Journal of Cellular Biochemistry 24:113–120 (1984)
Protein Transport and Secretion 165–172

A Distinct Signal Peptidase for Prolipoprotein in Escherichia coli

Masao Tokunaga, Judith M. Loranger, and Henry C. Wu

Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

We have previously demonstrated the modification and processing of Escherichia coli prolipoprotein (Braun's) in vitro (Tokunaga M, Tokunaga H, Wu HC: Proc Natl Acad Sci USA 79:2255, 1982). Using this in vitro assay of prolipoprotein signal peptidase and globomycin selection, we have isolated and partially characterized an E coli mutant which contained a higher level of prolipoprotein signal peptidase activity. In contrast, the procoat protein signal peptidase activity was not increased in this mutant as compared to the wild-type strain. Furthermore, E coli strains containing cloned procoat protein signal peptidase gene were found to contain elevated levels of procoat protein signal peptidase, but normal levels of prolipoprotein signal peptidase. These two signal peptidase activities were also found to exhibit different stabilities during storage at 4°C. Thus biochemical, immunological, and genetic evidence clearly indicate that prolipoprotein signal peptidase is distinct from procoat protein signal peptidase in E coli.

Key words: signal peptidase, protein secretion, lipoprotein, globomycin, posttranslational modification and processing

Most, if not all, outer membrane and periplasmic proteins in E coli are first synthesized with amino-terminal signal sequences. This peptide is removed by the so-called signal (leader) peptidase during or immediately after the translocation of the exported protein across the cytoplasmic membrane. One of this group of unique endopeptidases responsible for the processing of precursor proteins, the M13 procoat protein signal peptidase or SPase I, has been purified from E coli and extensively characterized by Wickner and his co-workers [1–4]. The molecular weight of SPase I was found to be 39,000 [1]. In addition to M13 procoat protein, this enzyme can also process several other precursor forms of outer and periplasmic proteins [3].

The murein lipoprotein of E coli contains a unique structure, N-acyl diglyceride-cysteine, at its N terminus [5]. Murein lipoprotein is first synthesized as a precursor protein, prolipoprotein [6]. The biogenesis of the mature lipoprotein involves successive modification and processing reactions. In vitro studies have shown that the

Abbreviations: SPase II, prolipoprotein signal peptidase; SPase I, procoat protein signal peptidase.

Received September 8, 1983; accepted September 28, 1983.

© 1984 Alan R. Liss, Inc.

processing of prolipoprotein by signal peptidase requires prior modification of prolipoprotein with glycerol or glyceride [7]. This novel requirement for the prolipoprotein signal peptidase (SPase II) suggests that glyceride-modified cysteine and its neighboring amino acid sequence constitute a unique recognition site for SPase II. The characterization of SPase II is essential to the understanding of the biosynthesis and assembly of lipoproteins in bacteria.

In this paper we describe the isolation of E coli mutants which overproduce SPase II. Thus, both biochemical and genetic evidence strongly indicate that there are at least two distinct signal peptidases in E coli, SPase I (for nonlipoproteins) and SPase II (specific for lipoproteins).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Media

Bacterial strains JA200 ($F^+ \triangle$*trp*E5, *rec*A, *thr*, *leu*, *lac*Y), SM31 (F^-, *sup*E, *ton*A, *thr*, *leu*, rk^-, mk^+, *rec*BC) and JE5505 (F−, *lpo*, *pps*, *his*, *pro*A, *arg*E, *thi*, *gal*, *lac*, *xyl*, *mtl*, *tsx*) were used. From Clark and Carbon's collection [8], JA200 (pLC7-46) and JA200 (pLC7-47) were used. JA200 (pTD101) containing cloned procoat protein signal peptidase gene [2] was provided by W. Wickner.

L-Broth, PPBE (proteose peptone beef extract) broth and M9-0.2% glucose minimal medium were used throughout this study. Ampicillin (30 μg/ml for agar plate, 100 μg/ml for liquid culture) was used for JA200 (pTD101).

Assay of Signal Peptidases

Prolipoprotein signal peptidase (SPase II) was measured in a reaction mixture (10 μl) containing glyceride-modified prolipoprotein (20,000 cpm, prepared according to [9]), 0.25% Nikkol, 50 mM Tris-HCl buffer (pH 7.4), 0.25% β-mercaptoethanol, and cell envelope fraction as the enzyme source.

Procoat protein signal peptidase (SPase I) was assayed in a reaction mixture (10 μl) containing ^{35}S-methionine-labeled procoat protein (10,000 cpm, provided by W. Wickner), 1% Triton X-100, 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$ and cell envelope fraction as the enzyme source.

Procedures used for the termination of enzyme reactions, SDS-polyacrylamide gel electrophoresis and SDS/urea-polyacrylamide gel electrophoresis, were described previously [9].

Quantitative Determination of Globomycin Sensitivity of E coli Strains

Overnight cultures in L-broth were diluted ten times with the same media and 5 μl of the diluted cultures were inoculated into Linbro wells containing 45 μl of L-broth and various amounts of globomycin. After a 5- to 10-hr incubation at 37°C, the turbidity of the cells in each microtiter well was either estimated by visual inspection or measured with a spectrophotometer.

Chemicals

^{35}S-Cysteine (942.2 Ci/mmol) and Nikkol (octaethylene glycol mono-n-dodecyl ether) were obtained from New England Nuclear and Nikko Chemical (Tokyo), respectively. Globomycin was a generous gift from M. Arai (Sankyo, Tokyo). Other chemicals were of the best grade commercially available.

RESULTS

In Vitro Assay of Prolipoprotein Signal Peptidase

The maturation of murein lipoprotein requires four successive modification and processing reactions—modification of cysteine residue with glycerol and with O-acyl groups, processing of signal peptide, and acylation of the amino group of glyceride-cysteine. We have succeeded in demonstrating all these modification and processing reactions of prolipoprotein in vitro, using (2-^{3}H)glycerol-labeled JE5505 cell envelope (lacking murein-lipoprotein [10]) and ^{35}S-cysteine-labeled MM18 cell envelope (containing unmodified prolipoprotein) as the donor and acceptor of glyceryl moiety, respectively (Fig. 1-1) [7]. By using this in vitro enzyme assay, we have shown that the processing of prolipoprotein with SPase II requires prior modification of prolipoprotein with glycerol or glyceride [7]. The experiments are shown schematically in Figure 2. At pH 9.0, glyceryl transferase and SPase II are both active so that unmodified prolipoprotein was modified and processed to lipoprotein. However, at pH 5.0, glyceryl transferase is inactive so that no modification (and consequently, no processing) took place, even though SPase II is active at pH 5.0.

The cyclic antibiotic globomycin was reported to inhibit SPase II activity in vivo [11] and in vitro [7] and resulted in the accumulation of glyceride-modified prolipoprotein in globomycin-treated cells [12]. We have purified glyceride-modified prolipoprotein by immunoprecipitation with antilipoprotein antiserum as the substrate

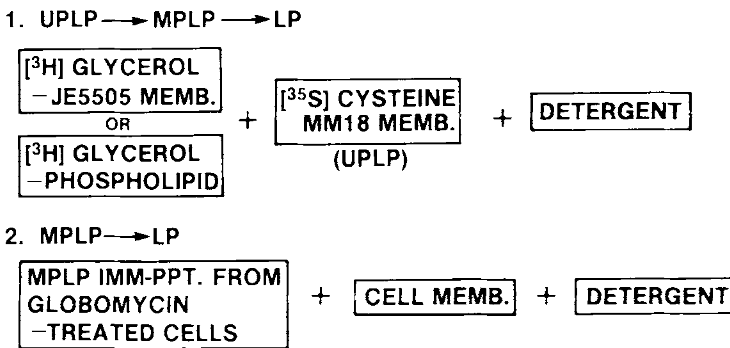

Fig. 1. In vitro assays for the modification and processing of prolipoprotein. UPLP, unmodified prolipoprotein; MPLP, modified prolipoprotein; LP, lipoprotein.

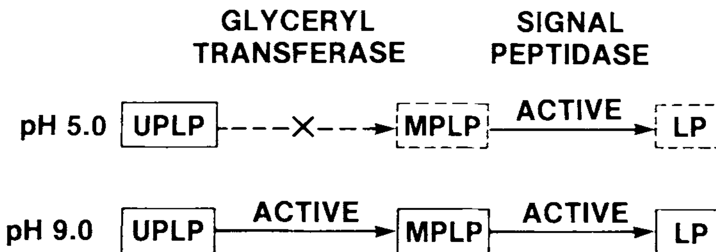

Fig. 2. Processing of prolipoprotein requires prior modification of cysteine residue by glyceride.

of SPase II and have established an in vitro assay for SPase II activity (Fig. 1-2) [9]. The fact that SPase II requires prior modification of prolipoprotein by glyceride and that SPase II activity, but not SPase I, is inhibited by globomycin suggests that SPase II is distinct from SPase I.

Isolation of E coli Mutant Overproducing Prolipoprotein Signal Peptidase

It is reasonable to assume that E coli mutants containing increased levels of SPase II would exhibit increased resistance to globomycin. E coli strain SM31 was grown in PPBE-broth containing 50–100 μg of globomycin, and several spontaneous globomycin-resistant mutants were obtained. Among 20 globomycin-resistant mutants, we found two mutants, SM31-2B4 and SM31-1D5, containing higher SPase II activities in the in vitro SPase II assay (Fig. 3). The globomycin sensitivity and SPase II activity of SM31-2B4, as compared with those of parental strain SM31, are shown in Table I. Mutant SM31-2B4 cells grew in the presence of 120 μg/ml of globomycin and were found to contain three times higher SPase II activity than that of the parental strain SM31.

Genetic Evidence That Prolipoprotein Signal Peptidase Is Distinct From Procoat Protein Signal Peptidase

The mutant SM31-2B4 was found to contain three times higher SPase II activity. As shown in Figure 4, the SPase I activity was present in the same level in SM31-2B4 as that of the parental strain SM31.

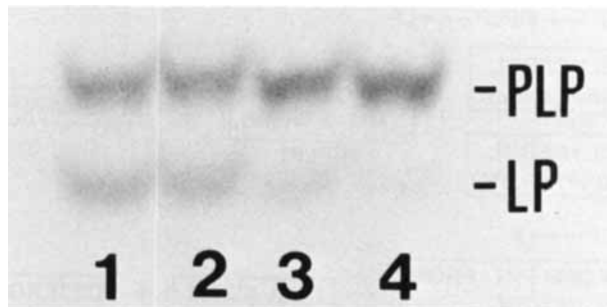

Fig. 3. Isolation of prolipoprotein signal peptidase-overproducing mutants. Cells were grown in L-broth overnight and cell envelope fraction was prepared for SPase II assay. Three micrograms of membrane protein were used. Lane 1, SM31-2B4; lane 2, SM31-1D5; lane 3, SM31; lane 4, no enzyme control.

TABLE I. The Globomycin Sensitivity and Prolipoprotein Signal Peptidase Activity in Mutant SM31-2B4

Strain	Globomycin[a] (μg/ml)	Prolipoprotein[b] signal peptidase (unit)
SM31	20	0.156
SM31-2B4	120	0.470

[a]Maximum globomycin concentrations which allow bacterial growth.
[b]Cell envelope fraction (5 μg protein) was used as the enzyme source.

The strains containing plasmid pLC7-47 or pTD101 have been reported to overproduce SPase I [2]. SPase II and SPase I activities were measured in the cell envelope fraction of E coli JA200 harboring plasmids pLC7-47 or pTD101 (Fig. 5). As shown in Figure 5, SPase II activities were not elevated in the strains containing plasmids pLC7-47 or pTD101, whereas SPase I activities were clearly elevated. These data indicate that the structure genes for SPase II and SPase I are distinct and not very closely linked. Recently, we have succeeded in the cloning [13] and mapping of the structural gene for SPase II *(lsp)* (Regue et al, manuscript in preparation). The *lsp* gene is present in pLC3-13 of the Carbon-Clarke collection of E coli genomic library [8] and is located at 0.5 min of the E coli chromosome. In contrast, the structural gene for SPase I *(lep)* is contained in pLC 7-47 [2] and located at 54 min of the E coli chromosome [4].

Several lines of biochemical data also suggest that SPase II is distinct from SPase I. Figure 6 shows the stability of signal peptidase activities upon storage at 4°C. SPase II activity was less stable than SPase I activity in the JE5505 cell envelope solubilized with 0.25% Nikkol during storage at 4°C. The difference of enzyme stability between SPase II and SPase I further suggests that the SPase II activity is

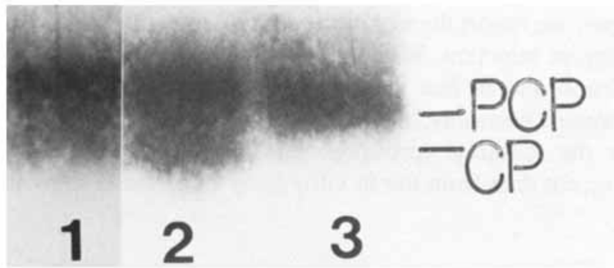

Fig. 4. Procoat protein signal peptidase activity in mutant SM31-2B4. Cell envelope fraction was prepared from overnight culture and used for procoat protein signal peptidase assay. One microgram of membrane protein was used. PCP, procoat protein; CP, coat protein. Lane 1, SM31-2B4; lane 2, SM31; lane 3, no enzyme control.

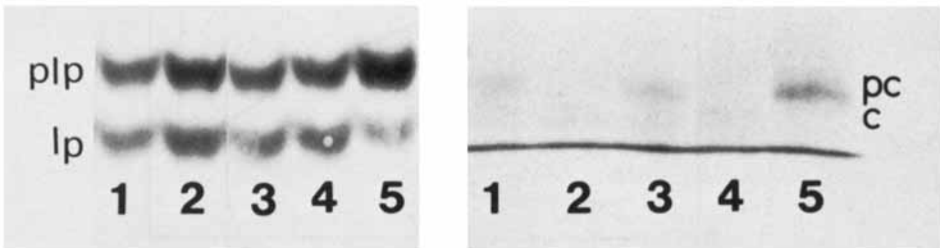

Fig. 5. Prolipoprotein signal peptidase and procoat protein signal peptidase activities in procoat protein signal peptidase-overproducing strains. Cell envelope fraction was prepared and used for prolipoprotein signal peptidase assay (3 μg protein) and for procoat protein signal peptidase assay (1 μg protein). JA200 (pLC7-46) was used as a control for the JA200 (pLC7-47). Lane 1, JA200 (pLC7-46); lane 2, JA200 (pLC7-47); lane 3, JA200; lane 4, JA200 (pTD101); lane 5, no enzyme control; plp, Prolipoprotein; lp, lipoprotein; pc, procoat protein; c, coat protein.

present on different molecules from those of SPase I. Table II summarizes the biochemical, genetic, and immunochemical data which suggest that SPase II is distinct from SPase I.

DISCUSSION

Many important observations about the biogenesis of lipoprotein have rapidly accumulated since the discovery of the novel cyclic antibiotic, globomycin. Globomycin was first discovered by Arai and his co-workers [14] as a specific inhibitor of the processing of prolipoprotein [11]. Mizushima and his co-workers have reported that 1) prolipoprotein accumulated in globomycin-treated cells contain glyceride-modified cysteine [12], and 2) there are several "new" lipoproteins in E coli cell envelope that are structurally distinct from murein lipoprotein [15]. Giam et al [16] and Wu et al [17] reported the isolation of E coli mutants containing structure-altered lipoproteins by globomycin selection. In addition, Lai et al [18] isolated globomycin-resistant mutants which either lack murein lipoprotein or contain modification- and processing-deficient prolipoproteins. Finally, Yamagata et al [19] obtained a mutant that contains temperature-sensitive SPase II by the selection of temperature-sensitive globomycin, followed by the isolation of temperature-resistant revertants.

In this paper, we report the isolation of E coli mutants which overproduce SPase II by the globomycin selection. While globomycin has provided us a strong selection for mutant isolation and in fact globomycin has been extensively employed in the studies of lipoprotein assembly, the precise interaction between globomycin and its target(s), either the substrate (prolipoprotein) or the enzyme (SPase II), remains unknown. Our recent data from the in vitro assay of SPase II show that a molar ratio

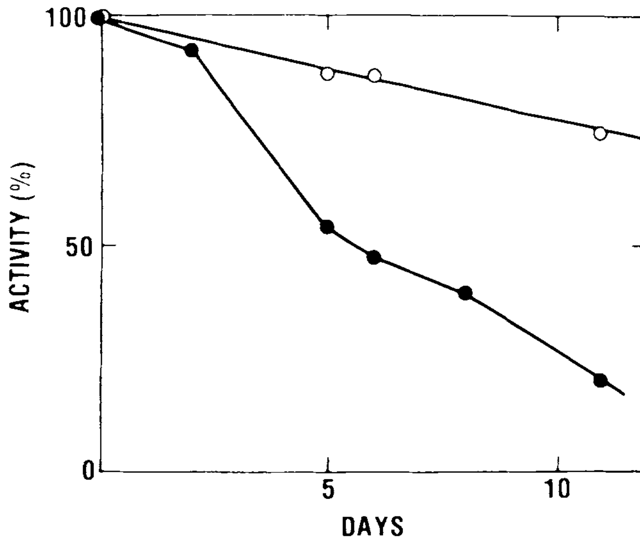

Fig. 6. Stability of prolipoprotein signal peptidase and procoat protein signal peptidase in storage at 4°C. JE5505 cell envelope fraction was solubilized with 50 mM Tris-HCl buffer (pH 7.4) and 0.25% Nikkol and stored at 4°C for 11 days. Prolipoprotein signal peptidase (●) and procoat protein signal peptidase (○) activities were measured.

TABLE II. Comparison of Prolipoprotein Signal Peptidase and Procoat Protein Signal Peptidase

Properties	Prolipoprotein signal peptidase (SPase II)	Procoat protein signal peptidase (SPase I)
Stability at 4°C	Unstable	Stable
Activity at pH 5.0	Active	Inactive
Globomycin	Sensitive	Insensitive
Antiprocoat protein signal peptidase antiserum	Not reacted	Reacted
Subcellular localization	Inner membrane	Inner and outer membrane
Cleavage site	Glycylglyceride cysteine	Alanine-X
Activity in SM31-2B4	Overproduced	Normal
Activity in JA200 (pTD101)	Normal	Overproduced
Activity in JA200 (pLC 7-47)	Normal	Overproduced
Elution from chromatofocusing	pH 6.5	pH 6.0
Map position of structural gene	0.5 min *(lsp)*	54 min *(lep)*

of globomycin/substrate of 1:400 results in the half inhibition of SPase II activity (manuscript in preparation). These data suggest that globomycin binds to the enzyme rather than the substrate. More definitive information awaits the kinetic studies of the purified SPase II.

The original observation by Inukai et al [11] that globomycin inhibited the processing of prolipoprotein but not that of other major outer membrane proteins provides the first indication that the SPase II is unique from that for other secreted proteins. We have now been able to clearly distinguish SPase II from SPase I by the biochemical, immunological, and genetic data summarized in Table II.

ACKNOWLEDGMENTS

We are most grateful to Dr W. Wickner for the gift of ^{35}S-methionine-labeled procoat protein and strain JA200 (pTD101), and Dr M. Arai for the gift of globomycin.

This investigation was supported by United States Public Health Service grant GM-28811 and American Heart Association grant 81-663.

REFERENCES

1. Zwizinski C, Wickner W: J Biol Chem 255:7973, 1980.
2. Date T, Wickner W: Proc Natl Acad Sci USA 78:6106, 1981.
3. Wolfe PB, Silver P, Wickner W: J Biol Chem 257:7898, 1982.
4. Silver P, Wickner W: J Bacteriol 154:569, 1983.
5. Braun V: Biochim Biophys Acta 415:355, 1975.
6. Inouye S, Wang S, Sekizawa J, Halegoua S, Inouye M: Proc Natl Acad Sci USA 74:1004, 1977.
7. Tokunaga M, Tokunaga H, Wu HC: Proc Natl Acad Sci USA 79:2255, 1982.
8. Clarke L, Carbon J: Cell 9:91, 1976.
9. Tokunaga M, Loranger JM, Wolfe PB, Wu HC: J Biol Chem 257:9922, 1982.
10. Hirota Y, Suzuki H, Nishimura Y, Yamada S: Proc Natl Acad Sci USA 74:1417, 1977.

11. Inukai M, Takeuchi M, Shimizu K, Arai M: J Antibiot 31:1203, 1978.
12. Hussain M, Ichihara S, Mizushima S: J Biol Chem 255:3707, 1980.
13. Tokunaga M, Loranger JM, Wu HC: J Biol Chem 258:12102, 1983.
14. Nakajima M, Inukai M, Haneishi T, Terahara A, Arai M, Kinoshita T, Tamura C: J Antibiot 31:426, 1978.
15. Ichihara S, Hussain M, Mizushima S: J Biol Chem 256:3125, 1981.
16. Giam CZ, Hayashi S, Wu HC: Annual Meeting of the American Society for Microbiology, 1983, p 189.
17. Wu HC, Lai JS, Hayashi S, Giam CZ: Biophys J 37:307, 1982.
18. Lai JS, Philbrick WM, Hayashi S, Inukai M, Arai M, Hirota Y, Wu HC: J Bacteriol 145:657, 1981.
19. Yamagata H, Ippolite C, Inukai M, Inouye M: J Bacteriol 152:1163, 1982.