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Processing of Lipid-Modified Prolipoprotein Requires Energy and *sec* Gene Products In Vivo

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The kinetics of processing of glyceride-modified prolipoprotein that accumulated in globomycin-treated *Escherichia coli* has been found to be affected by *sec* mutations, i.e., *secA*, *secE*, *secY*, *secD*, and *secF*, and by metabolic poisons which affect proton motive force (PMF). The effect of *sec* mutations on processing of glyceride-modified prolipoprotein in vivo was not due to a secondary effect on PMF. Neither a *secF* mutation nor metabolic poisons affected the processing of previously accumulated proOmpA protein in vivo, suggesting that the requirements for functional *sec* gene products and PMF are specific to the processing of lipoprotein precursors by signal peptidase II.

Among prokaryotes, the mechanism of protein export has been extensively studied in the gram-negative bacterium *Escherichia coli*. The majority of outer membrane and periplasmic proteins are synthesized in the cytoplasm as precursors with amino-terminal signal sequences and are exported across the cytoplasmic membrane with the cleavage of signal sequences by signal peptidases. Efficient protein export requires a number of proteins which fall into three categories. Members of the first group (primarily SecB) play a role at the initial step of protein export as chaperones, which maintain the translocation-competent conformation of the preproteins and facilitate their targeting to the export machinery (18). The second group comprises at least five *sec* gene products—SecA (22), SecD (9), SecE (26), SecF (9), and SecY (16)—which have been postulated to form a multimeric protein complex as the protein secretion machinery or protein translocase for the general protein export (2). The third group of proteins corresponds to the signal peptidases (SPases), which cleave the signal sequences of precursor proteins so that cleaved proteins are released from the cytoplasmic membrane (6, 12). There are at least two SPases in bacteria, SPase I for nonlipoproteins (31) and SPase II for lipoproteins (7). The latter enzyme is specifically inhibited by globomycin (14).

Lipoproteins are distinct from nonlipoproteins in that their precursors utilize lipoprotein-specific SPase (SPase II) and undergo lipid modification prior to the processing by SPase II (28). The major outer membrane (Braun's) lipoprotein of *E. coli* is the prototype of bacterial lipid-modified proteins (3). It is synthesized as a precursor form (prolipoprotein); this is followed by glyceride modification and processing by SPase II (10, 28). Studies focused on the Braun's lipoprotein have shown that the export of lipoprotein requires functional *sec* gene products except the SecB protein, i.e., the SecA, SecD, SecE, SecF, and SecY proteins, suggesting that the export of lipoproteins utilizes the general protein export machinery (27, 29). Furthermore, it has been shown that functional Sec proteins are required at steps prior to the lipid modification and processing of prolipoproteins (27, 29). The divergence of the secretion pathways of lipoproteins and nonlipoproteins appears to occur in the cytoplasmic membrane during or immediately after the translocation of precursor proteins (10). To investigate the dynamics of the processing of prolipoproteins in vivo, kinetic studies with a globomycin-treated wild-type strain and *sec* mutants were carried out at both permissive and nonpermissive temperatures and in the presence or absence of metabolic poisons. Our results indicate that efficient processing of lipid-modified prolipoprotein requires functional Sec proteins and proton motive force (PMF).

MATERIALS AND METHODS

Materials. [^{35}S]methionine (1,000 to 1,200 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, Calif.). L-[2,3,4,5-^{3}H]proline (103 Ci/mmol) was from Amersham Corp. (Arlington Heights, Ill.). Other chemicals used were obtained from commercial sources. Globomycin was a generous gift from M. Arai (Sankyo Co., Tokyo, Japan).

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Luria-Bertani medium was used for subculture, and M9 minimal medium supplemented with 0.4% glucose and thiamine (100 μg/ml) was employed for labeling experiments.

Labeling experiments. A pulse-chase experiment was used to study the kinetics of the processing of lipoprotein and OmpA precursors. To study the processing of lipid-modified prolipoprotein in *sec* mutants, cultures were grown in M9 minimal medium at the permissive temperature (30°C for *secA*, *secB*, and *secY* mutants and 37°C for *secD*, *secE*, and *secF* mutants) to the mid-logarithmic phase of growth (A_{600} = 0.4 to 0.5), incubated with globomycin (100 μg/ml) for 20 min, and labeled with [^{35}S]methionine for 5 min. After being washed, the cells were shifted to the nonpermissive temperature (42°C for *secA*, *secB*, and *secY* mutants; 25°C for *secD*, *secE*, and *secF* mutants) in M9 glucose minimal medium containing 0.4% methionine and were chased for 40 to 50 min. One-milliliter aliquots of cultures were withdrawn at 5-min intervals, and trichloroacetic acid was added to a final concentration of 10%. The trichloroacetic acid precipitates were washed with acetone, solubilized with 1% sodium dodecyl sulfate (SDS) in 10 mM sodium phosphate buffer (pH 7.0) at 100°C, and immunoprecipitated with antiserum against purified lipoprotein or OmpA protein as described previously (11).

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source (reference)
MC1000	F⁻ *araD139* Δ(*ara-leu*)*7679 galE galK* Δ(*lac*)*X74 rpsL thi*	J. Beckwith (8)
CG-2	MC1000 *phoR*	J. Beckwith (8)
MC4100	F⁻ *lacU169 relA rpsL thi araD139*	M. Casadaban (4)
MM52	MC4100 *secA51*(Ts)	J. Beckwith (22)
CK1699	MC4100 *secB7*	J. Beckwith (19)
KJ173	MC1000 *phoR secD29*(Cs) *zaj*::Tn*5*	J. Beckwith (9)
KJ188	MC1000 *phoR secE501*(Cs) *zijRK498*::Tn*5*	J. Beckwith (24)
KJ184	MC1000 *phoR secF62*(Cs) *zaj*::Tn*10*	J. Beckwith (9)
AD97	F⁻ *ara139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR phoA* Δ*E15 lon100 tsx*::Tn*5 secY24 zhd33*::Tn*10*	K. Ito (1)
CK1801	F⁻ Δ(*lac*)*U169 araD139 thiA rpsL* Δ(*uncBC*)	P. C. Tai (5)
IT41	W3110 *lep9 zff*::Tn*10*	Y. Nakamura (13)
KJ18441	MC1000 *phoR secF62*(Cs) *lep9 zff*::Tn*10*	This study

PAGE. Lipoprotein and OmpA proteins were analyzed by two different polyacrylamide gel electrophoresis (PAGE) systems, the gel system of Ito et al. for OmpA protein (15) and the Tricine-SDS-PAGE system for lipoprotein (25). After electrophoresis, the gel was dried, and an autoradiogram was made with Kodak X-Omat film, using an intensifying screen at −80°C. The gel was also scanned with PhosphorImager. The percentage of processing of modified prolipoprotein (MPLP) was calculated from the scanning of autoradiograms or PhosphorImager scans.

[^{3}H]proline uptake. The PMF was assessed by measuring [^{3}H]proline uptake (17). Cells were grown in minimal glucose medium, and chloramphenicol (50 μg/ml) was added 0.5 min prior to the addition of 1 μCi of [^{3}H]proline per ml. One-milliliter aliquots were removed at 1-min intervals for 7 min, collected on 0.45-μm-pore-size filters, washed with 10 ml of ice-cold M9 medium, and dried, and the radioactivity was measured by liquid scintillation counting. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (60 μM final concentration), sodium azide (1 mM final concentration), and arsenate (5 mM final concentration) were added separately immediately prior to the addition of [^{3}H]proline.

RESULTS

Processing of MPLP was affected by *sec* mutations. We studied the effects of *secA*, *secB*, *secD*, *secE*, *secF*, and *secY* mutations on the processing of MPLP in globomycin-treated *E. coli* cells. Wild-type cells grown at 37°C and each of the *sec* mutants grown at the permissive temperature were incubated with globomycin (100 μg/ml) for 20 min and labeled with [^{35}S]methionine for 5 min. After the removal of globomycin by washing, the cells were chased at the nonpermissive temperature (30 or 42°C). As shown in Fig. 1, MPLP that accumulated in globomycin-treated cells was rapidly processed in the wild-type strain upon the removal of globomycin at both temperatures. However, the processings of the MPLP in *secA*, *secD*, *secE*, *secF*, and *secY* mutants were significantly inhibited at the nonpermissive temperature, in contrast to the rapid processing of MPLP at the permissive temperature. The processing of MPLP was not affected by the *secB* mutation (data not shown). The requirements for SecA, SecY, SecE, SecD, and SecF proteins but not SecB protein for the processing of MPLP are reminiscent of similar requirements or the lack thereof for the export of unmodified prolipoprotein in *E. coli* (27, 29). To confirm the requirement for SecA protein of MPLP processing, we employed sodium azide as an inhibitor of SecA ATPase

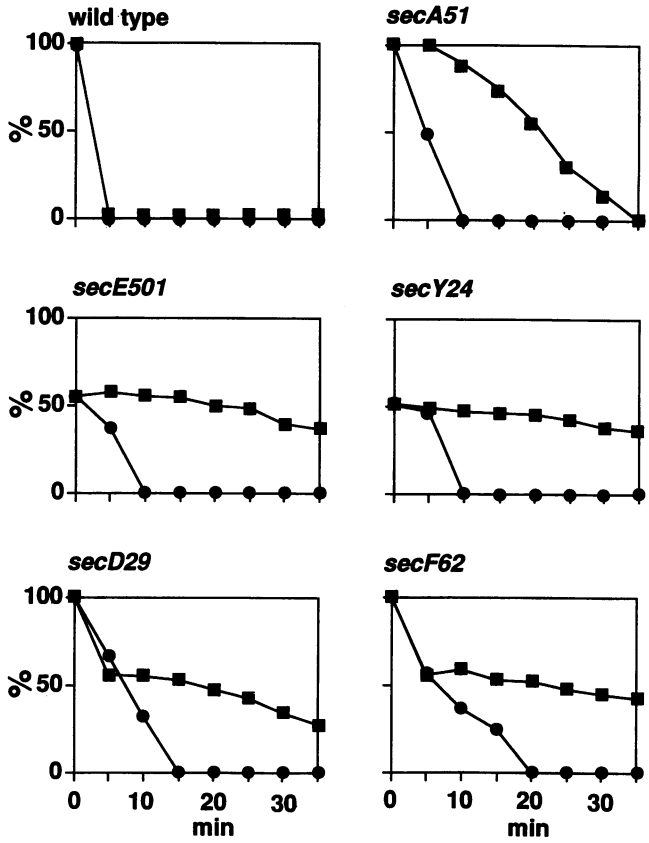

FIG. 1. Kinetics of lipoprotein processing in the wild-type strain and in *secA*, *secE*, *secY*, *secD*, and *secF* mutants. Cells grown at the permissive temperature (30°C for *secA* and *secY* mutants; 37°C for the wild type strain and *secD*, *secE*, and *secF* mutants) were incubated with globomycin (100 μg/ml) for 20 min and labeled with [^{35}S]methionine for 5 min. After the globomycin was removed by washing, the cells were chased at the permissive temperature or the nonpermissive temperature (42°C for *secA*, *secB*, and *secY* mutants; 25°C for *secD*, *secE*, and *secF* mutants) in M9 glucose minimal medium containing 0.4% methionine for 40 to 50 min. One-milliliter aliquots of culture were withdrawn at 5-min intervals and precipitated by trichloroacetic acid. The precipitated samples were further analyzed by immunoprecipitation and SDS-PAGE as described previously (11). From the scanned gel, the percentage of lipoprotein species as MPLP was calculated and is shown. Symbols: ■, nonpermissive temperature; ●, permissive temperature.

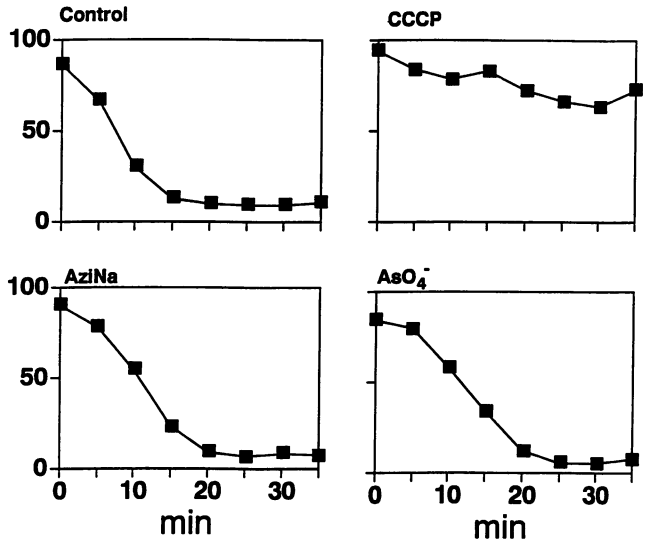

FIG. 2. Effect of metabolic poisons on the processing of globomycin-accumulated glyceride-modified prolipoprotein in strain CK1801. Cells grown at 37°C were incubated with globomycin (100 μg/ml) for 20 min and labeled with [^{35}S]methionine for 5 min. After the globomycin was removed by washing, the cells were chased in the presence or absence of metabolic poisons at 37°C in M9 glucose minimal medium containing 0.4% methionine for 40 to 50 min. One-milliliter aliquots of culture were withdrawn at 5-min intervals and precipitated by trichloroacetic acid. The precipitated samples were further analyzed by immunoprecipitation and SDS-PAGE as described previously (11). From the scanned gel, the percentage of lipoprotein species as MPLP was calculated and is shown.

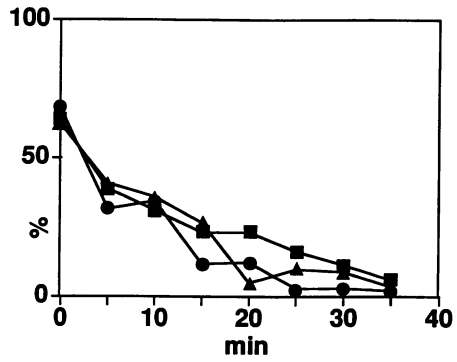

FIG. 3. Effect of metabolic poisons on the processing of accumulated proOmpA protein in an SPase I mutant (IT41). IT41 cells grown at 30°C were shifted to 42°C for 90 min and labeled with [^{35}S]methionine for 2 min. After centrifugation, the cells were chased in the presence or absence of metabolic poisons at 30°C in M9 glucose minimal medium containing 0.4% methionine for 40 min. One-milliliter aliquots of culture were withdrawn at 5-min intervals and precipitated by trichloroacetic acid. The precipitated samples were further analyzed by immunoprecipitation and SDS-PAGE as described previously (11). From the scanned gel, the percentage of OmpA protein as proOmpA was calculated and is shown. Symbols: ■, control; ●, 1 mM sodium azide; ▲, 60 μM CCCP.

activity (23); azide at 1 mM has been shown to inhibit the secretion of OmpA and maltose-binding protein (23). Surprisingly, sodium azide (1 mM) did not affect the kinetics of processing of the MPLP (data not shown; see Fig. 2).

We examined whether the defect in the processing of MPLP in *sec* mutants was due to the jamming of the export machinery and consequently could be relieved by an inhibition of de novo protein synthesis. The *secF* mutant labeled in the presence of globomycin was chased at the nonpermissive temperature in the presence or absence of chloramphenicol (30 μg/ml). The inhibition of MPLP processing was not reversed by the addition of chloramphenicol (data not shown), suggesting that the defect in MPLP processing is not due to the jamming of the secretion machinery by the accumulation of precursor proteins in *sec* mutants.

PMF is required for the processing of the globomycin-accumulated MPLP. To ascertain whether the processing of MPLP requires metabolic energy, wild-type cells containing MPLP were chased in the presence or absence of CCCP (60 μM), 2,4-dinitrophenol (0.5 mM), or AsO_4^- (5 mM). The processing of the MPLP was completely inhibited by CCCP and partially inhibited by 2,4-dinitrophenol or AsO_4^- (data not shown). These data suggest that the processing of accumulated MPLP requires energy. In order to determine whether ATP or PMF is the primary energy source for MPLP processing, the effects of these inhibitors on MPLP processing were studied in an *E. coli* Δ*unc* mutant, CK1801 (5). CCCP treatment resulted in a significant delay of the processing, while AsO_4^- and sodium azide had no effect on the processing of the MPLP (Fig. 2). We also measured the PMF indirectly as a function of proline uptake in CK1801. [^{3}H]proline uptake was measured as described in Materials and Methods, and the rate of transport was calculated from the increase in disintegrations per minute between 2 and 4 min after the addition of [^{3}H]proline. The results showed that CCCP (60 μM) inhibited the uptake of [^{3}H]proline completely, while AsO_4^- (1 mM) did not (22 and 16,396 dpm of [^{3}H]proline transported per min, respectively; the control value was 18,457 dpm/min). Furthermore, the uptake of [^{3}H]proline was not affected in *sec* mutants by the shift to the nonpermissive temperature (data not shown). These results suggest that the PMF is important for the processing of MPLP in vivo; furthermore, the inhibition of MPLP processing in *sec* mutants is not due to a secondary effect of the *sec* mutations on the PMF.

Processing of proOmpA is not affected by CCCP or the *secF* mutation. To determine whether the requirements of MPLP processing for *sec* gene products and PMF also extend to the processing of nonlipoprotein precursors in vivo, we measured the kinetics of processing of previously accumulated proOmpA in *sec* mutants or in the presence of metabolic poisons. We employed the temperature-sensitive SPase I mutant (IT41 *lep9*), which accumulates precursors of the OmpA protein and maltose-binding protein at the nonpermissive temperature (42°C) (13). IT41 was grown at the permissive temperature to logarithmic phase, shifted to the nonpermissive temperature for 90 min, labeled with [^{35}S]methionine for 2 min, and chased. As shown in Fig. 3, the previously accumulated proOmpA was processed upon the shift to the permissive temperature (30°C). Neither CCCP (60 μM) nor sodium azide (1 mM) affected the kinetics of proOmpA processing in vivo. To study the effect of the *secF* mutation on proOmpA processing, the *lep9* mutation was transduced into the *secF* mutant by P1 transduction. The transductant (KJ18441) grew slowly at 30°C but did not grow at 23 or 42°C. KJ18441 was grown at 30°C to the logarithmic phase, shifted to 42°C for 90 min, and labeled with [^{35}S]methionine for 1 min. The culture was divided into three portions and chased at 42, 30, and 23°C, respectively. As shown Fig. 4, the kinetics of processing of previously accumulated proOmpA was not reduced at 23°C, the non-

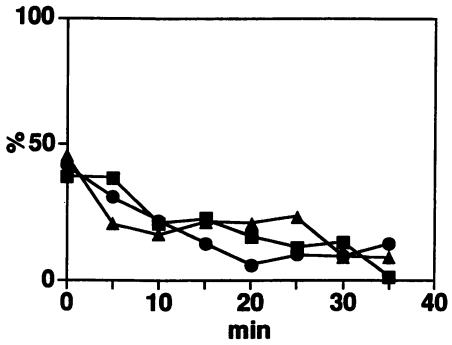

FIG. 4. Effect of the *secF* mutation on the processing of accumulated proOmpA protein in an SPase I mutant carrying the *secF* mutation (KJ18441). KJ18441 cells grown at 30°C were shifted to 42°C for 90 min and labeled with [^{35}S]methionine for 1 min. After centrifugation, the cells were divided into three groups and chased at 30, 37, and 42°C, respectively, in M9 glucose minimal medium containing 0.4% methionine for 40 min. One-milliliter aliquots of culture were withdrawn at 5-min intervals and precipitated by trichloroacetic acid. The precipitated samples were further analyzed by immunoprecipitation and SDS-PAGE as described previously (11). From the scanned gel, the percentage of OmpA protein as proOmpA was calculated and is shown. Symbols: ■, 23°C; ●, 30°C; ▲, 42°C.

permissive temperature for the *secF* mutant. These results indicate that the processing of previously accumulated proOmpA protein does not require PMF or functional SecF protein.

DISCUSSION

The current model of lipoprotein export postulates that the translocation of unmodified prolipoprotein by *sec* gene products takes place prior to its modification and processing to form mature lipoprotein. This conclusion is based on the observation that it is unmodified prolipoprotein which accumulates in each of the *secA*, *secY*, *secE*, *secD*, and *secF* mutants at the nonpermissive temperature (27, 29). Similarly, unmodified prolipoprotein accumulates in maltase-induced *E. coli* MM18, in which the export machinery is jammed (28). In genetic terms, the *sec* genes are epistatic over those encoding prolipoprotein modification and processing enzymes. It is therefore surprising that the processing of lipid-modified prolipoprotein is affected by *secA*, *secY*, *secE*, *secD*, and *secF* mutations at the nonpermissive temperature compared with the wild-type cells. This inhibition of MPLP processing in *sec* mutants does not appear to result from an accumulation of precursor proteins in general and/or jamming of the secretion machinery. The processing of MPLP was affected by the *secA* mutation but not by 1 mM sodium azide in vivo. SecA protein has been postulated to have multiple catalytic and regulatory functions during protein translocation (30). This observation suggests that the requirement of MPLP processing in vivo for functional SecA protein is not at the step of SecA ATPase, which is inhibited by sodium azide. It may involve the interaction of SecA with SecY/SecE complex for the assembly of a functional export machinery or protein translocase. A second unexpected finding in the present study is the apparent requirement for intact PMF for the processing of MPLP. The effect of *sec* mutations on MPLP processing in vivo does not result from a secondary effect on PMF. The requirements for functional *sec* proteins and PMF for MPLP processing appear to be specific. No such requirement is demonstrated for the processing of previously accumulated proOmpA in vivo.

Both SPase I and SPase II of *E. coli* have been purified and characterized (7, 31). While they exhibit different membrane topologies (20, 21) and substrate specificities (7, 31), the purified enzymes catalyze the processing of the respective precursors in vitro without an apparent requirement for other protein components or metabolic energy (7, 31). The apparent difference in their in vivo requirements for PMF and functional *sec* gene products for the processing of previously accumulated proOmpA and MPLP may be a reflection of both the complexity of cellular events occurring in the cytoplasmic membrane of *E. coli* and a difference in the interactions of these two groups of precursor proteins with their respective processing enzymes. The catalytic domain of SPase I is presumably located in the periplasmic space, with the enzyme and its substrates in similar membrane topologies, being anchored to the inner membrane via their N-terminal hydrophobic sequences (20). In contrast, SPase II is an integral membrane protein with four membrane-spanning segments (21), with its substrates anchored to the inner membrane via both the N-terminal signal sequence and the covalently attached diglyceride. The requirements for functional *sec* proteins and PMF for the processing of MPLP may be an indication of the topological differences of these two processing reactions in vivo. It is conceivable that the access of MPLP to SPase II requires functional *sec* proteins and PMF. A resolution of this difference between in vivo and in vitro requirements of MPLP processing may be attempted by using inverted membrane vesicles to study the dependence of MPLP processing on PMF or functional *sec* proteins.

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