Mechanism of Signal Peptide Cleavage in the Biosynthesis of the Major Lipoprotein of the Escherichia coli Outer Membrane*

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On treatment of Escherichia coli cells with globomycin, a glyceride-containing precursor of the major outer membrane lipoprotein accumulates in the cytoplasmic membrane (Hussain, M., Ichihara, S., and Mizushima, S. (1980) J. Biol. Chem. 255, 3707-3712). When the envelope fraction from such cells was incubated in a suitable buffer, this precursor could be processed to the mature lipoprotein. The processing involved removal of the signal peptide and subsequent acylation of the NH2 terminus thus bared. Two types of peptidase and an acylation enzyme(s) were found to be involved in these processes. The enzyme that cleaves the signal peptide, called signal peptidase in this paper, had many unique properties: being highly resistant to high temperature, having a wide optimum pH range, and being highly sensitive to detergents. The other peptidase(s), called signal peptide peptidase in this paper, was assumed to be responsible for the digestion of the signal peptide that had been cleaved from the precursor lipoprotein. This enzyme was rather heat-sensitive. Thus the processing from the precursor to the mature lipoprotein at a high temperature resulted in accumulation of a peptide that was most probably the intact signal peptide. The third enzyme(s) involved in the processing was the one that is responsible for acylation of the newly exposed NH2-terminal of the lipoprotein. The enzyme activity was also lost at 80 °C. In the light of these findings, the biosynthetic pathway of the lipoprotein is discussed.

The envelope of Gram-negative bacteria consists of cytoplasmic and outer membranes with a network of peptidoglycan in between (1). The most abundant outer membrane protein, numerically, in Escherichia coli, is a lipoprotein discovered by Braun et al. (2, 3). In this paper this lipoprotein is called Braun's lipoprotein. In the outer membrane it is present in two different forms, one-third as a bound form and the other two-thirds as a free form (4, 5). The BLP1 of E. coli is first synthesized as a precursor form that possesses a signal peptide of 20 amino acid residues extending from the NH2-terminus of BLP (6). The BLP consists of 58 amino acid residues with cysteine at the NH2 terminus. This cysteine residue is linked to diglyceride through the thiol group and to fatty acid through the terminal amino-group (3).

We have reported that on treatment of E. coli cells with a cyclic peptide antibiotic called globomycin (7, 8), a precursor form of BLP, termed pro-BLP in this paper, accumulated in the cytoplasmic membrane (9). The cytochrome residue of this pro-BLP had already been modified by a glyceride similar to that of pro-BLP. This pro-BLP thus accumulated could be chased in vitro to BLP. This in vitro processing would involve 3 changes, namely, (i) cleavage of the signal peptide from the pro-BLP, (ii) digestion of the cleaved signal peptide, and (iii) acylation of the amino-group of the newly exposed NH2-terminal cytochrome residue.

Using this in vitro processing system we tried to characterize the enzymes involved. We have found that at least two types of peptidase are involved in the processing of pro-BLP: an enzyme which carries out cleavage of the signal peptide (signal peptidase) and an enzyme which carries out digestion of the cleaved signal peptide (signal peptide peptidase). We have also been able to show the presence of a third enzyme which is responsible for acylation of the newly exposed NH2-terminal cytochrome residue. Furthermore, we have identified the signal peptide released from pro-BLP. These findings help in understanding the mode of export, processing, and assembly of lipoproteins in E. coli cells.

EXPERIMENTAL PROCEDURES

Materials—Globomycin was a gift from Dr. M. Arai of Sankyo Co. Ltd. L-[5(n)H]Arginine (specific activity, 20 Ci/mmol), L-[U-14C]arginine (specific activity, 345 mCi/mmol), L-[U-14C]leucine (specific activity, 342 mCi/mmol), L-[35S]methionine (specific activity, 1455 Ci/mmol), and [9,10(2)H]palmitic acid (specific activity, 590 mCi/mmol) were purchased from Amersham International Ltd. Acrylamide was from Eastman Kodak Co. and Nikkô BL 9EX (polyoxyethylene lauryl ether) was from Nikko Chemicals Ltd., Tokyo. The following protease inhibitors were a gift from Dr. T. Aoyagi of the Institute of Microbial Chemistry: antipain, leupeptin, chymostatin, elastatinal, pepstatin, bestatin, and phosphoramidon. All other reagents were of reagent grade.

Bacterial Strain and Media—E. coli B (the wild type strain) was used throughout the study. Cells were grown in M-9/glucose (0.2% w/v) medium at 37 °C with shaking.

Preparation of Envelope Fractions—To exponentially growing cells (3 x 108 cells/ml) was added a 0.5% (w/v) solution of globomycin in methanol to a final concentration of 5 µg/ml. After 5 min, [3H]arginine (2 µCi/35 nmol/ml), [14C]arginine (2 µCi/6 nmol/ml), [14C]leucine (2 µCi/6 nmol/ml), or [35S]methionine (5 µCi/4 pmol/ml) was added. For double labeling, [14C]arginine (0.1 µCi/35 nmol/ml) and [3H]palmitic acid (5 µCi/9 nmol/ml) were added simultaneously. After 15 min of labeling, cells were chilled and harvested. Envelope fractions were prepared as described by Inouye and Guthrie (10). An in vitro chase was performed as described earlier (9). After the chase, samples labeled with [14C]arginine and [3H]palmitic acid were washed 3 times with chloroform/methanol (2:1) as described (5).

Immunoprecipitation—Envelope fractions were solubilized in 1% SDS and immunoprecipitated with anti-BLP antisera as described by Hagleoua et al. (11).


**RESULTS**

**In Vitro Conversion of Pro-BLP to BLP—**It has previously been shown that pro-BLP that accumulates in the envelope fraction of *E. coli* cells labeled with [3H]arginine. As the numbers of arginine in pro-BLP and BLP are the same, quantitative conversion of pro-BLP to BLP was seen more clearly. For the conversion of pro-BLP to BLP, removal of globomycin from the envelope fraction was essential. When the envelope fraction was incubated in a small amount of buffer (e.g., envelope fraction from 8 x 10⁶ cells in 10 μl) at 37 °C, the conversion did not take place even after repeated washing of the envelope fraction at 4 °C. On the other hand, the peptidase reaction took place when the envelope fraction was incubated in a large volume of buffer (e.g., envelope fraction from 8 x 10⁶ cells in 2 ml) even at 37 °C. The results can be interpreted as follows. The interaction of globomycin with the envelope is so strong at lower temperatures that the drug cannot be removed even after repeated washing, while the interaction is weakened at an elevated temperature. Therefore, incubation of the envelope fraction in a large volume of buffer at a higher temperature stimulates the dissociation of the drug from the envelope, which in turn, initiates the peptidase reaction. As described later, we found that the peptidase was active at a temperature as high as 80 °C. At this temperature the reaction took place even when the envelope fraction was suspended in a small volume of buffer. This may be explained by efficient removal of the drug from the envelope at the extremely high temperature.

In order to isolate the peptidase(s) responsible for the removal of the signal peptide, solubilization of the activity with detergents was attempted. Hereafter this enzyme will be tentatively called signal peptidase. The envelope fraction was mixed with a detergent in 100 mM Tris-Cl buffer (pH 7.1), incubated at 30 °C for 30 min, and examined on polyacrylamide gel for the processing. However, the treatment with the following detergents resulted in complete inactivation of the signal peptidase activity; 0.1% SDS, 2% sodium deoxycholate, 0.5% sodium sarcosinate, 2% Triton X-100, and 0.5% Nikkol. The activity could not be restored even when the detergent-treated envelope fraction was dialyzed against 10 mM Tris-Cl, 5 mM MgCl₂, 0.02% NaN₃ (pH 8.0) at 25 °C for 2 days to remove the detergent according to the method of Yamada and Mizushima (17). The latter experiment was not carried out with Nikkol or Triton X-100, since these detergents are hardly removed by dialysis. Therefore, the following studies on the signal peptidase activity were carried out using the intact envelope. The envelope fraction could be stored in an ice box for at least a week or at −80 °C for more than a month without loss of the peptidase activity.

**Effect of pH on the Signal Peptidase Reaction—**The effect of pH on removal of the signal peptide upon incubation of the envelope fraction is shown in Fig. 2. It is notable that the processing took place to almost the same extent over the pH range of 4.0 to 11.0. Although the processing did not take place at pH 1.0 or 2.3, the activity was restored when the envelope fraction was resuspended in 10 mM sodium phosphate buffer at pH 7.0. Contrary to this, treatment at an extreme alkaline pH resulted in irreversible inactivation of the peptidase reaction.

**Effect of Temperature on the Signal Peptidase Reaction—**It is evident from Fig. 3 that the processing activity of the envelope fraction was stable over a broad temperature range. Even at 80 °C the envelope fraction showed considerable activity. The enzyme activity was totally lost on incubation at 96 °C for 2 min. There was no processing below 20 °C either. It is uncertain whether the lack of processing below 20 °C is due to inactivity of the enzyme or to incomplete dissociation of globomycin from the envelope. The processing activity was inhibited by globomycin at all temperatures. This indicates that the processing of pro-BLP at higher temperatures was

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**FIG. 1.** *In vitro* chase of pro-BLP to BLP. Cells of *E. coli* B were grown in M-9/glucose (0.2% w/v) medium and labeled with [3H]arginine. Envelope fractions were solubilized in 10 mM sodium phosphate buffer (pH 7.1), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol at 100 °C for 5 min, and analyzed on SDS-polyacrylamide gel (9). Slot 1 was a control sample without globomycin treatment. The envelope fraction from globomycin treated cells (8 x 10⁶ cells) (slot 2) was washed with and suspended in 2 ml of 10 mM sodium phosphate buffer (pH 7.1) at 4 °C and incubated at 37 °C for 2 h in the absence (slot 3) or presence (slot 4) of globomycin (5 μg/ml). After incubation, the envelope fraction was pelleted at 100,000 x g for 30 min and analyzed by gel electrophoresis as mentioned above. The gel was processed for fluorography. Each slot contained 30,000 cpn. The x-ray film was exposed for 3 days. The positions of BLP and pro-BLP are indicated.

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**SDS-Polyacrylamide Gel Electrophoresis—** Gel electrophoresis in 0.5% SDS with 10% (w/v) acrylamide gels was carried out as described (9). Sometimes 17.5% (w/v) acrylamide gel was used as described by Anderson et al. (12). Fluorography was performed as described by Bonner and Laskey (13). Kodak X-OMAT R films were exposed to an electronic flashlight immediately before use (14). After developing the x-ray films, densitometric tracing was done with a Joyce Loebel densitometer at 610 nm (filter 5-042). The relative amount of protein (radioactivity) was estimated from the weight of the peak on the densitometric tracing. The radioactivity was counted in a liquid scintillation counter immediately before use.

**Other Methods—** Protein was estimated by the method of Lowry et al. (15). Edman degradation of anti-BLP immunoprecipitates was carried out as described (9). For direct determination of radioactivity, gel regions located by fluorography were cut out and digested in NCS tissue solubilizer (American International Ltd.), and radioactivity was counted in a liquid scintillation counter as described (15).
not merely a chemical reaction but was catalyzed by an enzyme.

It should be stressed here that the peptidase reaction was assayed using an envelope fraction that had an organized membranous structure, and the substrate as well as the enzyme are proteins. Therefore, inactivation of the peptidase activity does not necessarily mean inactivation of the enzyme itself. It may also be due to denaturation of the substrate (pro-BLP) or the membrane structure.

Effect of Inhibitors on the Signal Peptidase Reaction—The signal peptidase activity in the envelope fraction was not affected by any of the protease inhibitors tested. The latter included 5 mM antipain, 5 mM leupeptin, 5 mM chymostatin, 5 mM elastatin, 2.5 mM pepstatin, 2 mM bestatin, 0.5 mM amastatin, 5 mM phosphoramidon, 25 mM benzamidine, 5 mM $N^\prime$-p-tosyl-L-lysine chloromethyl ketone and 1.7% (w/v) soybean trypsin inhibitor. The peptidase activity was also not affected by 0.4 mM N-ethylmaleimide or 1 mM p-chloromercuri-benzenesulfonyl acid.

Acylation of the Terminal Amino Group of BLP after Signal Peptidase Cleavage—The mature form of BLP contains three fatty acid residues at the NH$_2$-terminal glycerclycteine, two being ester-linked to glycerol and the other being amido-linked to the terminal amino-group (3). It is known that the pro-BLP that accumulates in the globomycin-treated cells contains the ester-linked fatty acids at the glycerclycteine residue, the residue that becomes the NH$_2$ terminus of BLP (9). To determine whether the BLP formed by _in vitro_ processing can also be acylated at the newly exposed terminal amino group, the increase of acyl residues during the conversion of pro-BLP to BLP was measured. Cells were treated with globomycin and labeled with both $[^3H]$palmitic acid and $[^14C]$arginine. As the numbers of arginine in pro-BLP and BLP are the same, $[^14C]$arginine was used as an internal standard for BLP. In _E. coli_ B, the palmitic acid contents in the amido-linked and ester-linked fatty acids are about 65% and 45%, respectively (18). Provided that $[^3H]$palmitic acid is not converted to other species of fatty acid, it is assumed that if acylation of the NH$_2$ terminus takes place, the $[^3H]/[^14C]$ ratio should increase by 70% upon conversion of pro-BLP to BLP. The envelope fraction was isolated and incubated at 37 or 80°C as described in the legend to Fig. 4. The envelope fraction was then delipidated with chloroform/methanol (2:1), solubilized in 1% SDS and immunoprecipitated with antiserum against BLP. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and a fluorogram was taken (Fig. 4A). Samples which were not incubated (slot 2) or incubated in the presence of globomycin (slots 6 and 8) contained pro-BLP only. Incubation at 37°C for 10, 20, and 60 min gave rise to increasing amounts of BLP as expected (slots 3, 4, and 5). Incubation at 80°C for 7 min also gave rise to a substantial amount of BLP (slot 7). Although lipopolysaccharide, another fatty acid-containing molecule, should give a characteristic band, if it exists, at a position near that of pro-BLP in the gel system employed here (19), such a band can not be seen in Fig. 4, indicating that these immunoprecipitated samples were not contaminated by lipopolysaccharide. The bands of pro-BLP and BLP were cut out from the gel, digested, and the $[^3H]$ and $[^14C]$ counted. No significant changes were observed in the $[^3H]/[^14C]$ ratio of pro-BLP during the incubation, while a nearly 50% increase in the $[^3H]/[^14C]$ ratio of the corresponding BLP bands was observed upon conversion from pro-BLP at 37°C, suggesting that the terminal NH$_2$ group of BLP thus formed had been modified by fatty acid. In contrast to the increase in $[^3H]/[^14C]$ ratio upon the processing at 37°C, the ratio did not increase at 80°C (Fig. 4A, slot 7). This indicates that at 80°C, cleavage of the signal peptide was not accompanied by acylation of the NH$_2$ terminus. This further suggests that the cleavage of the signal peptide and the acylation of the new NH$_2$ terminus is a 2-step process, the signal peptidase being active at 80°C, and the acylation enzyme(s) being inactive at this temperature.

Difference between BLP Formed at 37°C and That Formed at 80°C—The results mentioned above suggest that BLP formed at 80°C should be smaller in molecular weight than that formed at 37°C. The band of BLP formed at 80°C (Fig. 4, slot 7) seemed to be in a position slightly below the position of native BLP or BLP formed at 37°C. To confirm this we analyzed the same samples in a 17.5% polyacrylamide slab gel with a discontinuous buffer system (12); the pattern is shown in Fig. 5. The BLP formed at 37°C moved to the
subjected to one-cycle Edman degradation. Liberation of the \(^1H\) liberation even with the 80 °C-chased
5180
of the I\(^4C\) count during the one-cycle Edman degradation was
interpretation of the results in Fig. 1, and a fluorogram was taken. The immunoprecipitated samples shown
also show that the signal peptide of BLP was only labeled with \[^1\text{H}\]arginine or \[^1\text{C}\]methionine not with \[^1\text{C}\]arginine. It is known that the signal
peptide region of pro-BLP has no arginine residue. These results strongly suggest that the new band was the signal
peptide of pro-BLP. The signal peptide of pro-BLP has only one methionine residue that is present at the NH\(_2\) terminus.
As this new band was significantly labeled with \[^3\text{S}\]methionine, the NH\(_2\) terminus of pro-BLP was suggested to
be conserved in the signal peptide released during \textit{in vitro} processing. It should be mentioned, however, that the signal
peptide thus identified is assumed to contain a small amount of signal peptides derived from minor lipoproteins discovered
recently (19). This will be discussed later.

The results in Fig. 6 also show that the signal peptide of BLP is metabolized by at least two types of peptidase; a heat-
resistant endopeptidase that removes the signal peptide from pro-BLP (signal peptidase) and a heat-sensitive peptidase
that digests the cleaved signal peptide (signal peptide peptidase).

![Fig. 4. NH\(_2\)-terminal acylation of BLP processed \textit{in vitro}
from pro-BLP. A, envelope fractions were isolated from cells labeled with both \[^1\text{H}\]palmitic acid and \[^1\text{C}\]arginine in the presence of globomycin, suspended in 10 mM sodium phosphate buffer (pH 7.1)
(slot 2), and incubated at 37 °C for 10, 20, and 60 min, or at 80 °C for 7 min (slots 3, 4, 5, and 7, respectively). Each tube contained the
envelope fraction of 8 × 10\(^6\) cells suspended in 2 ml of the buffer. To slots 6 and 8 globomycin (5 \(\mu\)g/ml) was added before incubation at 37 °C for 60 min and at 80 °C for 7 min, respectively. The pellet
deposited fractions were then extracted 3 times with chloroform/methanol (2:1), solubilized in 1% SDS, and immunoprecipitated with
antiserum against BLP. The immunoprecipitates were solubilized and subjected to SDS-polyacrylamide gel electrophoresis, as described in
Fig. 1, and a fluorogram was taken. Slot 1 was the immunoprecipitate of the envelope fraction from control cells (no globomycin treatment).
Radioactivity in individual slots was 9,600 cpm (slots 1, 4, and 6), 8,000 cpm (slots 2–4), and 10,000 cpm (slots 7 and 8). B, the bands of
pro-BLP and BLP were cut out from the gel and digested with NCS tissue solubilizer as described (15), and the radioactivity was counted.
The \(^1\text{H}/^4\text{C}\) ratios of pro-BLP (■) and BLP (○) are shown. A background value was obtained by determining the radioactivity in an
approximately equal sized slice from an indicated area (bkg); this was subtracted from the radioactivity in the pro-BLP and BLP bands. In
cases the background radioactivity was very low.

![Fig. 5. Comparison of mobilities of BLPs processed from
pro-BLP at 37 and 80 °C. The immunoprecipitated samples shown
in Fig. 4 were analyzed in the gel system of Anderson et al. (12). Slot 1
was control cells (no globomycin treatment); slot 2 was the envelope
fraction of globomycin-treated cells incubated at 37 °C for 60 min;
slot 4 was the envelope fraction of BLP released during \textit{in vitro}
processing. It should be mentioned, however, that the signal
peptide thus identified is assumed to contain a small amount of signal peptides derived from minor lipoproteins discovered
recently (19). This will be discussed later.

The results in Fig. 6 also show that the signal peptide of BLP is metabolized by at least two types of peptidase; a heat-
resistant endopeptidase that removes the signal peptide from pro-BLP (signal peptidase) and a heat-sensitive peptidase
that digests the cleaved signal peptide (signal peptide peptidase).

same position as the native BLP, while the BLP formed at
80 °C moved faster. This was evident when both the samples
were run together. Fig. 5 also shows that the immunoprecipi-
tated samples contained only BLP and pro-BLP, and were
essentially free from any radioactive contaminants. The modi-
fication by fatty acid of the new NH\(_2\) terminus was further
investigated by using the same samples as follows. If the
amino group of the terminal glycine-bearing cysteine residue
of BLP formed \textit{in vitro} has been modified by fatty acid, this
terminus should be resistant to Edman degradation. These
anti-BLP immunoprecipitated samples were, therefore,
subjected to one-cycle Edman degradation. Liberation of the
\(^3\text{H}\) count from the 37 °C-chased sample was only 8%, while
that from the 80 °C-chased sample was about 30%. Liberation of the
\(^4\text{C}\) count during the one-cycle Edman degradation was
almost negligible. These results further substantiated the
interpretation of the results in Fig. 4. The relatively low
efficiency of the \(^3\text{H}\) liberation even with the 80 °C-chased
sample may be due to the unusual structure of the NH\(_2\)
terminus.

Identification of the Signal Peptide Cleaved from Pro-
BLP—During processing, the signal peptide region of pro-
BLP could either be digested from one end or clipped between the
glycine\(^20\) and cysteine\(^31\) residues. In the latter case, the signal peptide released could further be digested by another
peptidase(s). With the hope that the second peptidase is inactivated at 80 °C, identification of the signal peptide of
BLP released during \textit{in vitro} processing was attempted. Cells were labeled with \[^1\text{C}\]arginine, \[^4\text{C}\]leucine, or \[^3\text{S}\]methio-
nine in the presence of globomycin. Envelope fractions were isolated and an \textit{in vitro} chase was performed at 37 °C for 60
min or at 80 °C for 7 min, and then analysis by SDS-poly-
acylamide gel electrophoresis was carried out. The gel pattern
of samples processed at 37 °C contained no new bands (data
not shown), while the same samples processed at 80 °C showed a new band below the position of BLP (Fig. 6). This
new band was observed concurrently with the processing of
pro-BLP to BLP. When the processing was inhibited by the
addition of globomycin, the new band did not appear. More-
over, this band was labeled with \[^4\text{C}\]leucine or \[^3\text{S}\]methio-
nine but not with \[^1\text{C}\]arginine. It is known that the signal
peptide region of pro-BLP has no arginine residue. These
results strongly suggest that the new band was the signal
peptide of pro-BLP. The signal peptide of pro-BLP has only
one methionine residue that is present at the NH\(_2\) terminus
(6). As this new band was significantly labeled with \[^3\text{S}\]
methionine, the NH\(_2\) terminus of pro-BLP was suggested to
be conserved in the signal peptide released during \textit{in vitro}
processing. It should be mentioned, however, that the signal
peptide thus identified is assumed to contain a small amount of signal peptides derived from minor lipoproteins discovered
recently (19). This will be discussed later.

The results in Fig. 6 also show that the signal peptide of BLP is metabolized by at least two types of peptidase; a heat-
resistant endopeptidase that removes the signal peptide from
pro-BLP (signal peptidase) and a heat-sensitive peptidase
that digests the cleaved signal peptide (signal peptide pepti-
dase).
Fig. 6. Identification of the signal peptide cleaved from pro-BLP. Envelope fractions from globomycin-treated cells labeled with [3H]arginine (slots 2, 5, and 8), [3H]leucine (slots 3, 6, and 9) or [35S]methionine (slots 4, 7, and 10) were isolated. A part of them was solubilized and analyzed on SDS-polyacrylamide gel (slots 2-4) as described in Fig. 1. The rest was incubated at 80 °C for 7 min in the absence (slots 5-7) or present (slots 8-10) of globomycin (5 μg/ml). After incubation, the envelope fractions were solubilized and analyzed by SDS-polyacrylamide gel electrophoresis. Slot 1 was the envelope fraction from control cells (no globomycin treatment) labeled with [35S]methionine. The gel was then fixed and dried, and an autoradiogram was taken. Each slot contained 1.8-2.0 × 10⁶ cpm and the x-ray film was exposed for 9 days. The positions of pro-BLP and BLP are indicated. SP, signal peptide. The band indicated by an arrow is a cytoplasmic membrane protein (24).

DISCUSSION

Biosynthesis and assembly of BLP in E. coli is a complicated process. The BLP is first synthesized as a precursor form that possesses a signal peptide of 20 amino acid residues extending from the NH2 terminus (6). Then it is converted to the mature form through cleavage of the signal peptide and modification of the newly bared NH2-terminal cysteine residue. During these processes, BLP is exported through the cytoplasmic membrane and assembled into the outer membrane.

With the aid of an antibiotic called globomycin, we found pro-BLP that had already been modified by glyceride in the cytoplasmic membrane (9). This indicates that the modification by glyceride takes place before the cleavage of the signal peptide. We have also presented evidence showing that pro-BLP can be exported through the cytoplasmic membrane while it still retains the signal peptide that is, most probably, held in the cytoplasmic membrane (20). This indicates that the cleavage of the signal peptide is not required for the export of the major part of the protein.

In the present work, we further found that at least two types of peptidase are involved in the processing of pro-BLP; an enzyme that removes the signal peptide (signal peptidase) and an enzyme that digests the cleaved signal peptide (signal peptide peptidase). We have also been able to show a third process activity which is responsible for acylation of the newly exposed NH2 terminus of the cysteine residue. Fig. 7 is a summary of these processes which are discussed in detail below.

Signal Peptidase—In the present work, we were able to identify the signal peptide that was released from pro-BLP upon incubation of the globomycin-treated envelope. The signal peptide thus identified has methionine at the NH2 terminus and its molecular weight was roughly estimated to be 2,000 daltons or higher.¹ The results indicate that the signal peptidase responsible for pro-BLP is an endopeptidase that clips the glycylglycylcysteine bond. It should be noted that the signal peptide thus identified might have contained that from the minor lipoproteins (19), since precursors of these lipoproteins accumulate in globomycin-treated cells, and at least some of them could be chased to the mature form after the removal of globomycin. However, the total amount of these lipoproteins, numerically, is very small compared to that of BLP (19). Therefore, a greater part of the signal peptide is supposed to be that from pro-BLP. The signal peptide has been isolated in quantity and detailed characterization is now in progress in this laboratory.

The signal peptidase in the envelope fraction has many unique properties as a protease; being highly resistant to heat, having a wide optimum pH range, and being highly sensitive to detergents. These results suggest that a hydrophobic environment is important for the peptidase reaction; in other words, the expression of the peptidase activity may be closely related to the membrane structure. The signal peptidase is also unique in that its activity is not inhibited by the many protease inhibitors listed in the text, while it is inhibited by globomycin that does not inhibit cleavage of the signal peptide of other major outer membrane proteins (9). But it may be possible that the signal peptidase was not accessible to the protease inhibitors, especially to the hydrophilic ones, because of the localization of the enzyme in the membrane. As discussed in a previous paper (19), this signal peptidase is supposed to be specific for lipoproteins that include BLP and other minor lipoproteins. Wickner and his associates purified and characterized a signal peptidase for phage M13 procoat protein (21). This signal peptidase is apparently different from the signal peptidase for pro-BLP in that it is resistant to Triton X-100.

Signal Peptidase Peptidase—Under the usual conditions, the

² M. Hussain, S. Ichihara, and S. Mizushima, unpublished experiments.

Fig. 7. Biosynthesis of BLP. The S above Cys belongs to the cysteine residue. The number and length of the digested products of the signal peptide is not known.
signal peptidase reaction did not result in accumulation of the signal peptide, indicating that the signal peptide must have been digested into smaller fragments immediately after release from pro-BLP. Incubation at 80 °C resulted in the appearance of a signal peptide on a gel, indicating that the E. coli cells possess a rather heat-sensitive protease that is responsible for digestion of the cleaved signal peptide. We tentatively named it signal peptidase, although it is unclear whether the enzyme is specific for the digestion of signal peptides or not. It should be noted that pro-BLP was quite stable in the laboratory.

The results shown in Fig. 4 strongly suggest that cleavage of the signal peptide in vitro is accompanied by the acylation. The acylation was prevented when the cleavage reaction was carried out at 80 °C, suggesting that the cleavage of the signal peptide and the modification by fatty acid are sequential but independent reactions, only the enzyme(s) for the latter reaction being inactivated at 80 °C. The BLP formed by in vitro processing at 37 °C is most probably NH2-acylated as mature BLP is, since it migrated to the same position as that of the mature BLP, and the NH2-terminal cysteine residue was resistant to Edman degradation. These results also indicate that neither an external supply of energy nor additional cofactors are required for the modification by fatty acid as well as for the cleavage of the signal peptide. It has been reported that the acetyl moiety of phospholipids is the precursor for the NH2-terminal fatty acid residue in BLP (22).

It should be noted that the in vitro system employed in the present work is a very simple one, and yet it most likely allows the entire biochemical reactions involved in the conversion of pro-BLP to BLP to occur. The relationship between these biochemical events and the translocation and assembly of BLP into the outer membrane is now being studied in this laboratory.

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