig. 4, Fig. S2). R139 is conserved in γ-proteobacteria and is substituted by K in some species. One species (Tolumonas) has M at this position. Position 136 in E. coli Lnt and the corresponding location in the orthologues contains a conserved acidic residue, usually an E and less frequently a D. F358 and M362 are less conserved but are nevertheless shown to be essential in Lnt of E. coli. Interestingly, several Lnt sequences have F358 replaced by valine and M362 replaced by isoleucine, substitutions that lead to inactivation of Lnt of E. coli. Other poorly conserved residues are probably required to act together with F358 and M362 for Lnt to function correctly. In the structural model the carboxyl group of L338 interacts with M362 (Vidal-Ingigliardi et al., 2007). Substitution of L338 by alanine did not affect Lnt function, since the amino group of L338 does not play a role in the M362-L338 interaction (Vidal-Ingigliardi et al., 2007). P353 is also poorly conserved and is only essential in combination with an additional mutation (L392H). This supports the idea that conformational changes take place in the catalytic domain that involves movement of flexible loops to facilitate binding of substrate.

Essential residues are located on flexible loops in the catalytic periplasmic domain

We have previously reported a structural model for the catalytic periplasmic domain of Lnt (Vidal-Ingigliardi et al., 2007). The α-β-β-α fold is characteristic for members of the nitrilase, or carbon-nitrogen hydrolase, family (Pace & Brenner, 2001). The localization of the essential residues on a revised version of the structural model
obtained with PHYRE2 (Kelley & Sternberg, 2009) is shown in Fig. 5A. The less conserved essential residues are located on flexible loops. The structural modeling program I-TASSER (Zhang, 2008) did not lead to a more precise or different secondary structure prediction than that obtained with PHYRE2 (data not shown). Essential residue T481 is part of a α-helix on top of the α-β-β-α folded structure of Lnt. Replacement of T481 by arginine probably results in a conformational change in this α-helix affecting the overall structure of Lnt. Levels of mutant Lnt (T481R) detected by immunoblotting are lower compared to wild type, suggesting that the mutated protein is less stable (Fig. S1, Fig. 6). G447 and L392 are found close to the active site triad. P353, F358 and M362 are located on a flexible loop β5/β6 (residues N336-L382) facing away from the active site in the model. E343 was previously identified as essential and required for the first step in the reaction. It is located on loop β5/β6 in close proximity to P353, F358 and M362 (Fig. 5A). Essential residue W237 is located on loop β1/α1 (Vidal-Ingigliardi et al., 2007). Similar structural models were predicted for the catalytic domain of Lnt from C. glutamicum (NCgl_1424), M. smegmatis (MSMEG_3860), M. bovis (BCG_2070c), S. coelicolor (SCO_1014) and S. scabies (SCAB_83111) (Fig. 5B). Whereas P340-E343 is highly conserved, other essential residues located on this loop, including P353, F358 and M362, are not. These residues probably have a role in correctly positioning the conserved residues and/or substrate(s) to efficiently N-acylate lipoproteins.

Essential residues located on the cytoplasmic loop CL-2 and the β5/β6 periplasmic loop in the catalytic domain are involved in the second step of the Lnt reaction
A stable thioester acyl-enzyme intermediate is formed as part of the two-step reaction catalyzed by Lnt (Buddelmeijer & Young, 2010). The effect of the various amino acid substitutions resulting in non-functional Lnt on the formation of the thioester acyl-enzyme intermediate was assessed by alkylation of C387 by malPEG. The acyl group bound to C387 can be specifically cleaved by neutral hydroxylamine (HA), after which the free thiol group is accessible for alkylation by malPEG. Whole cells from strains expressing lnt variants from an arabinose-controlled promoter were treated with or without hydroxylamine and after protein precipitation samples were treated with malPEG. Wild type Lnt was alkylated by malPEG after HA treatment, corresponding to the presence of a thioester acyl-enzyme intermediate. Lnt (T481R) was alkylated both in the absence and presence of HA, suggesting that a thioester acyl-enzyme intermediate was not formed (Fig. 6). The amount of Lnt protein in this mutant is lower compared to Lnt. All other mutants tested were only alkylated in the presence of HA and thus all Lnt variants form a stable thioester acyl-enzyme intermediate (Fig. 6). This suggests that the essential residues identified in this study, including those located in CL-2, do not affect the first step of the N-acyltransferase reaction. We analyzed endogenous Lpp in the presence of non-functional Lnt variants in the lnt depletion strain by Western blotting to confirm that accumulation of apo-Lpp occurred under restrictive growth conditions. Since basal level expression of lnt-myc2 from a strong pTrc promoter was sufficient to observe complementation in the case of wild type Lnt we did not add IPTG to avoid overexpression effects. The results shown in Fig. 7 demonstrate that apo-Lpp accumulates in strains where Lnt with R139A, P147L, F358V, M362I, T481R or P353S/L392H substitutions is the only form of enzyme present. Mature Lpp was detected in the presence of Lnt with G447S substitution. This residue probably plays a role in the efficiency of the
reaction. This demonstrates that the non-functional variants are affected in the second step of the apolipoprotein N-acyltransferase reaction.
Two acyltransferases and one signal peptidase are involved in the post-translational modification of lipoproteins but little is known about the molecular mechanism of the catalytic reactions. In this study we searched for essential residues that are either involved in the first step (auto-acylation of Lnt) or in the second step (N-acylation of apolipoprotein) of the N-acyltransferase reaction with the hope to identify residues involved in recognition or binding of substrate.

We identified essential residues and regions in Apolipoprotein N-acyltransferase (Lnt) of E. coli using random and site-directed PCR mutagenesis. A loop (CL-2) located between TMS-IV and TMS-V containing two hydrophobic segments and a conserved region, is located in the cytoplasm. Secondary structure predictions of CL-2 of Lnt demonstrated that the hydrophobic segments are α-helices with high hydrophobicity and with a length of 18-20 amino acids that resemble transmembrane segments, however, our results demonstrate that these segments do not completely span the cytoplasmic membrane but may partially enter into the lipid bilayer. Reentrant loops are structural motifs that go halfway through the membrane and then turn back to the side of entry (Yan & Luo, 2010). These loops are found in transporters such as ion-channels and undergo conformational changes following substrate binding (Grunewald et al., 2002, Yagur-Kroll et al., 2009). In general, reentrant loops are shorter and less hydrophobic than transmembrane segments, they have very low hydrophobicity around the turning point and the two sides usually have different secondary structure (Yan & Luo, 2010). Since CL-2 does not have these characteristics, we hypothesize that the α-helices form a tilted fold in the membrane.
as described for intra-membrane proteases like GlpG in *E. coli* (Ben-Shem et al., 2007).

The first hydrophobic segment (TMS-IVa) has a pair of amino acids with opposite charges (E136 and R139 in *E. coli*), an arrangement found in all Lnt enzymes. Immediately downstream of this hydrophobic segment a conserved sequence (T144GFPWL149) was identified. In a previous study we have replaced several of these conserved amino acids by alanine and found that all Lnt variants were functional (Vidal-Ingigliardi et al., 2007). Here we identified P147 as an important residue for Lnt function since replacement by leucine resulted in an inactive variant. By increasing the hydrophobicity of this region or by eliminating the proline-imposed turn, it is likely that the orientation of CL-2 changed resulting in an inactive conformation. Substitution of R139 by alanine also resulted in non-functional Lnt but no effect was observed when E136 was replaced by alanine. This result was surprising since E136 is as conserved as R139. **Association of CL-2 with the membrane positions residues R139 and P147 in close proximity to the lipid bilayer.**

Other essential residues were located in the catalytic periplasmic domain of Lnt and several on flexible loops facing away from the active site triad in the structural model predicted using PHYRE2. The degree of amino acid conservation was high for G447 and T481 but low for P353, F358, M362 and L392 in sequences more than 15% identical to Lnt from *E. coli*. Residues P353, F358 and M362 are located on the flexible loop β5/β6 between N336 and L382. Two other highly conserved residues, W237 and E343, were previously identified as being essential for Lnt activity. E343 is also located on the β5/β6 loop and W237 is found on loop β1/α1 between G229 and G240 (Vidal-Ingigliardi et al., 2007). All residues, except E343 (Buddelmeijer & Young, 2010), are involved in the second step of the reaction since the thioester acyl-
enzyme intermediate was formed but apolipoprotein accumulated and mature lipoprotein was not formed. The loops likely adopt a secondary structure upon substrate binding that brings the $\alpha$-amino group of S-diacylglyceryl-cysteine of the substrate in close proximity to the active site cysteine. **We hypothesize that the CL-2 region and the catalytic flexible loops are in close proximity and together contribute to efficient transfer of fatty acids onto apolipoprotein.** One likely possibility is that the six transmembrane segments surround the partially membrane-embedded periplasmic and cytoplasmic loops adopting a barrel shape where the active site cavity becomes into close contact with the carbonyl group of PE. The bottom of this barrel shape is closed by CL-2 that sticks into the membrane towards the active site. This model suggests that access of the apolipoprotein substrate to the thioester-acyl group of Lnt occurs via lateral opening of the barrel.

The CL-2 region was targeted separately in an error-prone mutagenesis approach but essential residues were not identified. Lnt is a low abundant enzyme that we estimated to exist in about 100-200 molecules per cell (Hillmann et al., 2011). This might explain why mutations resulting in slightly less efficient enzyme were not identified and that only mutations leading to a severe phenotype were obtained in our screen. The fact that an increase in protein level of non-functional Lnt enzymes restores growth of the *int* depletion strain supports this hypothesis.

Together with the catalytic triad, residues E389 and E343 are conserved even in the more distantly related actinobacterial Lnt-s (Widdick et al., 2011). Lipoproteins from *C. glutamicum* are modified with a $N$-acyl (C16:0) and S-diacylglyceryl (C16:0, C18:1) moiety like in *E. coli* (Mohiman et al., 2012), whereas lipoproteins in *Mycobacteria* contain tuberculostearic acid (C19:0) as part of their S-diacylglyceryl moiety and palmitic or tuberculostearic acid as $N$-acyl group (Brülle et al., 2010,
Tschumi et al., 2009, Brülle et al., 2013). The fatty acid composition of the N-acyl group of lipoproteins in *S. scabies* varies from C15 to C19, but details about the N-acyl and S-diacyl composition of lipoproteins are not known. It appears likely that Lnt attaches palmitic acid and tuberculostearic acid onto apolipoprotein. E343 is involved in the first step of the Lnt reaction, formation of the thioester acyl-enzyme intermediate between fatty acid and Lnt (Buddelmeijer & Young, 2010), suggesting that it is involved in the interaction and/or hydrolysis of phospholipid. Lnt of *M. smegmatis* could not complement a *lnt* depletion strain of *E. coli* (Tschumi et al., 2009). It is conceivable that the enzyme is not inserted into the *E. coli* membrane in an active conformation and therefore, unable to correctly interact with the apolipoprotein substrate. Alternatively, the lipid moiety of the substrate might not be correctly fitted into the enzyme for N-acylation to occur. Interestingly, the *sn*-1 position of mycobacterial phospholipids is occupied by C18 related fatty acids and *sn*-2 by palmitic acid (Okuyama et al., 1967). This implies that mycobacterial Lnt uses fatty acids only from *sn*-2 and not from *sn*-1, as is the case for the *E. coli* enzyme (Jackowski & Rock, 1986, Hillmann et al., 2011, Nakayama et al., 2012). The substrate specificity for both the phospholipid and the apolipoprotein substrates is different for Lnt of different species. The 3D structural models for the catalytic domain of all Lnt enzymes identified to date are highly similar and illustrate that the mechanism of N-acyltransferase is identical. However, several residues located on the disordered β5/β6 loop are essential but poorly conserved and may play a role in apolipoprotein substrate specificity. Sortases are membrane-associated enzymes that covalently link proteins to peptidoglycan or polymerize proteins to construct pili in Gram-positive bacteria (Spirig et al., 2011). Structural analyses have demonstrated that the sortase catalytic mechanism involves opening and closing of loops upon
binding of substrate (Suree et al., 2009) and that the loops are involved in substrate recognition (Bentley et al., 2007). We hypothesize that the β5/β6 flexible loop in Lnt is involved in recognition of the S-diacylglyceryl-cysteine of apolipoprotein and that binding of substrate leads to conformational changes and a substrate-bound closed form of Lnt similar to sortases. Our data suggest that cytoplasmic and periplasmic membrane-associated loops are required for N-acylation of lipoproteins and that specific amino acids in these loops may be involved in substrate recognition and/or binding. Ongoing three-dimensional structural analyses of full-length Lnt enzymes are expected to highlight differences between various species and to establish how the enzyme interacts with its substrates.
Experimental Procedures

Bacterial strains and growth conditions

Strains and plasmids are listed in Table S3. Cells were grown in LB medium supplemented with ampicillin (100 µg/ml) when required. Induction of gene expression from a pBAD promoter was obtained by addition of L-arabinose (0.2%) and from pTrc promoter with IPTG (1 mM).

Standard DNA manipulation

Silent sites were introduced in int flanking the cytoplasmic loop 2 (CL-2) coding sequence using the QuickChange II site-directed mutagenesis protocol (Stratagene). The procedure included a two-step PCR reaction where large oligomers were synthesized in the first PCR reaction that contained only one of the two mutant primers and synthesis of the entire mutated plasmid in the second reaction that contained the large oligomers and DNA template. A PmlI site was introduced at codon 120 (T120) with primers T120-PmlI_for and T120-PmlI_rev, an NheI site at L185 with primers L185-NheI_for and L185-NheI_rev, and a StuI site at A186 with primers Del_StuI-5’ and Del_StuI-3’ (primers are listed in Table S3). The PmlI and NheI sites were combined in pCHAP9610 and the CL-2 segment was removed by deleting the PmlI-StuI fragment, resulting in pCHAP9609.

The gene encoding int was amplified by PCR using primers 5’-cutE and IntXbaIrev and was inserted between the EcoRI and XbaI sites of pUC18, the 3’ end of int is in frame with a double c-myc tag, resulting in pCHAP7547. Site-directed mutagenesis
was used to create alanine substitutions for cysteines (C23 and C62) on pCHAP7547 resulting in plasmid pCHAP7556 (encoding Lnt$^{C23AC62A}$) (Buddelmeijer & Young, 2010). C387 was replaced by serine or alanine as described previously (Buddelmeijer & Young, 2010, Vidal-Ingigliardi et al., 2007). An EcoRI-HindIII fragment from pCHAP7556 was inserted into pDSW204 and pBAD18, digested with the same enzymes, resulting in pCHAP7721 and pCHAP7626, respectively.

Screening for non-functional Lnt variants

Random PCR mutagenesis was performed on lnt using GeneMorph II EZ Clone (Agilent) on plasmid pCHAP7547 or pCHAP7721 as template for full-length lnt and on plasmid pCHAP9515 (Hillmann et al., 2011) as template for the CL-2 region of lnt. Plasmid DNA from mutagenesis on pCHAP7547 or pCHAP7721 was either directly transformed into the lnt depletion strain (PAP9502) and cells were selected for growth on LB agar plates containing ampicillin and L-arabinose, or DNA was transformed into DH5α and lnt fragments re-cloned into a new vector backbone before transformation into PAP9502. Colonies were re-streaked on LB agar plates containing ampicillin with or without L-arabinose. In the case of mutagenesis of the CL-2 region, fragments were first re-cloned in template pCHAP9610 using PmlI and NheI sites, and then screened for non-complementing phenotype in PAP9502 on plates lacking L-arabinose.

For detailed analysis of the mutants, strains were grown in medium without 0.2% L-arabinose starting from one colony grown on a fresh plate with L-arabinose. Cells were grown for two hours at 37°C and serial diluted from $10^{-1}$ to $10^{-6}$. Cells were
spotted on plates with or without L-arabinose in the presence or absence of 1 mM IPTG.


**Determination of thioester-acyl enzyme intermediate**

The procedure to determine the acylation state of Lnt was described previously (Buddelmeijer & Young, 2010). Briefly, whole cells were treated with 1 M neutral hydroxylamine (HA) that cleaves specifically thioester bonds resulting in the case of Lnt in a free thiol group of active site residue C387. Control samples were incubated with 1 M Tris-HCl (pH 7.0). Subsequent labeling of free SH groups with maleimide-polyethylene glycol (malPEG) resulted in alkylation of SH by maleimide and in an increase of molecular weight by 5 kDa.

**Cysteine protection assay**

Accessibility of cysteine residues in whole cells was determined using the substituted cysteine accessibility method (SCAM) as described previously (Bogdanov et al., 2005, Pailler et al., 2012). In summary, whole cells were resuspended in phosphate buffer (PB) containing EDTA and treated with the membrane-impermeable reagent (2-sulfonatoethyl)-methane thiosulfonate) (30 mM MTSES). The reaction was quenched with 100 mM L-Cysteine and cells were washed twice with PB and resuspended in PB. Proteins were precipitated with either trichloroacetic acid (10% TCA) or methanol/chloroform. Pellets were resuspended in PEG buffer (1 M Tris-HCl pH 7.0, 10 M urea, 1% SDS, 1 mM EDTA). 0.2 mM MalPEG was added to all
samples and incubated for 60 minutes at room temperature. Proteins were precipitated with TCA or MeOH/CHCl$_3$ and analyzed by SDS-PAGE and Western blotting.

*Gel electrophoresis and Western blotting*

Protein samples were solubilized in SDS sample buffer containing 4 mM DTT or 5% β-mercaptoethanol (β-ME) and were heated at 100°C for 5 minutes. Samples were separated on polyacrylamide gels (Biorad) and transferred onto nitrocellulose membranes. Lnt was detected by chemiluminescence (Pierce) using anti-c-myc antibodies (Sigma) and secondary anti-rabbit antibodies conjugated with horseradish peroxidase (HRP). Lpp was detected with antibodies against Lpp (a gift from H. Tokuda).

*Bioinformatics analyses*

The presence of Lnt orthologs was investigated in all bacteria and separately in the γ-proteobacteria taxonomic class using *E. coli* Lnt (NP_415190) as bait as follows. For each genus, a single representative and sequenced genome originating from the NCBI repository (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/) was selected alphabetically and submitted to TBLASTN analysis with Lnt using an in-house C# script. Taxonomic relationships between individual species were determined with the SyntTax web service (Oberto, 2013). Normalized genomic BLAST scores were used to measure the degree of orthology as described (Lerat *et al.*, 2003). The threshold was set empirically at the value of 15% in agreement with the annotations of the selected genomes. Lnt orthologs with a score equal or greater than 15% were collected and
submitted to multiple protein sequence alignment using the Clustal Omega web service (https://www.ebi.ac.uk/Tools/msa/clustalo/).
Acknowledgements

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33
**Figure legends**

Fig. 1. Membrane topology, essential residues and protein sequence of cytoplasmic loop 2 (CL-2) of Lnt from *E. coli*. Lnt has six transmembrane segments (TMS) indicated as I-VI and is located in the cytoplasmic membrane (CM). Cytoplasmic loop 2 (CL-2) corresponds to residues R$_{114}$-R$_{193}$. Positively (+) and negatively (-) charged residues are indicated. Residues substituted by cysteine are indicated as white circles. Amino acid sequence of CL-2 is shown including TMS-IVa and TMS-IVb (undelined). Essential residues (red), important residues (bleu) and the active site triad (green) are indicated.

Fig. 2. Complementation spot-dilution assay of Lnt mutants in the *lnt* depletion strain. Strains were depleted from Lnt by growing the cells in the absence of L-arabinose in liquid medium. Serial dilutions were spotted on plates with or without L-arabinose (0.2%) (+ARA) in the presence or absence of IPTG (1 mM). Lnt(C387S) is an active site mutant of Lnt.

Fig. 3. Accessibility to MTSES of substituted cysteine residues in whole cells. Western blot analysis of Lnt-myc$_2$ from cell lysates treated with (+) or without (-) MTSES and subsequent alkylation by malPEG. Lnt enzymes containing active site cysteine 387 (+) or C387S substitution are included as controls (-). Individual cysteine substitutions are introduced in C387S variant of Lnt. All Lnt proteins contain C23A and C62A substitutions. Cysteine substitutions were introduced in known cytoplasmic and periplasmic loops of Lnt. Protein bands corresponding to Lnt-c-myc$_2$ (Lnt, 57
kDa) and the alkylated form of Lnt-c-myc₂ (Lnt-malPEG) are indicated. Proteins were detected with antibodies against c-myc.

Fig. 4. LOGO representation of conserved residues in Lnt of γ-proteobacteria. Lnt of γ-proteobacteria were aligned by Clustal Omega and conserved residues were identified (see also Fig. S2). Active site residues are shown in red. Essential and important residues identified in this study are indicated in the LOGO coordinates in red and blue, respectively. Amino acid substitutions of all mutants identified here are shown in bold and in blue. The transmembrane segments are shown in bold as TMS-I to VI, including membrane associated sequences TMS-IVa and TMS-IVb. Asterisks indicate gaps in the alignment, some of which result in conserved residues excluding the E. coli sequence. The predicted secondary structure of the periplasmic catalytic domain is indicated (see also Fig. 5).

Fig. 5. Localization of essential residues on a structural model of Lnt. A structural model for the catalytic periplasmic domain of Lnt (residues T221-N488) was obtained using PHYRE2 program (A). The catalytic triad (E267-K335-C378) is indicated in red, essential residues are shown in green. W237, E343 were identified previously (Vidal-Ingigliardi et al., 2007). Loop β5/β6 is colored blue. Structural models of the catalytic domain of Lnt orthologues are shown (B). Color indications are the same as for E. coli.

Fig. 6. Acylation state of C387 in non-functional Lnt variants. Western blot analysis of cell lysates from cells treated with (+) or without (-) hydroxylamine (HA) and subsequent alkylation with malPEG. Detection of Lnt-c-myc₂ (Lnt) and alkylated Lnt-
c-myc₂ (Lnt-malPEG) was obtained with anti-myc antibodies. Lnt^{C23AC62A} corresponds to Lnt with C23A and C62A substitutions but containing active site C387.

Fig. 7. Accumulation of apo-Lpp in presence of non-functional Lnt. Western blot analysis of Lpp in whole cell lysates from the lnt depletion strain expressing non-functional lnt variants as described in Fig. 6 under depletion conditions (in the absence of L-arabinose). Lysates from a wild type strain and a Δlpp mutant were loaded as controls. Detection of Lpp was obtained with anti-Lpp antibodies, mature Lpp (Lpp) and the apo form of Lpp (apo-Lpp) are indicated.
FIGURE 2

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<th>F358V</th>
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Dilution factor
### FIGURE 3

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[Image of a gel or blot with Lnt and Lnt-malPEG labels]
FIGURE 5

A

B

C. glutamicum  M. smegmatis  M. bovis  S. coelicolor  S. scabir
### FIGURE 6

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**Legend:**
- Lnt-malPEG
- Lnt

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832
833
834