MINI-REVIEW

Lipoproteins in Bacteria

Shigeru Hayashi[1,2] and Henry C. Wu[1]

Abstract

Covalent modification of membrane proteins with lipids appears to be ubiquitous in all living cells. The major outer membrane (Braun's) lipoprotein of *E. coli*, the prototype of bacterial lipoproteins, is first synthesized as a precursor protein. Analysis of signal sequences of 26 distinct lipoprotein precursors has revealed a consensus sequence of lipoprotein modification/processing site of Leu-(Ala, Ser)-(Gly, Ala)-Cys at − 3 to + 1 positions which would represent the cleavage region of about three-fourth of all lipoprotein signal sequences in bacteria. Unmodified prolipoprotein with the putative consensus sequence undergoes sequential modification and processing reactions catalyzed by glyceryl transferase, O-acyl transferase, prolipoprotein signal peptidase (signal peptidase II), and N-acyl transferase to form mature lipoprotein. Like all exported proteins, the export of lipoprotein requires functional SecA, SecY, and SecD proteins. Thus all precursor proteins are exported through a common pathway accessible to both signal peptidase I and signal peptidase II. The rapidly increasing list of lipid-modified proteins in both prokaryotic as well as eukaryotic cells indicates that lipoproteins comprise a diverse group of structurally and functionally distinct proteins. They share a common structural feature which is derived from a common biosynthetic pathway.

Key Words: Lipoprotein; posttranslational modification; signal pepidase II; fatty acylation; glyceride modification; phospholipids; acyl transferase; protein translocation.

Introduction

The cell envelopes of Gram-negative bacteria are composed of three distinct layers: the outer membrane, the peptidoglycan (or murein) layer, and the

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[1] Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799.
[2] Present address: Pfizer Central Research, Groton, CT 06340.

inner (or cytoplasmic) membrane. The murein lipoprotein of *E. coli* was discovered by Braun and his coworkers (Braun and Rehn, 1969). Their pioneering studies not only demonstrated the covalent attachment of lipoprotein to the peptidoglycan layer by a peptide linkage between the COOH group of meso-diaminopimelic acid in peptidoglycan and the ε-NH_2 group of the carboxy-terminal lysine (Braun and Wolff, 1970), but also elucidated the structure of the novel lipo-amino acid at the amino terminus of the lipoprotein as N-acyl diglyceride-cysteine, i.e., glycerylcysteine containing two ester-linked fatty acids and one amide-linked fatty acid (Hantke and Braun, 1973). The ester-linked fatty acids in murein lipoprotein are similar in composition to those present in bulk phospholipids, including the presence of cyclopropane fatty acids in murein lipoprotein as well as in phospholipids. Of amide-linked fatty acids present in the lipoprotein, 65% is palmitic acid. Murein lipoprotein also exists as a free form which is one of the major outer membrane proteins in *E. coli* (Inouye *et al.*, 1972).

A major advance in our understanding of the biogenesis of the outer membrane lipoprotein was the identification of a precursor form of lipoprotein, the prolipoprotein, which contains an extra 20 amino acids extension (the signal sequence) at the NH_2-terminus (Inouye *et al.*, 1977). The structure of prolipoprotein differs from that of the mature lipoprotein in a number of ways. Consequently, a series of modification and processing reactions must occur prior to the assembly of lipoprotein into the outer membrane. In addition, like other exported outer membrane or periplasmic proteins in *E. coli*, prolipoprotein must be translocated across the cytoplasmic membrane with the signal sequence proteolytically cleaved during the export process.

While the *E. coli* outer membrane lipoprotein is the most extensively studied lipoprotein over the past two decades, it is not the only membrane protein with covalently attached lipid. The number of lipoproteins in bacteria has been increasing steadily in recent years. This review will summarize our current understanding of the structures, functions, and biogenesis of lipoproteins in bacteria.

The Signal Sequences of Lipoprotein Precursors in Bacteria

Table I summarizes the signal sequences of lipoprotein precursors in bacteria. These proteins have been shown or proposed to be lipid-modified proteins by one of the following criteria: (1) examination of the putative signal sequence for the existence of a lipoprotein consensus sequence, i.e., Leu-Ala(Ser)-Gly(Ala)-Cys at the C-terminal region of the signal sequence; (2) inhibition of the processing of [^{35}S]methioine-labeled precursor protein by

globomycin, a specific inhibitor of prolipoprotein signal peptidase (Inukai *et al.*, 1978); (3) metabolic labeling of the putative lipoprotein with $[2^3\text{-H}]$glycerol and/or $[^3\text{H}]$palmitate; and (4) identification of $[2\text{-}^3\text{H}]$glyceryl-$[^{35}\text{S}]$cysteine sulfone in the acid hydrolysate of performic acid-oxidized lipoprotein (Hayashi and Wu, 1988).

A prototypical signal sequence contains three distinct regions: a positively charged amino terminal region (the *n*-region), a central hydrophobic region (the *h*-region), and a polar carboxy-terminal region (the *c*-region) that specifies the processing site between the signal sequence and the mature protein. For nonlipoprotein precursors, the putative cleavage site by signal peptidase can be deduced with the so-called $(-1, -3)$ rule of von Heijne (1983). The amino acids at the -1 and -3 positions from the cleavage site are usually small neutral amino acids such as Ala, Gly, and Ser (von Heijne, 1983, 1985; Perlman and Halvorson, 1983). In a previous review, we analyzed the signal sequences of 16 lipoprotein precursors, and suggested a consensus sequence of Leu-X-Y-Cys for prolipoproteins with X and Y being predominantly neutral small amino acids such as Ala, Gly, and Ser (Wu, 1987). Two recent reports have addressed the same question of the consensus sequence in lipoprotein precursors based on 16 lipoprotein sequences (Klein *et al.*, 1989; von Heijne, 1989). Klein *et al.* (1989) have concluded that (1) the signal sequences of lipoprotein precursors tend to be shorter, more hydrophobic, and bulkier than those of nonlipoprotein precursors; (2) the lipoprotein precursors are predicated to have a β turn secondary structure immediately following the cleavage site at the $+2$ or $+3$ positions; and (3) most lipoproteins lack a net negative charge in the N-terminal region of the mature proteins. On the other hand, statistical analysis of lipoprotein and nonlipoprotein signal sequences by von Heijne (1989) reveals that the only significant difference between these two classes of exported proteins is in the region close to the signal peptidase cleavage site: an apolar region with the sequence of (Leu, Val, Ile)-(Ala, Ser, Thr, Gly)-(Gly, Ala)-Cys defines a lipoprotein cleavage site between Gly/Ala and modified Cys (diglyceride-cysteine), while small neutral residues at -3 and -1 define the cleavage site in nonlipoprotein precursors.

We have analyzed the signal sequences of bacterial lipoproteins listed in Table I. While there are 35 lipoproteins of which the primary structures are deduced from DNA sequences of the cloned genes, several lipoproteins are homologs from different bacterial species, e.g., the murein or Braun's lipoproteins in Gram-negative bacteria, the bacteriocin release proteins or lysis proteins encoded by various colicinogenic plasmids, TraT proteins, and pullulanases. These homologous proteins are grouped as a single lipoprotein with partial score given to any residue at a given position. All together, we have analyzed the frequencies of amino acid residues at positions -4 to $+3$

Table I. Signal Sequences of Lipoprotein Precursors in Bacteria

Lipoprotein	Sequence	References
Braun's lipoprotein (lpp)		
E. coli	MK$^+$ATK$^+$LVLGAVILGSTLLAGCSSNA	Nakamura and Inouye, 1979
S. marcescens	MNR$^+$TK$^+$LVLGAVILGSTLLAGCSSNA	Nakamura and Inouye, 1980
E. amylovora	MNR$^+$TK$^+$LVLGAVILGSTLLAGCSSNA	Yamagata *et al.*, 1981
M. morganii	MGR$^+$SK$^+$IVLGAVVLASALLAGCSSNA	Huang *et al.*, 1983
P. mirabilis	MK$^+$AK$^+$IVLGAVILASGLLAGCSSSN	Ching and Inouye, 1986
New lipoproteins in *E. coli*		
E. coli lpp-28	MK$^+$LTTHHLR$^+$TGAALLLAGILLAGCD·QSS	Yu *et al.*, 1986
TraT^1	MK$^+$MK$^+$K$^+$LMMVALVSSTLALSGCGAMST	Ogata *et al.*, 1982
TraT^2	MK$^+$HNVK$^+$LMAMTAVLSSVLVLSGCGAMST	Finlay and Paranchych, 1986
Pal	MQLNK$^+$VLK$^+$GLMIALPVMAIAACSSNK$^+$NASN	Chen and Henning, 1987
RplA	MR$^+$K$^+$QWLGICIAAGMLAACTSD$^-$D$^-$GQQQ	Takase *et al.*, 1987
RplB	MR$^+$YLATLLSLAVLITAGCGWHLR$^+$D$^-$	Takase *et al.*, 1987
OsmB	MFVTSK$^+$K$^+$MTAAVLAITLAMSLSACSNWSK^{1}R$^+$	Jung *et al.*, 1989
NlpB (*dapA nlpB purC*)	MAYSVQK$^+$SR$^+$LAK$^+$VAGVSLVLLLAACSSD$^-$··SR$^+$YK$^+$R$^+$	P. Stragier, personal communication
Orf17 (*btuCED orf17*)	MR$^+$FCLILITALLLAGCSHHK$^+$AP	R. Kadner, personal communication
Lysis proteins		
Pro-H	MK$^+$K$^-$AK$^+$AIFLFILIVSGFLLVACQANY	Hakkart *et al.*, 1981
Pro-Kil	MR$^+$K$^+$K$^+$FFVGIFAINLLVGCQANY	Oka *et al.*, 1979
Pro-CelB	MK$^+$K$^+$ITGIILLLAVIILSACQANY	Cole *et al.*, 1985
Pro-Hic	MK$^+$K$^+$ITGIILLLAVIIHLSACQANY	Watson *et al.*, 1984
Pro-Cal	MK$^+$K$^+$IIICVILLAIMLLAACQVNN	Cavard *et al.*, 1985
Lipoproteins in other Gram-negative bacteria		
Pullulanases		
K. pneumoniae	MLR$^+$YTCNALFLGSLILLSGCD$^-$NSS	Chapon and Raibaud, 1985
K. aerogenes	MLR$^+$YTCHALFLGSLVLLSGCD$^-$NSS	Katsuragi *et al.*, 1987
Pullulanase secretion		
Pul S (*K. pneumoniae*)	MR$^+$NFILFPMMAVVLLSGCQQNR$^+$P	d'Enfert and Pugsley, 1989
Chitobiase		
V. harveyi	MLK$^+$HSLIAASVITTLAGCSSLQSSE·QQ	Soto-Gil and Zyskind, 1989

β-1,4-Endoglucanase		
Pseudomonas solanacearum	MR$^+$R$^+$CMPLVAASVAALMLAGCGGGD$^-$	Huang *et al.*, 1989
H. influenzae		
Pal	MNK$^+$FVK$^+$SLLVAGSVAALAACSSSNND$^-$AAG	Deich *et al.*, 1988
Pcp	MK$^+$K$^+$TNMALALLVAFSVTGCANTD$^-$IFSGD	Deich *et al.*, 1988
Pseudomonas aeruginosa		
OprI	MNNVLK$^+$FSALALAAVLATGCSSHSK$^+$E$^-$TE	Duchene *et al.*, 1989; Cornelis *et al.*, 1989
Rps. viridis		
Cytochrome subunit	MK$^+$QLIVNSVATVALASLVAGCFE$^-$PP	Weyer *et al.*, 1987
Lipoproteins in Gram-positive bacteria		
B. licheniformis		
PenP	MK$^+$LWFSTLK$^+$LK$^+$K$^+$AAAVLLFSCVALAGCANNQTNA	Neugebauer *et al.*, 1981
B. cereus		
Type III PenP	MFVLNK$^+$FFTNSHYK$^+$K$^+$IVPVVLLSCATLIGCSNSNTQS	Hussain *et al.*, 1987
S. aureus		
PenP	MK$^+$K$^+$LIFLIVIALVLSACNSNS	McLaughlin *et al.*, 1981
S. pneumoniae		
MalX	MSSK$^+$FMK$^+$STAVLGTVTLASLLLVACGSK$^+$TAD$^-$	Gilson *et al.*, 1988
AmiA	MK$^+$K$^+$NR$^+$VFATAGLVLLAAGVLAACSSSK$^+$SSD$^-$	Gilson *et al.*, 1988
Lipoproteins in other bacteria		
Treponema pallidum		
34-kDa antigen	MK$^+$R$^+$VSLLGSAAIFALVFSACGGGGE$^-$HQHG	Swancutt *et al.*, 1989
TpmA	MNAH$^+$TLVYSGVALACAAMLGSCASGAK$^+$E$^-$	Hansen *et al.*, 1985
Mycoplasma hyorhinis		
P37	MLK$^+$K$^+$LK$^+$NFILFSSIFSPIAFAISCSNTGVVK$^+$Q	Dudler *et al.*, 1988
Rickettsia rickettsii		
17-kDa antigen	MK$^+$LLSK$^+$IMIIALATSMLQACNGPGGMNKQGT	Anderson *et al.*, 1987

Table II. Consensus Sequence for Prolipoprotein Modification/Processing Enzymes

Frequencies of amino acid residues at position						
-4	-3	-2	-1	$+1$	$+2$	$+3$
L(0.37)	L(0.75)	A(0.47)	G(0.55)	C(1.00)	S(0.38)	S(0.38)
M(0.15)	V(0.08)	S(0.25)	A(0.38)		G(0.19)	N(0.23)
	A(0.08)					
V(0.13)		I(0.08)	S(0.08)		A(0.12)	G(0.12)
	T(0.04)	T(0.08)				
A(0.10)	F(0.04)				Q(0.08)	Q(0.08)
		V(0.05)			D(0.08)	
S(0.08)	I(0.02)				N(0.08)	A(0.07)
T(0.08)		G(0.04)				
		Q(0.04)			T(0.04)	E(0.04)
I(0.05)					F(0.04)	W(0.04)
						H(0.04)
F(0.04)						
						V(0.01)

of 26 distinct lipoprotein precursors, and the results are shown in Table II. Cys at $+1$ is the only invariable. The absolute requirement of Cys at the $+1$ position is not surprising, in view of the proposed pathway for the biosynthesis of lipoproteins in bacteria (see below). Gly (55%) and Ala (38%) are strongly favored at the -1 position, while Ala (47%) and Ser (25%) are favored at -2. Leu predominates at -3 with 75% frequency. Amino acids at -4, $+2$, and $+3$ are much more variable; Leu is favored at -4 (37%), Ser is present in 38% at both $+2$ and $+3$ positions, followed by Gly at $+2$ (19%) and Asn at $+3$ (23%), respectively. The amino acid residues at $+2$ and $+3$ positions are enriched with those having a high propensity to form β turn secondary structure such as Ser, Gly, Asn, and Gln. In addition, a notable feature at the junction of the signal sequence and the mature lipoprotein is the absence of positively charged residues in this region. Based on the available data, the consensus sequence of lipoprotein precursors can be best defined as Leu-(Ala, Ser)-(Gly, Ala)-Cys from -3 to $+1$, which would account for about three-fourths of all lipoprotein signal sequences in bacteria.

We have also analyzed the length of the *h* plus *c* segment of the signal sequences in lipoprotein precursors. The range is 13–19 residues with a mean of 16 ± 1.61 residues. This value is slightly smaller than that of nonlipoprotein precursors (18.1, von Heijne, 1989), and may reflect the difference in the topology of the two signal peptidases, signal peptidase I and II for non-lipoprotein and lipoprotein precursors, respectively, in the cytoplasmic membrane (see below). The presence of a β turn secondary structure immediately following the cysteine residue in Braun's lipoprotein has been noted previously (Giam *et al.*, 1984), and *lpp* mutant proliproproteins with

decreased probability for a β turn structure in this region were found to be defective in prolipoprotein modification (Inouye *et al.*, 1986).

The minimum requirement for lipoprotein modification and processing can be defined by two additional approaches. Inouye and his coworkers have used oligonucleotide-directed site-specific mutagenesis to alter the signal sequence of Lpp prolipoprotein in *E. coli* (Pollitt and Inouye, 1986; see also Gennity *et al.*, this volume). Their results are consistent with the conclusion derived from the statistical analysis of signal sequences from distinct lipoproteins that the lipoprotein box is best described by Leu-Leu-Ala-Gly-Cys, with Gly replaceable by small neutral amino acids (Ser or Ala) but not by bulkier residues such as Thr, Val, and Leu. The second approach is to use gene fusions and truncated lipoproteins to ascertain whether lipoprotein specificity is also contained in the mature portion of lipoproteins. A Lpp-Bla hybrid protein containing the signal sequence of Lpp plus nine amino acids of the mature Lpp followed by β-lactamase is modified and processed like Lpp (Ghrayeb and Inouye, 1984). Similarly, hybrid proteins containing 0, 2, and 11 amino acid residues from the lipoproteins, i.e, BRP-Bla (Luirink *et al.*, 1989), Orf17-PhoA (Kadner, Hayashi, and Wu, unpublished data), and Pul-PhoA (d'Enfert and Pugsley, 1987) are modified and processed by SPase II. Thus there does not seem to be any essential sequence contained in the mature portion of lipoproteins which is required for the recognition by prolipoprotein glyceryl transferase and/or signal peptidase II. The results of studies using truncated lipoproteins are less clear. Luirink *et al.* (1989) have reported that a truncated mutant BRP containing the signal sequence plus four amino acids from the mature BRP is lipid-modified and processed by SPase II. On the other hand, truncated mutant Cal proteins containing the signal sequence plus 16 or 18 amino acid residues of the mature Cal protein are neither modified nor processed (Howard *et al.*, 1989). The reason for this difference is not clear.

Structures, Functions, and Localization of Lipoproteins in Bacteria

Covalent modification of membrane proteins with diacylglycerol via a thioether linkage to cysteine and with fatty acid by an amide bond appears to be ubiquitous for prokaryotic cells. To date, lipid-modified proteins in a manner analogous to Braun's lipoprotein of *E. coli* have been found in many bacterial species (Table I). They include Braun's lipoprotein homologs in various Gram-negative bacteria; extracellular enzymes (β-lactamases from *Bacillus licheniformis*, *Staphylococcus aureus* and *B. cereus*; pullulanases from *Klebsiella pneumoniae* and *K. aerogenes*; chitobiase of *Vibrio harveyii*; and β-1,4-endoglycanase from *Pseudomonas solanacearum*); plasmid-encoded

TraT proteins (required for conjugation) and lysis proteins for the release of bacteriocins; cytochrome subunit in *Rhodopseudomonas viridis*; proteins with proposed functions in the excretion of pullulanase from the outer membrane into the medium (PulS in *K. pneumoniae*), in the transport of maltodextrin or oligopeptides in Gram-positive bacteria (MalX and AmiA of *Streptococcus pneumoniae*) or in high-affinity transport system of an unknown ligand (p37 in *Mycoplasma hyorhinis*); surface antigens in *Treponema pallidum* and *Rickettsia rickettsii*; and lipoproteins of unknown functions [Pal, RplA, RplB, Lpp-28, NlpB, and Orf17 in *E. coli*; PAL and PAL cross-reacting protein (or PCP) in *Hemophilus influenzae*; OprI of *Pseudomonas aeruginosa*; and lipoproteins of unknown structures in *E. coli, B. cereus, B. licheniformis* and *Mycoplasma capricolum*].

It is clear that there is no common function for such a diverse group of bacterial membrane proteins collectively called lipoproteins. What they have in common are twofold. They share the common structural feature of having a signal sequence with the lipoprotein consensus sequence of Leu-(Ala, Ser)-(Gly-Ala)-Cys at the modification/processing site which confers the lipoprotein modification specificity. Once they are modified to contain the lipid moieties at their N-termini, they are anchored to the surface membrane where they function as structural proteins (e.g., murein lipoprotein) or catalytic proteins (membrane-bound enzymes or transport proteins). Those lipoproteins which function as enzymes (e.g., β-lactamases) or presumptive high-affinity binding proteins (e.g., MalX, AmiA, or p37) in Gram-positive bacteria or mycoplasma would presumably be located in the outer surface of the plasma membrane, with the lipid anchor interacting with the hydrophobic interior in the outer leaflet of the lipid bilayer. The topology of most membrane lipoproteins in Gram-negative bacteria is yet to be determined. Braun's lipoprotein is most likely located in the periplasm, with the fatty acids interacting with the inner leaflet of the outer membrane plus both covalent and noncovalent interactions between the C-terminal region of the lipoprotein with the peptidoglycan layer. This conclusion is based on a study of the localization of this protein in intact cells of varying LPS chemotypes using an immunological detection of lipoprotein (Braun *et al.*, 1976), and on the studies of OmpF-Lpp chimeric proteins lacking the lipid moiety at its N-terminus (Choi *et al.*, 1986). The lysis proteins appear to have a dual subcellular localization: they are presumably involved in the release of colicins through the cytoplasmic membrane and in the activation of phospholipase A located in the outer membrane. Subcellular localization experiments have revealed that pCloDF13 encoded bacteriocin release protein is located in both the cytoplasmic and outer membranes of *E. coli* cells (Oudega *et al.*, 1984; Luirink *et al.*, 1987). Whether they are enriched in the zones of adhesions remains to be determined. Using chemical crosslinking studies in

intact cells, Rothfield and his coworkers (Leduc *et al.*, 1989) have shown that nine [³H]leucine-labeled polypeptides are crosslinked to peptidoglycan, two of which are identified to be OmpA protein and Braun's lipoprotein, confirming previous reports that OmpA and free-form lipoprotein can be chemically crosslinked to peptidoglycan (Palva, 1979). Five of these nine polypeptides are membrane lipoproteins, as evidenced by their labeling with [³H]palmitate; they appeared to correspond to several of the new lipoproteins described by Ichihara *et al.* (1981). This interesting finding suggests a close association of membrane lipoproteins of *E. coli* with the murein layer of the cell envelope, and this association may play an important role in the structural integration of inner and outer membranes with the murein layer of the cell envelope.

Among the 26 distinct lipoproteins, those functioning as membrane-bound and extracellular enzymes such as β-lactamases or pullulanases are presumably nonessential proteins. The major outer membrane lipoprotein in *E. coli* is also nonessential for the growth and division of *E. coli* cells, since an *E. coli* mutant deleted for the *lpp* gene has few phenotypes except a weakened association of the outer membrane with the underlying cell envelope (Hirota *et al.*, 1977; Suzuki *et al.*, 1978). Both Lpp-28 (*nlp*A) and NlpB (or Nlp-3) are also not essential since null mutations in these genes are not lethal (Yamaguchi and Inouye, 1988; Stragier, P., personal communication). Two lipoproteins appear to be essential: the peptidoglycan-associated protein (PAL) of *E. coli* and *H. influenzae* and the PCP (PAL cross-reacting protein) of *H. influenzae*. Attempts to isolate null mutations in these genes have been unsuccessful (Chen and Henning, 1987; Green, B., personal communication). Similar approaches may be used to ascertain the functions of other lipoproteins in bacteria.

Lipp-28 is one of the new lipoproteins identified by Ichihara *et al.* (1981) which is located in the inner membrane of the *E. coli* cell envelope (Yu *et al.*, 1986). It has been proposed that the topogenic information targeting lipoproteins to the outer membrane or inner membrane resides in the second amino acid residue of the mature lipoprotein, with Asp and Ser as topological determinants for lipoproteins targeted for the inner and outer membranes, respectively. This hypothesis is supported by studies of Lpp::Bla hybrid proteins in which the localization of the lipo-β-lactamases is correlated with the nature of the second amino acid residue of the mature enzyme (Yamaguchi *et al.*, 1988). However, examination of the sequences shown in Table I has revealed a more complex picture. For example, NlpB is presumably the same protein as Nlp-3 of Ichihara *et al.* (1981) which is located in the inner membrane of the *E. coli* cell envelope, and the mature protein has an N-terminal sequence of Cys-Ser-Ser-Asp (P. Stragier, personal communication). Likewise, pullulanases from *K. pneumoniae* and *K. aerogenes* contain Asp at the second position of the mature enzymes, and they are found in the

outer membrane prior to the secretion into the medium in both *K. pneu-moniae* and in *E. coli* (Pugsley *et al.*, 1986; d'Enfert *et al.*, 1987; Katsuragi *et al.*, 1987). Moreover, a number of lipoproteins which are located in the outer membrane do not contain Ser at their second positions, such as the TraT protein (Cys-Gly) (Minkley, 1984), PulS protein (Cys-Gln) (d'Enfert and Pugsley, 1989), *B. licheniformis* β-lactamase (Cys-Ala) (expressed in *E. coli*, Sarvas and Palva, 1983), and BRP-β-lactamase (Cys-Gln) (Luirink *et al.*, 1987). Further confirmation of this provocative suggestion awaits additional characterization of lipoproteins known to be localized in the inner or outer membrane exclusively.

Biosynthesis of Lipoproteins in Bacteria

Murein lipoprotein is one of the most extensively studied proteins in *E. coli*, and most of our knowledge concerning the biosynthesis of bacterial lipoproteins is based on the studies of this protein. Both *in vivo* and *in vitro* studies led to the establishment of the biosynthetic pathway shown in Fig. 1. The protein is first synthesized as unmodified prolipoprotein which under-goes extensive covalent modifications before it is processed and translocated to the outer membrane. Diglyceride-prolipoprotein is processed by pro-lipoprotein signal peptidase (or signal peptidase II) to form apolipoprotein and the signal peptide, the latter being further degraded by the combined actions of membrane-bound and soluble peptidases (Suzuki *et al.*, 1987; Novak and Dev, 1988). Globomycin, a cyclic peptide antibiotic, specifically inhibits SPase II (Inukai *et al.*, 1978) by binding to this enzyme in a noncom-petitive manner with an apparent K_i of 36 nM (Dev *et al.*, 1985). The apolipo-protein is further modified at the α-NH_2 group of glyceride-cysteine residue with palmitate by the enzyme designated as N-acyl transferase to form mature free-form lipoprotein.

Modification of Prolipoprotein with Glycerol and Fatty Acids

Both *in vivo* and *in vitro* studies have revealed that the glycerol moiety in phosphatidylglycerol (PG) is transferred to the sulfhydryl group of the cysteine in prolipoprotein (Chattopadhyay and Wu, 1977; Chattopadhyay *et al.*, 1979; Tokunaga *et al.*, 1982). Likewise, studies using intact cells or crude cell envelope have led to the conclusion that both the ester-linked and the amide-linked fatty acids in lipoprotein are derived from the acyl moieties in phospholipids (Lai *et al.*, 1980; Tokunaga *et al.*, 1982). Fusion of [³H]pal-mitate-labeled phospholipid vesicles with intact cells of an *E. coli fad*D mutant defective in acyl CoA synthetase have provided further support for
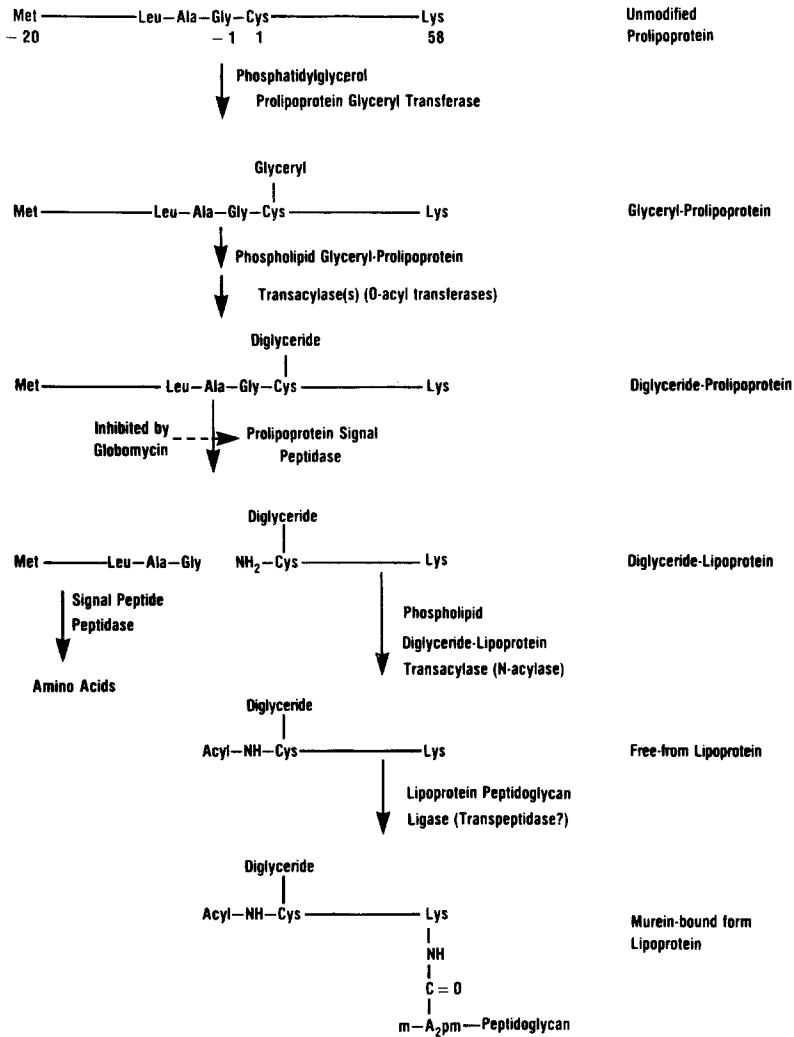
Fig. 1. Biosynthetic pathway of murein lipoprotein in *E. coli*. A$_2$pm, meso-diaminopimelic acid.

the hypothesis, and among the major glycerophosphatides in *E. coli* (PE, PG, CL), no specificity was observed regarding the efficacy of each individual phospholipid as the fatty-acyl donor (Lai and Wu, 1980).

The key step in the prolipoprotein modification pathway is the transfer of glyceryl moiety from phosphatidylglycerol to unmodified prolipoprotein. Recent work from Shibuya's and Dowhan's laboratories on the phenotype of null mutation in the *pgs*A gene (encoding phosphatidylglycerol phosphate synthetase) is interesting and relevant to lipoprotein maturation (Miyazaki

et al., 1985; Heacock and Dowhan, 1989). The *pgs*A3 allele isolated by Shibuya and his coworkers is lethal in a *lpp*⁺ host but not in a *lpp*⁻ (*lpo*) host. In fact, unknown to Shibuya, the wild-type strain from which they isolated the *pgs*A3 mutant is in fact *lpp*⁻. It appears that *E. coli* cells require a minute amount of acidic phospholipids (PG plus CL). The most plausible explanation for the lack of lethality of a *lpp*⁻ *pgs*A3 double mutant is that the lipoprotein defect spares the mutant cells from further depleting the low levels of acidic phospholipids as a result of prolipoprotein modification. This interpretation is supported by our observation that the *lpp*D14 *pgs*A3 double mutant containing unmodified mutant prolipoprotein is viable, and *pgs*A3 mutant containing P_{lac} *lpp*⁺ is IPTG-sensitive (Yamamoto and Wu, unpublished data).

An *in vitro* system has been developed for the modification and processing of prolipoprotein in *E. coli* (Tokunaga *et al.*, 1982). At least four of the enzyme activities involved in lipoprotein maturation were demonstrated in this system: glyceryl transferase, O-acyl transferase, SPase II, and N-acyl transferase. Recently, an *in vitro* transcription/translation/translocation system with inverted membrane vesicles was established to study prolipoprotein secretion *in vitro*. [³⁵S]Methionine-labeled unmodified prolipoprotein was first synthesized in a Zubay's system using *lpp* DNA or mRNA. The unmodified prolipoprotein was efficiently converted to diglyceride-modified prolipoprotein, apolipoprotein, and mature lipoprotein by the addition of inverted membrane vesicles (Tian *et al.*, 1989; Hayashi and Wu, unpublished data).

Processing of Glyceride-Modified Prolipoprotein by SPase II

The discovery of globomycin represents a major breakthrough in the biochemical studies of lipoprotein maturation. It provides the first clue that there exist at least two distinct signal peptidase in *E. coli*, SPase I and SPase II for nonlipoprotein and lipoprotein precursors, respectively. Accumulation of lipid-modified prolipoprotein in globomycin-treated cells provides the evidence that modification of prolipoprotein precedes the processing reaction. Both enzymes have been extensively purified (Wolfe *et al.*, 1982; Dev and Ray, 1984), and the genes encoding these two enzymes have been cloned and sequenced (Wolfe *et al.*, 1983; Tokunaga *et al.*, 1983; Innis *et al.*, 1984; Yamagata *et al.*, 1983; Yu *et al.*, 1984). The structural genes for SPase I (*lep*) and SPase II (*lsp*) have been located at the 55 min and 0.5 min of the *E. coli* map, respectively (Silver and Wickner, 1983; Regue *et al.*, 1984). The molecular weights of SPase I and SPase II as determined by SDS-PAGE are 37,000 and 18,000, respectively. The proposed topologies of SPase I and SPase II in the cytoplasmic membrane are shown in Fig. 2. SPase I is
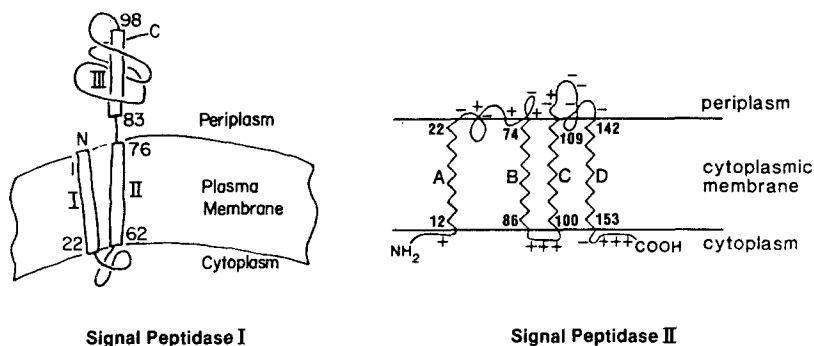
Signal Peptidase I   **Signal Peptidase II**

Fig. 2. Postulated transmembrane structure of signal peptidase I and signal peptidase II of
E. coli.

presumed to be anchored in the inner membrane by two hydrophobic segments
near the N-terminus with the bulk of the polypeptide localized in the peri-
plasm. This model has been supported by recent experiments using lep-phoA
gene fusions (Millan et al., 1989). SPase II is an inner membrane enzyme, and
its deduced amino acid sequence predicts a highly hydrophobic integral
membrane protein. There are four major hydrophobic domains (A–D) which
correspond to regions predicted to favor β-sheet conformation. Our working
model predicts that this enzyme contains four transmembrane segments, with
both its amino- and carboxy-termini residing inside the inner membrane.
Studies using Lsp-LacZ and Lsp-PhoA fusion proteins have provided sup-
port for this model; Lsp-PhoA fusions between domains A and B are highly
active for the PhoA activity, whereas Lsp-LacZ fusion between domain B
and C is highly active for the LacZ activity which is enriched in the cell
envelope fraction (Munoa, Goto, and Wu, unpublished data).

　　Determination of the nucleotide sequence of the lsp gene of E. coli
reveals that the lsp gene overlaps with ileS which encodes isoleucyl tRNA
synthetase (Innis et al., 1984; Yu et al., 1984). Analysis of the mRNA of ileS
and lsp genes indicate that the lsp gene in E. coli is part of a five-gene
mixed-functions operon consisting of x-ileS-lsp-orf149-orf316 (Miller et al.,
1987). Recent studies indicate that the same genomic organization of these
five genes exists in Enterobacter aerogenes (Isaki et al., 1990) and in Pseudo-
monas fluorescens (Isaki, Beers, and Wu, unpublished data). The physiologi-
cal significance of the organization of ileS and lsp with these three genes
of unknown functions into an operon in diverse bacterial species including
E. coli and P. fluorescens remains obscure.

　　The E. coli SPase II contains 164 amino acid residues, and the Enter-
obacter aerogenes enzyme consists of 165 amino acid residues with a 96%

amino acid sequence homology with the *E. coli* enzyme (Isaki *et al.*, 1990). The *P. fluorescens* SPase II is longer, consisting of 170 amino acid residues with 63% amino acid sequence homology with the SPase II from *E. coli*. The four putative transmembrane domains of the *E. coli* enzyme is also preserved in SPase II from *P. fluorescens* (Isaki, Beers, and Wu, unpublished data).

Apolipoprotein N-Acyl Transferase

Both *in vivo* and *in vitro* studies using intact cells or cell envelope fraction have led to the conclusion that the acyl donor for apolipoprotein N-acyl transferase is glycerophosphatides (Lai *et al.*, 1980; Tokunaga *et al.*, 1982). Based on *in vivo* studies of the turnover of 1-acyl moiety in phosphatidyl-ethanolamine, Jackowski and Rock (1986) have shown that the amide-linked fatty acid (predominantly palmitate) in lipoproteins is derived from the 1-acyl moiety of PE, and apolipoprotein is converted to the mature free-form lipoprotein with the concomitant formation of lyso-phosphatidylethanola-mine. Turnover of 1-acyl residue of PE required *de novo* synthesis of lipoproteins. This work has not only provided the clue for the nature of the acyl donor of the apolipoprotein N-acyl transferase, but has also clarified the function of the enzyme first identified by Cronan and his coworkers (Ray and Cronan, 1976; Rock and Cronan, 1979).

Mizushima and coworkers have shown that SPase II is thermostable while apolipoprotein N-acyl transferase is not (Hussain *et al.*, 1982). Based on this finding, we have established an *in vitro* assay for this enzyme (Gupta and Wu, unpublished data). Labeled apolipoprotein was prepared from diglyceride-modified prolipoprotein accumulated in globomycin-treated cell by incubation of the cell envelope for 45 min at 80°C. In a second incubation, apolipoprotein was converted to the mature free-form lipoprotein with detergent-solubilized cell envelope fraction. The development of an *in vitro* assay of this enzyme should facilitate further biochemical and genetic studies of this modification enzyme concerning the role of amide-linked fatty acid in the functions and assembly of bacterial lipoproteins.

While most of the studies on the modification and processing of lipopro-tein precursors have been performed with the *E. coli* major outer membrane lipoprotein, similar enzymes must exist in other bacteria including *B. subtilis*. The *E. coli lpp* gene has been expressed in *B. subtilis* resulting in the formation of mature-free form lipoprotein (Hayashi *et al.*, 1985). As in *E. coli* cells, the processing of lipid-modified prepenicillinase is inhibited by globomycin in *B. subtilis* (Hayashi and Wu, 1983). Furthermore, internalized signal sequence with the consensus sequence of Leu-Ala-Gly-Cys far removed from the N-terminus is modified and processed to form mature lipoprotein both in *E. coli* and in *B. subtilis* (Hayashi *et al.*, 1985). Thus the lipoprotein

maturation pathway is conserved among diverse groups of bacteria. On the other hand, this pathway is most likely absent in eukaryotic cells. *In vitro* translation and translocation of the *E. coli* Lpp :: β-lactamase hybrid protein with pancreatic microsomes have revealed that the precursor protein is processed by a signal peptidase at a site distinct from the authentic processing site without lipid modification (Garcia *et al.*, 1987). Thus, there is an apparent dichotomy between eukaryotic cells and prokaryotic cells with regard to both the structures of the lipid moieties and the nature of the covalent linkages in fatty acylated proteins. In eukaryotic cells, proteins are modified by N-myristoylation of N-terminal glycine by N-myristoyl trans-ferase (Towler *et al.*, 1987) which is an essential enzyme in yeast (Duronio *et al.*, 1989), by O- or S-acylation of membrane proteins with palmitate attached to threonine/serine or cysteine (Magee and Schlesinger, 1982), by attachment of preformed glycophospholipid to the C-terminal portion of membrane proteins by a putative transpeptidase (Ferguson and Williams, 1988), or by the attachment of a farnesyl moiety to the SH group of cysteine in a C-terminal Cys-Ali-Ali-Xaa sequence via a thioether linkage (Anderegg *et al.*, 1988; Casey *et al.*, 1989). In contrast, the modification of membrane proteins with diacylglycerol via thioether linkage to cysteine and with palmitate by an amide linkage is unique for prokaryotic cells. At the functional level, it is conceiva-ble that these lipid-modified proteins from both prokaryotic and eukaryotic cells share a common requirement of anchoring an otherwise hydrophilic polypeptide to the membrane via hydrophobic lipid moieties in a structurally diverse group of proteins in order to function at the membrane/aqueous interface.

Secretion of Lipoproteins in Bacteria

Like lipoprotein maturation, protein secretion in bacteria is a multistep process. In addition to the structural genes for SPase I and SPase II (*lep* and *lsp*, respectively), several genes have been identified whose functions are essential for protein secretion in general. These gene products include the SecA protein (Oliver and Beckwith, 1981), the SecB protein (Kumamoto and Beckwith, 1983), the SecD protein (Gardel *et al.*, 1987), the SecE protein (Schatz *et al.*, 1989) and the SecY protein (Ito *et al.*, 1983). Lipoproteins differ from nonlipoproteins in the nature of the signal peptidase as well as the preceding modification reactions unique for lipoprotein precursors. Since the processing of precursor proteins is a late event in protein secretion, it is likely that the initial events in protein secretion are common to lipoprotein and nonlipoprotein precursors alike. *In vivo* studies using conditionally lethal mutants defective in protein secretion in general (Hayashi and Wu, 1985;
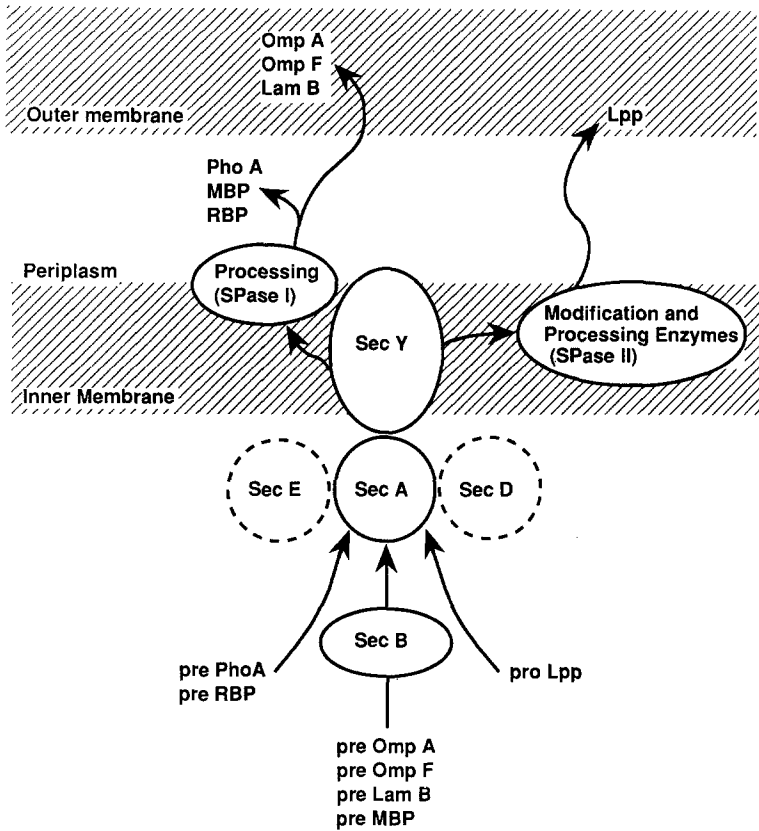
Fig. 3. A common secretory pathway for prolipoproteins and nonlipoprotein precursors in *E. coli.*

Watanabe *et al.,* 1988) have provided support for the model depicted in Fig. 3. The export of Braun's lipoprotein occurs normally in an *E. coli* *secB* :: Tn*5* mutant (Watanabe *et al.,* 1988). Thus the major outer membrane lipoprotein belongs to the so-called SecB-independent group of exported proteins which include ribose-binding protein and alkaline phosphatase. It implies that the export of prolipoprotein is so rapid that the antifolding activity of the SecB protein is not required (Collier *et al.,* 1988). On the other hand, the export of an OmpF :: Lpp hybrid protein, consisting of the OmpF signal sequence plus 11 amino acid residues of the mature OmpF protein followed by in-frame fusion of mature lipoprotein without the lipoprotein modification site, is affected by the *sec*B mutation (Watanabe *et al.,* 1988). This result is consistent with the notion that the SecB requirement in protein export is a function of the kinetics of folding of precursor proteins. It would

not be surprising if one of the many lipoproteins in bacteria would require the SecB protein for its export. Like all exported proteins, the export of lipoprotein requires functional SecA, SecY, and SecD proteins, and the synthesis of Lpp is reduced in a *secC* mutant (Hayashi and Wu, 1985; Watanabe *et al.*, 1988). Precursors which are totally defective in modification may be processed by signal peptidase I to form mature nonlipoproteins (Hayashi *et al.*, 1984; Hayashi *et al.*, 1986; Ghrayeb *et al.*, 1985). These data strongly suggest that the translocation of lipoproteins utilizes the same export machinery as nonlipoproteins. The export pathways for lipoproteins and nonlipoproteins are not compartmentalized in the cytoplasmic membrane, and all precursor proteins are exported through a common pathway accessible to both SPase I and SPase II. The divergence in the export process takes place in the cytoplasmic membrane due to the intervention of the highly efficient prolipoprotein modification and processing enzymes. The latter system is extremely efficient in *E. coli*, since kinetic studies have revealed rapid conversion of unmodified prolipoprotein to the mature form (Tokunaga and Wu, 1984). In *B. subtilis*, the kinetics of prolipoprotein modification and processing is much slower, with the intermediates in lipoprotein maturation readily detectable by steady-state labeling experiments (Hayashi *et al.*, 1985).

A highly efficient *in vitro* system to study translocation of precursor proteins into inverted membrane vesicles has been developed (Müller and Blobel, 1984; Chen *et al.*, 1985). This system has been recently extended to the studies of prolipoprotein export *in vitro* (Tian *et al.*, 1989). Unmodified prolipoprotein synthesized in a S-30 system of *E. coli* was incubated with inverted membrane vesicles from *E. coli* (Tian *et al.*, 1989) or *B. subtilis* (Hayashi and Wu, unpublished data). Based on both protection from protease digestion and the conversion of prolipoprotein to the mature lipoprotein, the overall translocation process is shown to require ATP hydrolysis, cytoplasmic translocation factors, and functional SecY protein. The initial step of prolipoprotein insertion into the membrane vesicles occurs without these apparent requirements, but is temperature-dependent. Using liposomes to study prolipoprotein insertion, it has been shown that prolipoprotein inserts most efficiently into liposomes made of negatively charged phospholipids. A role of acidic phospholipids in protein export in general has also been suggested (de Vrije *et al.*, 1988). Translocation of prolipoprotein into inverted membrane vesicles is also evidenced by the conversion of unmodified prolipoprotein in sequential reactions catalyzed by glyceryl transferase, O-acyl transferase, signal peptidase II, and N-acyl transferase to form the mature lipoprotein, and the various biosynthetic intermediates in the maturation of prolipoprotein are identified as distinct bands in SDS gel. Further studies using this *in vitro* system in conjunction with genetically defined mutants altered in one of the components required for lipoprotein

maturation or protein export will lead to a better understanding of the mechanism of lipoprotein export and maturation in bacteria.

Note Added in Proof

Two putative lipoproteins from *Borrelia burgdorferi* have been reported to have deduced amino acid sequences as shown below (Bergström *et al.*, 1989):

OspA   MK$^+$K$^+$YLLGIGLILALIACK$^+$QNV

OspB   MR$^+$LLIGFALALALIGCAQK$^+$G

Acknowledgment

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