Accumulation of Prolipoprotein in *Escherichia coli* Mutants Defective in Protein Secretion

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The export of lipoprotein has been found to be affected in both secA and secY mutants of *Escherichia coli* which are defective in the secretion of outer membrane and periplasmic proteins. The kinetics of accumulation of prolipoprotein upon a temperature shift to 42°C is indistinguishable from that of pre-OmpA protein accumulation in the secA mutant. In both secA and secY mutants, the accumulated prolipoprotein is unmodified with glyceride and localized in the cytoplasmic membrane. We conclude from these results that the early steps in protein export are common to prolipoprotein and non-lipoprotein precursors. The pathways for the export of these two groups of precursor proteins diverge with regard to the modification and processing reactions which are late events in the process.

The gram-negative bacterium *Escherichia coli* exports a large number of proteins from the cytoplasm to the periplasmic space and the outer membrane (15). These exported proteins are initially synthesized as precursor forms which have an amino-terminal extension consisting of an extra 20 to 30 amino acids, the so-called signal peptides (15). The precursor proteins are processed by a specific endopeptidase(s) or signal peptidase(s) during or immediately after the translocation of the nascent polypeptides through the cytoplasmic membrane.

Protein export is a complex process and presumably would require a number of gene products. Recently, several mutants defective in protein export have been isolated and partially characterized (13, 16, 20). They have been classified by genetic mapping into various loci on the *E. coli* chromosome (13, 16, 20). In these mutants, protein export is defective; consequently, the precursor forms of exported proteins accumulate in the mutant cells.

The major outer membrane lipoprotein is also synthesized as a precursor protein, the prolipoprotein. It differs from other outer membrane and periplasmic proteins in the specificity of the processing enzyme. The signal peptidase for prolipoprotein (SPase II) is distinct from that for M13 procac light (SPase I) (21), and this difference is related to the extensive posttranslational modification and processing reactions involved in the biosynthesis of membrane lipoproteins (27).

The processing of precursor proteins is a late event in protein export (12). It remains a distinct possibility that the initial events in protein export are common to prolipoproteins and non-lipoprotein precursors alike. The identification of genes required for protein export in general (secA, secY, and secB) (13, 16, 20) thus offers an opportunity to ascertain whether the products of these genes are also required for lipoprotein export. In this paper, we report the results on the biogenesis of outer membrane lipoprotein in *E. coli* mutants defective in protein export in general.

**MATERIALS AND METHODS**

**Bacterial strains and medium.** Bacterial strains used in this study were derived from strain MC4100 F− ΔacU169 araD139 rpsL150 thi ffbB5301 deoC7 ptsF25 relA1 (2), MM52 secA(Ts) (16), CK1699 secB7 (13), and IQ85 secY(Ts) (20). M9 minimal medium supplemented with 0.4% glucose and 100 μg of thiamine per ml was used for labeling experiments.

**Labeling experiments.** Pulse-labeling and pulse-chase experiments were carried out to study the lipoprotein maturation at both the permissive and nonpermissive temperatures for both secA and secY mutant strains. Samples of cultures (20 ml) grown at 30°C were harvested at mid-logarithmic phase of growth (absorbance at 600 nm = 0.5 to 0.6), suspended in 10 ml of the same medium, and incubated at 42°C for 2 or 3 h for secA or secY strains, respectively. For the secB7 mutants, mid-log-phase culture grown at 37°C was used for the labeling experiments. Cells were then pulse-labeled with 200 μCi of [35S]methionine for 15 s and chased for 15, 30, and 45 s after the addition of 10 μl of medium containing 0.4% methionine. Pulse-labeling (2 min) with 50 μCi of [35S]methionine was also carried out for the kinetic studies of the accumulation of OmpA and lipoprotein precursors. In both pulse-labeling and pulse-chase experiments, the incorporation of radioisotope into the cells was terminated by the addition of 50% trichloroacetic acid to a final concentration of 10%. The trichloroacetic acid precipitates were washed with acetone, dried, solubilized with 1% sodium dodecyl sulfate (SDS) in 10 mM sodium phosphate buffer (pH 7.0) at 100°C for 2 to 3 min, and immunoprecipitated with antisera against purified lipoprotein or OmpA protein. The procedure of immunoprecipitation was as described previously (6).

**Separation of outer membrane and inner membrane fractions.** To study the subcellular distribution of lipoprotein and prolipoprotein in the cell envelope, the mutant cells were labeled with 100 μCi of [35S]methionine for 2 min after 3 h of incubation at 42°C. Labeling was terminated by pouring the culture (5 ml) into a centrifuge tube containing 200 μg of chloramphenicol per ml, sodium azide (final concentration, 0.2%), and crushed ice. The cells were harvested by centrifugation at 10,000 × g for 5 min at 4°C, washed, and disrupted by sonication. The sonicated crude extract was centrifuged at 200,000 × g for 2 h at 4°C. The membranes were suspended in 100 μl of 10 mM sodium phosphate buffer (pH 7.0) with a bath sonicator. Membrane suspensions (75 μl) were mixed with 125 μl of water and 200 μl of 1% Sarkosyl solution in water (4). After 1 h of incubation at room
temperature, the suspensions were centrifuged for 2 h at 200,000 \times g at 4°C. The pellet was washed once with 10 mM sodium phosphate buffer (pH 7.0). The supernatant was precipitated by the addition of 400 \mu l of 10% trichloroacetic acid, washed once with acetone, and solubilized with 1% SDS in 10 mM sodium phosphate buffer (pH 7.0) at 100°C. Both the Sarkosyl-soluble and -insoluble fractions were immunoprecipitated with antiserum against purified lipoprotein and OmpA protein, respectively, and the distribution of lipoprotein, OmpA protein, and their respective precursors was determined by SDS-polyacrylamide gel electrophoresis. The separation of inner and outer membrane fractions was also achieved by the sucrose density gradient centrifugation method as described by Osborn et al. (17). A 5-ml culture of either secA or secY mutant cells was shifted to 42°C at mid-log phase of growth and labeled with 200 \mu Ci of \textsuperscript{[35]S}methionine for 2 min. The labeled cells were harvested by centrifugation and treated with EDTA-lysozyme, and the total cell envelope was fractionated into inner and outer membranes as previously described (17). \textsuperscript{35}S radioactivity in each fraction of the sucrose gradient was measured by liquid scintillation counting, and the light (inner membrane) and heavy (outer membrane) fractions were pooled.

**Other biochemical techniques and chemicals.** Procedures for the preparation of the cell envelope and for SDS-polyacrylamide gel electrophoresis have been reported previously (1, 9, 14, 26). \textsuperscript{[35]S}methionine (specific activity, 1064.1 Ci/mmole) was purchased from New England Nuclear Corp, Boston, Mass. Fixed Staphylococcus aureus cells were purchased from Calbiochem-Behring, La Jolla, Calif.

**RESULTS**

Prolipoprotein accumulation in secA and secY mutant cells but not in secB mutant cells. The maturation of lipoprotein in secretion-defective mutants was studied by pulse-chase experiments which were carried out at both the permissive and nonpermissive temperatures for both secA and secY mutants, respectively, as described above. About 55% of newly synthesized lipoprotein in a secA strain after a 15-s pulse was present as precursor forms, and this amount of prolipoprotein remained constant after a 45-s chase (Fig. 1). In contrast, only 6% of newly synthesized lipoprotein in the wild-type cells was recovered as prolipoprotein after a 15-s pulse-labeling and disappeared immediately during a 15-s chase. This result indicates that prolipoprotein accumulates in the secA strain at the nonpermissive temperature. Similar results were obtained with the secY mutant at 42°C (Fig. 1). After a 15-s pulse-labeling with \textsuperscript{[35]S}methionine, 50% of newly synthesized lipoprotein in the secY mutant cells was recovered as prolipoprotein, and the amounts of prolipoprotein decreased slowly during the subsequent chase, with 40% still remaining as prolipoprotein after a 45-s chase. At the permissive temperature (30°C), no accumulation of prolipoprotein was observed in either the secA or secY mutant (data not shown). The accumulation of prolipoprotein in the secY mutant grown at the nonpermissive temperature has been previously reported by Shibata et al. (20). Similar experiments were carried out for the secB mutant strain CK1699; no prolipoprotein accumulation was observed, even though a small but significant amount of pre-OmpA protein was accumulated (Fig. 2).

Kinetics of accumulation of prolipoprotein in secA and secY mutants upon a shift to the nonpermissive temperature. The results described in Fig. 1 represent the export of lipoprotein 2 to 3 h after the shift to the nonpermissive temperature. It is conceivable that the accumulation of prolipoprotein under this condition may be a secondary consequence of the accumulation of many precursor proteins, the export of which are directly dependent on the functions of SecA or SecY proteins. We therefore compared the kinetics of accumulation of prolipoprotein and pre-OmpA protein in secA or secY mutant cells upon a temperature shift to 42°C. The accumulation of both prolipoprotein and pre-OmpA protein began between 20 to 40 min after the temperature shift in the secA mutant (Fig. 3). The extent of precursor accumulation was related to the duration of temperature shift, with 62% of lipoprotein as prolipoprotein and 55% of OmpA protein as pre-OmpA protein in a 2-min pulse at 2 h after the temperature shift. It should be noted that the kinetics of prolipoprotein accumulation is indistinguishable from that of pre-OmpA protein. In contrast, the kinetics of prolipoprotein accumulation in the secY mutant appeared to be different from that of pre-OmpA protein; the accumulation of pre-OmpA protein preceded that of prolipoprotein. The nearly identical kinetics of precursor accumulation in the secA mutant was also reflected in the reversibility of the secA\textsubscript{ts} defect upon a temperature shift to 30°C with regard to the maturation of OmpA protein and lipoprotein. Mid-log-phase cells of both secA and secY mutants grown at 30°C were incubated at 42°C for 2 and 3 h, respectively, and the cultures were returned to 30°C. Pulse-labeling at various times after the temperature shift to 30°C were carried out to
FIG. 2. Effect of secB mutation on the maturation of pre-OmpA protein and prolipoprotein. (A) Wild-type (strain MC4100) and secB7 mutant (strain CK1699) cells were pulse-labeled at 37°C with \(^{35}\)S\)methionine for 2 min, and the trichloroacetic acid-precipitated samples were immunoprecipitated with antisera against purified OmpA protein and analyzed by SDS-12% polyacrylamide gel electrophoresis. (B) The secB7 mutant (strain CK1699) cells were pulse-labeled at 37°C with \(^{35}\)S\)methionine for 15 s and chased for 15, 30, and 45 s after the addition of excess unlabeled methionine. The trichloroacetic acid-precipitated samples were immunoprecipitated with antisera against purified lipoprotein and analyzed by SDS-12.5% polyacrylamide gel electrophoresis. PLP, Prolipoprotein; LP, mature lipoprotein.

compare the recovery of export of OmpA protein and lipoprotein (Fig. 4). In the case of the secA mutant, ca. 50% of newly synthesized lipoprotein was recovered as prolipoprotein after a 2-min pulse, and this block in lipoprotein maturation was gradually reduced so that only 12% of pulse-labeled lipoprotein was recovered as prolipoprotein 40 min after the temperature shift to 30°C. Identical kinetics was observed for the resumption of OmpA maturation in the secA mutant. These data indicate that functional SecA protein returns during the 40-min incubation at the permissive temperature, and the restoration of this function affects lipoprotein and OmpA protein maturation to a similar extent. In contrast, the particular is allele of the secY mutant used in the present study did not appear to be reversible upon a temperature shift to 30°C.

Prolipoprotein accumulated in both secA and secY mutants is unmodified with glyceride and localized in the inner membrane. To determine whether or not prolipoprotein which accumulates in the secA or secY mutants is modified with glyceride, we employed SDS-polyacrylamide gel electrophoresis in two different buffer systems, sodium phosphate buffer and Tris-glycine buffer. It has been shown previously that both the unmodified and the modified prolipoprotein migrate slower than does mature lipoprotein in SDS gel with the sodium phosphate buffer system, whereas unmodified prolipoprotein and mature lipoprotein migrate faster than does the modified prolipoprotein in the Tris-glycine-buffered SDS gel system (23). Pulse-labeled membrane proteins from secA or secY mutant cells were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis, with the mature lipoprotein and the modified prolipoprotein produced by globomycin treatment (7) as the standards (Fig. 5A

FIG. 3. Accumulations of prolipoprotein and pre-OmpA protein at various time after shifting to the nonpermissive temperature (42°C). The secA or secY mutant cells grown at 30°C were shifted to 42°C. Samples (5 ml) were taken at various times after the temperature shift, and cells were pulse-labeled for 2 min with 50 µCi of \(^{35}\)S\)methionine. OmpA and lipoprotein were isolated by immunoprecipitation and analyzed by SDS-14% and SDS-12.5% polyacrylamide gel electrophoresis, respectively. After autoradiography, bands corresponding to the mature and precursor forms were sliced; the total radioactivities were measured by liquid scintillation counting, and percentages as precursors were calculated and adjusted by a factor of 0.75 and 0.83 for prolipoprotein pre-OmpA protein, respectively, based on their respective methionine contents. Symbols: (Δ) the percentage of prolipoprotein in the secA strain; (●) the percentage of prolipoprotein in the secA strain; (○) the percentage of prolipoprotein in the secY strain; and (◊) the percentage of pre-OmpA protein in the secY strain.

FIG. 4. Kinetics of protein export recovery in the secA and secY mutants after a shift from the nonpermissive to the permissive temperature. The secA and secY mutant cells were grown at 30°C and shifted to 42°C at mid-log phase; the incubation was continued for 2 and 3 h, respectively. The cultures were shifted back to 30°C, and 5-ml samples were taken at various times. Two minutes of pulse-labeling was carried out with 50 µCi of \(^{35}\)S\)methionine to measure protein processing in vivo. Other experimental details are the same as described in the legend to Fig. 2. Symbols: (Δ) the percentage of prolipoprotein in the secA strain; (●) the percentage of prolipoprotein in the secA strain; (○) the percentage of prolipoprotein in the secY strain; and (◊) the percentage of pre-OmpA protein in the secY strain.

FIG. 5. Time after shifting to 42°C
FIG. 5. Identification of unmodified prolipoprotein in both the secA and the secY strains. secA and secY mutant cells were pulse-labeled with 50 μCi of [35S]methionine for 2 min after 2 and 3 h of incubation at 42°C, respectively. The mature form of lipoprotein was isolated from the wild-type after a pulse-labeling with 50 μCi of [35S]methionine for 2 min. The wild-type cells were also treated with globomycin (50 μg/ml) for 5 min and pulse-labeled with 50 μCi of [35S]methionine for 5 min. (A) SDS-12.5% polyacrylamide gel electrophoresis (sodium phosphate buffer system); (B) SDS-15% polyacrylamide gel electrophoresis (Tris-glycine buffer system). Lane 1, Wild-type (lane 1 and 2), the membrane with membrane of membrane fractions of the cell envelope; lane 2, Sarkosyl-insoluble fraction of the cell envelope; lane 3, inner membrane fraction of the cell envelope obtained by the sucrose gradient centrifugation method; and lane 4, outer membrane fraction of the cell envelope obtained by the sucrose gradient centrifugation method.

and B, lane 1 and 2). The prolipoproteins accumulated in both secA and secY mutants migrated at the same position as did the wild-type mature lipoprotein or unmodified prolipoprotein in the SDS gel with the Tris-glycine buffer system (Fig. 5B, lane 3 and 4), whereas the modified prolipoprotein in globomycin-treated cells migrated slower than did the mature lipoprotein. In SDS gel based on the sodium phosphate buffer system, the prolipoprotein accumulated in both secA and secY mutants migrated slightly faster than did the modified prolipoprotein but slower than did the mature lipoprotein (Fig. 5A, lane 3 and 4, cf. lane 2). These data indicate that the prolipoproteins accumulated in the secA and secY mutants were unmodified.

To determine the localization of the unmodified prolipoprotein accumulated in the mutant cells, we used Sarkosyl solubilization to distinguish inner membrane proteins from outer membrane proteins. The crude membrane fractions of [35S]methionine-labeled secA or secY mutant cells were treated with Sarkosyl. OmpA protein and lipoprotein were isolated from both Sarkosyl-soluble and -insoluble fractions by immunoprecipitation with antisera against lipoprotein and OmpA protein, respectively, and analyzed by SDS-polyacrylamide gel electrophoresis. In both mutants, pre-OmpA and unmodified prolipoprotein were recovered from the Sarkosyl-soluble fraction, i.e., the inner membrane fraction (Fig. 6, lane 1 and 2, Fig. 7, lane 1 and 2). Similar results were obtained when the inner and outer membrane fractions were separated by the sucrose gradient centrifugation method (17). In both secA and secY mutants, pre-OmpA and prolipoprotein were mainly recovered from the inner membrane fraction of the cell envelope (Fig. 6, lane 3 and 4, Fig. 7, lane 3 and 4). Pre-OmpA protein and prolipoprotein were also recovered in the soluble fraction, albeit in lesser amounts than those in the membrane fractions of both secA and secY mutant cells (data not shown).

DISCUSSION

Evidence is rapidly accumulating that the mechanism of protein export is similar for procaryotic and eucaryotic cells and that, within the same species, structurally diverse proteins may share a common secretory pathway. The work of Ito et al. indicated that a common step in the protein export process exists for proteins destined for different subcellular compartments (11). These investigators demonstrated the accumulation of several precursor proteins, including prolipoprotein, in MM18 cells under conditions in which the export machinery is jammed due to an abortive export of the MalE-LacZ hybrid protein. More recently, mutants defec-

FIG. 6. Subcellular localization of pre-OmpA protein and prolipoprotein in the secA mutant. Subcellular localization of precursor proteins was determined both by the differential Sarkosyl solubility of membrane proteins (4) and by the physical separation of inner and outer membrane fractions, and analysis for precursor proteins by SDS-polyacrylamide gel electrophoresis of immunoprecipitates were described in the text. (A) Region of the gel containing pre-OmpA and OmpA proteins; and (B) region of the gel containing prolipoprotein (PLP) and lipoprotein (LP). Lane 1, Sarkosyl-soluble fraction of the cell envelope; lane 2, Sarkosyl-insoluble fraction of the cell envelope; lane 3, inner membrane fraction of the cell envelope obtained by the sucrose gradient centrifugation method; and lane 4, outer membrane fraction of the cell envelope obtained by the sucrose gradient centrifugation method.

FIG. 7. Subcellular localization of pre-OmpA and prolipoprotein in the secY mutant. Subcellular localization of precursor proteins was determined both by the differential Sarkosyl solubility of membrane proteins (4) and by the physical separation of inner and outer membrane fractions, and analysis for precursor proteins by SDS-polyacrylamide gel electrophoresis of immunoprecipitates were described in the text. (A) Region of the gel containing pre-OmpA and OmpA proteins; and (B) region of the gel containing prolipoprotein (PLP) and lipoprotein (LP). Lane 1, Sarkosyl-soluble fraction of the cell envelope; lane 2, Sarkosyl-soluble fraction of the cell envelope; lane 3, inner membrane fraction of the cell envelope obtained by the sucrose gradient centrifugation method; and lane 4, outer membrane fraction of the cell envelope obtained by the sucrose gradient centrifugation method.
tive in the secretion of outer membrane and periplasmic proteins have been isolated, and the pleiotropic nature of these mutations is consistent with the notion that the export of various proteins shares a common export machinery (13, 16, 20).

The processing of prolipoproteins is distinct from that of non-lipoprotein precursors. Both SPase I (for non-lipoprotein precursors) and SPase II (for prolipoproteins) are localized in the cytoplasmic membrane, even though their topological orientations in the inner membrane might be different (8, 25). SPase I is a peripheral membrane protein anchored at the inner membrane through its NH₂-terminal hydrophobic segment, and the bulk of the SPase I molecule in intact spheroplasts appears to be accessible to protease digestion (25). This subcellular localization is consistent with the processing of signal peptides as a late event in protein export (12), and even for cotranslational processing, the nascent chain is not translocated (and thereby not processed) until ca. 80% of polypeptide synthesis is completed (19). In contrast, SPase II is postulated to be an integral inner membrane protein (8). This topological orientation may be related to the nature of its substrate, glyceride-modified prolipoproteins. Because processing of prolipoproteins requires prior modification (22), this is also a late event in a series of novel posttranslational modification and processing reactions.

The results reported in this paper can be illustrated by the schematic diagram of Fig. 8. The early steps for protein export are common for prolipoproteins and other precursor proteins. These steps presumably involve the recognition of signal peptides of precursor proteins by a putative export machinery analogous to the signal recognition particles in eucaryotic cells (24). That this hypothetical export machinery does not distinguish prolipoprotein signal sequences from those of non-lipoprotein precursor proteins is not surprising. The general features of signal peptides in prolipoproteins follow much the same general pattern of all signal peptides, i.e., a short positively charged hydrophilic segment at the NH₂-terminus, a hydrophobic core in the middle that is long enough to span the lipid bilayer, and finally, a stretch of neutral amino acids with short side-chains preceding the cleavage site (10). The major difference between the signal peptides in prolipoproteins and those of nonlipoprotein precursors lies in the sequence in the vicinity of the cleavage site. For SPase I, Ala-X-Ala is favored as the sequence immediately preceding the cleavage site (18). For prolipoproteins, Leu-Ala-Gly-Cys is recognized by the glycerol transferase, and the glyceride-modified cysteine in prolipoprotein is recognized by SPase II (27). It is therefore reasonable to postulate that the divergence of the export pathway for lipoproteins and non-lipoproteins occurs in the cytoplasmic membrane during or immediately after the translocation across the cytoplasmic membrane. The enzymes involved in the posttranslational modification or processing will convert precursors with Leu-Ala-Gly-Cys into lipid-modified precursor proteins, and they will be subsequently processed by SPase II. That these two pathways are not compartmentalized in the cytoplasmic membrane is demonstrated by the observation that a mutant pre- penicillinas (which is not modifiable due to the mutational conversion of Cys into Ser at the modification or cleavage site) can nevertheless be processed at two alternative sites, both preceded by an alanine (5). The rate or extent of alternative processings may also depend upon the overall conformations of the precursor proteins (5).

Although the effects of the secA mutation on OmpA protein and lipoprotein export are indistinguishable, the export of lipoprotein appears to be less affected than that of pre-OmpA protein in the secY mutant, and the effect on lipoprotein export occurs later than that observed for the export of the OmpA protein. secY is an allele of the prlA gene and is presumably involved in signal peptide recognition by virtue of the ability of prlA mutations to suppress signal sequence mutations (3). Whether this quantitative and kinetic difference in the export of OmpA protein and lipoprotein in the secY mutation reflects a subtle difference in the interaction of OmpA signal peptide or lipoprotein signal peptide with the defective SecY protein remains to be determined. In addition, we cannot deduce from the results presented here the sequence of events in which the secY and secA gene products participate in protein export. Unlike the export of OmpA protein, the export of lipoprotein appears to be unaffected in the secB mutant.

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