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Assembly of Outer Membrane Lipoprotein in an *Escherichia coli* Mutant with a Single Amino Acid Replacement Within the Signal Sequence of Prolipoprotein

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We have compared the rate of assembly of outer membrane proteins including the lipoprotein in a pair of isogenic *mlpA*$^+$ (*lpp*$^+$) and *mlpA* (*lpp*) strains by pulse-chase experiments. The rate of assembly of the mutant prolipoprotein into the outer membrane was slightly slower than that of the wild-type lipoprotein. The rate of assembly of protein I and protein H-2 was similar in the wild type and the mutant, whereas the rate of assembly of protein II* into the outer membrane was slightly reduced in the mutant strain. The organization of outer membrane proteins in the mutant cells appeared not to be grossly altered, based on the apparent resistance (or susceptibility) of these proteins toward trypsin treatment and their resistance to solubilization by Sarkosyl. Like the wild-type lipoprotein, the mutant prolipoprotein in the outer membrane was resistant to trypsin. On the other hand, the prolipoprotein in the cytoplasmic membrane fraction of the mutant cell envelope was susceptible to trypsin digestion. We conclude from these data that proteolytic cleavage of prolipoprotein is not essential for the translocation and proper assembly of lipoprotein into outer membrane.

We have isolated and characterized an *Escherichia coli* mutant altered in the structural gene for the murein lipoprotein (*mlpA* or *lpp*) (12-15). We have shown that the mutant lipoprotein contains a single amino acid substitution (glycine → aspartic acid) within the signal sequence of the prolipoprotein (7). As a result of this amino acid substitution, the mutant prolipoprotein is not processed to the mature form of lipoprotein, nor is it covalently attached to the murein sacculus. The cellular localization of the lipoprotein is also altered in this mutant. Unlike the wild-type lipoprotein which is exclusively located in the outer membrane, the mutant prolipoprotein could be detected in the soluble fraction, the cytoplasmic membrane, and the outer membrane fractions of the *mlpA* mutant cells (7, 12). These results suggest that the uncleaved prolipoprotein can be translocated into the outer membrane. The presence of significant amounts of the mutant prolipoprotein in the soluble fraction as well as in the cytoplasmic membrane fraction raises an interesting possibility that the kinetics of assembly of the mutant prolipoprotein into the outer membrane may be significantly altered.

In the present study, we have employed pulse-chase experiments to compare the rate of assembly of outer membrane proteins in both the wild-type and the *mlpA* mutant cells. The results indicate that the uncleaved prolipoprotein is assembled into the outer membrane more slowly than the mature wild-type lipoprotein. The rate of assembly of protein II* appears also to be reduced in the *mlpA* mutant. The prolipoprotein in the outer membrane of the mutant cells was indistinguishable from the wild-type lipoprotein in the resistance both to trypsin digestion and to the solubilization with Sarkosyl.

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MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* strains used in the present study were the wild-type strain E613 (*mlpA*$^+$) and the isogenic transductant strain E614 (*mlpA*) (15). Culture media included M9 minimal medium and proteose peptone beef extract broth (15).

Pulse-chase experiment. A 300-ml culture of M9 minimal medium supplemented with 0.4% glucose, 50 μg each of required amino acids per ml (except arginine at 10 μg/ml), and 5% (vol/vol) of proteose peptone beef extract broth medium was used for the growth and prelabeling of bacterial cells with 30 μCi of [^{14}C]arginine for 2.5 generations at 37°C. The cells were harvested in the mid-exponential phase of growth (absorbance at 600 nm, 0.4 to 0.5) by centrifugation (2 min, 16,300 × *g*, 25°C) and resuspended in 20 ml of the same medium. The cell suspension was then preincubated at 25°C in a water bath shaker for 5 min. A 1-mCi quantity of [^{3}H]arginine was added, and the culture was aerated vigorously for 10 s at 25°C. At this

time, 20 ml of unlabeled arginine (60 mg/ml) was quickly added to the culture, and then 10 ml of culture was quickly pipetted into a mixture of 10 g of crushed ice, 1 ml of 2% NaN_3, 1 ml of chloramphenicol (0.2%), and 1 ml of 0.1 M 2,4-dinitrophenol. This was taken as the pulse-labeled sample. The remainder was further incubated at 25°C, and samples were taken at 30, 60, and 120 s. At each time point, 10 ml of culture was pipetted into the same ice mixture as described above. The cells were collected immediately by centrifugation (1 min, 17,300 × *g*, 4°C) and quickly suspended in 3 ml of 0.75 M sucrose–10 mM Tris-acetate (pH 7.8)-2.5 mM dinitrophenol containing 200 μg of lysozyme. Preparation of spheroplasts and isolation of cytoplasmic and outer membranes were performed by the method described by Osborn et al. (8). The cytoplasmic and outer membranes were recovered from the sucrose gradient fractions by centrifugation at 275,000 × *g* for 2 h at 4°C. The pellets were dissolved in a small volume of 1% sodium dodecyl sulfate (SDS) in 1 mM sodium phosphate buffer at pH 7.0. A portion of each membrane fraction was analyzed in SDS-polyacrylamide gel electrophoresis.

Isolation of radioactive labeled membranes. Wild-type cells were labeled with either [^{14}C]arginine (0.5 μCi/ml) or [^{14}C]leucine (0.5 μCi/ml) for 2.5 generations; likewise, the mutant cells were labeled with either [^{3}H]arginine (1 μCi/ml) or [^{3}H]leucine (5 μCi/ml). Outer and cytoplasmic membranes were isolated from labeled cells. To compare the susceptibility of membrane proteins toward trypsin digestion or solubilization with Sarkosyl between the wild-type and mutant cells, the ^{14}C-labeled outer membrane from wild-type cells was mixed with equal amounts of radioactivity of ^{3}H-labeled outer membrane from mutant cells. Likewise, the cytoplasmic membranes from ^{14}C-labeled wild-type and ^{3}H-labeled mutant cells were mixed accordingly.

Trypsin treatment. The outer and cytoplasmic membranes were treated with 50 μg of trypsin per ml (192 U/mg, Worthington Co.) in 1 ml of 10 mM sodium phosphate buffer (pH 7.0) for 5 min or 30 min at 37°C. At the end of the incubation, 400 μg of soybean trypsin inhibitor (Sigma Chemical Co.) was added to terminate the reaction. The membranes were collected by ultracentrifugation (163,000 × *g*, 2 h, 4°C) and analyzed by 10% SDS-polyacrylamide gel electrophoresis or SDS-urea-polyacrylamide gel electrophoresis (11).

Sarkosyl treatment. The outer membranes were treated with 1 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.5% sodium lauryl sarcosinate (Sarkosyl) for 20 min at room temperature (4). The Sarkosyl-insoluble fraction was collected by ultracentrifugation (163,000 × *g*, 2 h, 4°C) and analyzed by 10% SDS-polyacrylamide gel electrophoresis.

Other biochemical techniques. Ten percent SDS-polyacrylamide gel electrophoresis was carried out by the method of Inouye and Guthrie (5). The polyacrylamide gel was sliced into 1-mm fractions by a Gilson gel slicer and extracted in 0.5 ml of a 2% SDS solution. A 7-ml amount of scintillation fluid (160 ml of Liquifluor, 500 ml of Bio-solv, and toluene made up to 4 liters) was added to each fraction. The radioactivity was measured in a Beckman LS-230 liquid scintillation counter. The procedure for immunoprecipitation of lipoprotein has been described previously (13).

Chemicals and radiochemicals. All chemicals were of reagent grade and were purchased from commercial sources. Radioactive chemicals used in the present study included L-[3-^{3}H(N)]arginine (27 Ci/mmol), L-[*U*-^{14}C]arginine (309 mCi/mmol), L-[4,5-^{3}H]leucine (42 Ci/mmol), and L-[*U*-^{14}C]leucine (270 mCi/mmol) and were purchased from either New England Nuclear Corp. or Schwarz/Mann.

RESULTS

Assembly of outer membrane proteins in the *mlpA* mutant. The wild-type (strain E613) and mutant (strain E614) cells prelabeled with [^{14}C]arginine for 2.5 generations were pulse-labeled for 10 s at 25°C with [^{3}H]arginine and chased for 30, 60, and 120 s in the presence of 30 mg of unlabeled arginine per ml. Proteins in the isolated outer and cytoplasmic membrane fractions were analyzed by 10% SDS-polyacrylamide gel electrophoresis, and the gel profiles for the mutant cells are shown in Fig. 1 and 2. To compare the rate of assembly of individual outer membrane proteins (I, H-2, II*, and lipoprotein) between the wild-type and mutant cells, the total counts of [^{3}H]arginine incorporated into each of the outer membrane proteins were integrated from the gel profiles and normalized with constant [^{14}C]arginine counts to allow for variation in the recovery. To rule out the possibility that immature polypeptides of other cytoplasmic membrane proteins contaminated the lipoprotein peak in the SDS gel, the cytoplasmic membrane fractions were immunoprecipitated by antisera against purified lipoprotein and analyzed by SDS gel electrophoresis. The ^{3}H/^{14}C ratio of lipoprotein in the immunoprecipitate was almost identical to that of the lipoprotein peak identified in the SDS gel of the total cytoplasmic membrane fraction (data not shown). This result suggests that there has not been significant contamination in the region of the gel containing the lipoprotein peak by incomplete polypeptides of other cytoplasmic membrane proteins. Figure 3 shows the rate of assembly of lipoprotein in wild-type and *mlpA* mutant cells. The assembly of mutant prolipoprotein differs from that of wild-type lipoprotein in several aspects. (i) The rate of assembly of the mutant prolipoprotein into the outer membrane (H fraction) was slightly slower than that of the wild-type lipoprotein even though we were unable to obtain the true initial rate of lipoprotein assembly. (ii) Pulse-labeled lipoprotein in the outer membrane of the wild-type cells decreased slightly at the end of the 2-min chase; such a decrease was not seen with the mutant cells. The decrease of [^{3}H]arginine-labeled lipoprotein after a 2-min chase in the wild-type cells was

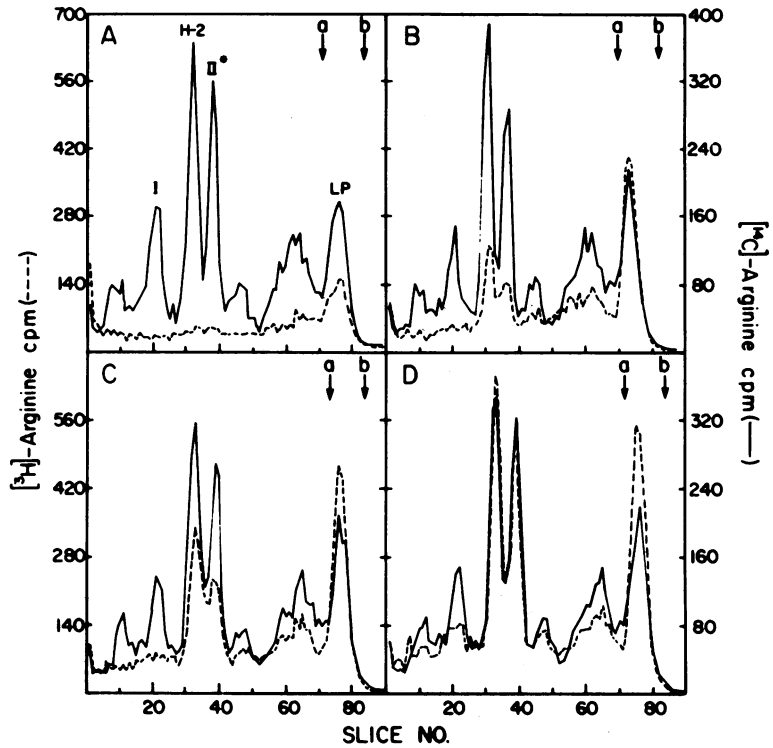

FIG. 1. *Assembly of outer membrane proteins in the mlpA mutant. [^{14}C]arginine was used to prelabel the mutant cells for 2.5 generations and [^{3}H]arginine was used for pulse-labeling. (A) 10-s pulse; (B) 30-s chase; (C) 60-s chase; (D) 120-s chase. The outer membrane fraction of each sample was analyzed by 10% SDS-polyacrylamide gel electrophoresis. a, cytochrome c; b, pyronine Y dye.*

most likely due to the conversion of the free-form lipoprotein into the murein-bound form. This conversion does not take place in the *mlpA* mutant. (iii) The wild-type lipoprotein transiently accumulated in the cytoplasmic membrane at the end of the 10-s pulse and could be chased into the outer membrane. The fact that wild-type lipoprotein in the cytoplasmic membrane fraction could not be completely chased into the outer membrane is most likely due to contamination of the L fraction by the H fraction. The pulse-labeled mutant lipoprotein persisted in the cytoplasmic membrane and did not decrease after the chase. These data taken together suggest that the assembly of lipoprotein into the outer membrane is altered in the *mlpA* mutant.

The assembly of outer membrane proteins I, H-2, and II* in both the wild type and the *mlpA* mutant is shown in Fig. 4. We have calculated the relative rate of assembly of individual outer membrane proteins, as shown in Table 1. The equation for the linear regression was used to calculate the slope from the plot of the ^{3}H/^{14}C ratio versus the chase period. This slope would represent the relative rate of assembly of each outer membrane protein. The coefficient of determination (r^2) ranged from 0.96 to 1.00. Therefore, the relative rate determined by this method represents the initial rate for all these proteins except lipoprotein. As can be seen in Table 1, the rates of assembly of protein I and H-2 are similar in the wild-type and the *mlpA* mutant. On the other hand, the rates of assembly of protein II* and lipoprotein in the mutant are 65.08 and 81.08% of that in the wild-type cells, respectively. Since we could not measure the true initial rate of assembly of lipoprotein for both the mutant and wild-type cells, this 20% reduction in the rate of assembly of the mutant prolipoprotein may be an underestimation.

Trypsin treatment of outer and cytoplasmic membranes of the mutant and wild-type cells. To ascertain whether or not the mutant prolipoprotein is assembled in a normal fashion, we have compared the susceptibility of the outer and cytoplasmic membrane proteins from ^{3}H-labeled mutant cells and ^{14}C-labeled wild-type cells toward trypsin digestion. It has been shown by Braun et al. that for the wild-

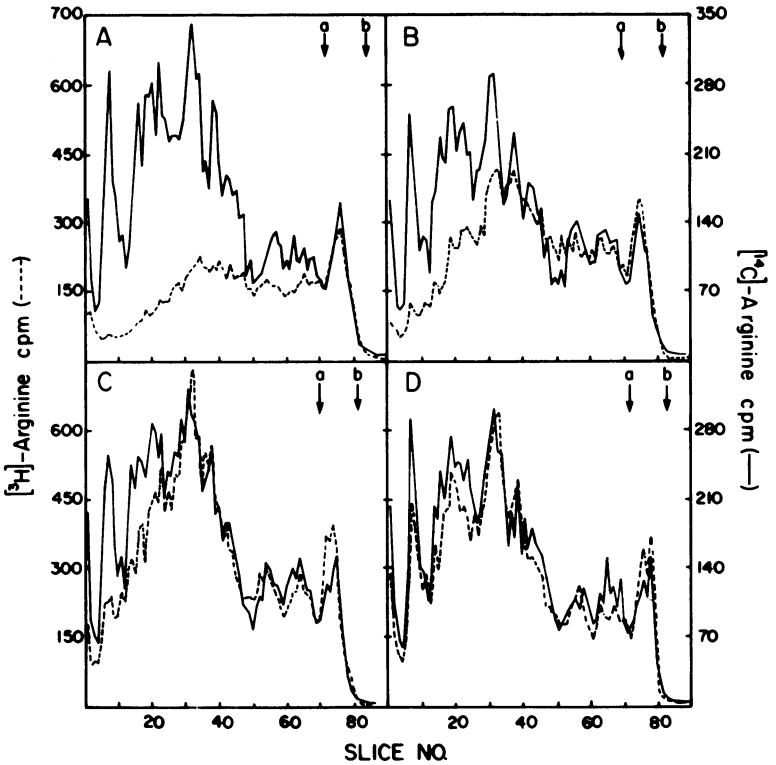

FIG. 2. *Assembly of cytoplasmic membrane proteins in the mlpA mutant. [^{14}C]arginine was used for prelabeling, and [^{3}H]arginine was used for pulse-labeling; (A) 10-s pulse; (B) 30-s chase; (C) 60-s chase; (D) 120-s chase. The cytoplasmic membrane fraction of each sample was analyzed by 10% SDS-polyacrylamide gel electrophoresis. a, cytochrome c; b, pyronine Y dye.*

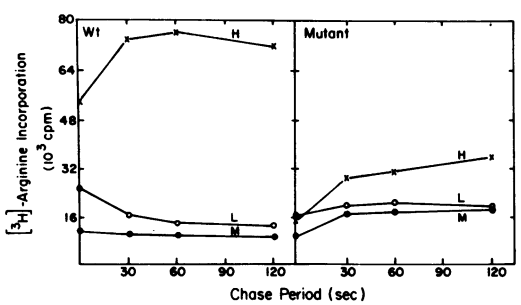

FIG. 3. *Rate of assembly of the lipoprotein in the wild-type (Wt) and mlpA mutant cells. H, outer membrane fraction; M, mixed membrane fraction; L, cytoplasmic membrane fraction.*

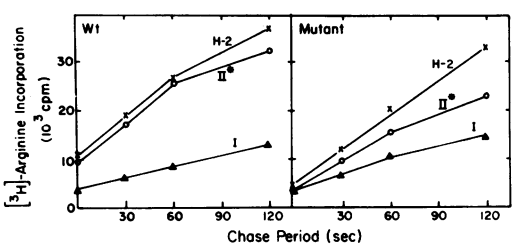

FIG. 4. *Rate of assembly of outer membrane proteins I, H-2, and II* in the wild-type and mlpA mutant cells.*

type lipoprotein, both in the native form present in the cell envelope (2) and in a purified state (1), two out of eight potential cleavage sites are exposed and accessible to trypsin. These two sites (lysine at the 55th position and arginine at residue 57) are both located near the COOH terminus of the molecule. We have observed that the mutant prolipoprotein in its purified form is much more sensitive to trypsin than the wild-type molecule (data not shown). Therefore, the apparent susceptibility of mutant prolipoprotein to trypsin may indicate that the mutant prolipoprotein is not properly assembled into the outer membrane. Isolated outer and cytoplasmic membranes were treated with trypsin, recovered by centrifugation at 163,000 × *g* for 2 h, and analyzed by 10% SDS-polyacrylamide gel electrophoresis. The ^{3}H/^{14}C ratio of individual proteins was calculated from the gel profile, and

the results are shown in Table 2. It should be noted that in the control (untreated) sample the $^3H/^{14}C$ ratio of the lipoprotein in the outer membrane was lower than that of the bulk of outer membrane proteins, whereas the $^3H/^{14}C$ ratio of lipoprotein in the cytoplasmic membrane was higher than that of the bulk of cytoplasmic membrane proteins. This is because of a selective enrichment of the mutant prolipoprotein in the cytoplasmic membrane (7). The $^3H/^{14}C$ ratio of each of these outer membrane proteins remained unchanged before and after the trypsin digestion, whereas the $^3H/^{14}C$ ratio of the lipoprotein in the cytoplasmic membrane decreases substantially after the trypsin treatment for 5 and 30 min, respectively.

It could be argued that the region of the gel containing the lipoprotein peak may be contaminated with the tryptic fragments of larger proteins, especially in the case of the cytoplasmic membrane. To rule out this possibility, we have immunoprecipitated lipoprotein from control and trypsin-treated samples with specific antisera, and the immunoprecipitates were analyzed by SDS-urea-polyacrylamide gel electrophoresis which gives a better separation for the small peptides. The results are shown in Fig. 5 to 7 and Table 2. As can be seen in Fig. 5A, 6A, and 7A, the mutant prolipoprotein migrated more

TABLE 1. *Relative rate of assembly of individual outer membrane proteins in the wild-type (E613) and the mutant (E614) strains*

Protein	Relative rate of assembly[a] (%)		
	Mutant (E614)	Wild-type (E613)	E614/E613
I[b]	0.50	0.43	116.28
H-2[b]	0.86	0.81	106.17
II*[b]	0.82	1.26	65.08
Lipoprotein[c]	1.80	2.22	81.08

[a] The relative rate of assembly is calculated from the slope of the straight line for each outer membrane protein shown in Fig. 3 and 4 and is expressed as increase in $^3H/^{14}C$ ratio per minute.

[b] The initial rate is calculated from the first three points of the chase in Fig. 4.

[c] This initial rate calculated from the first two points of the chase shown in Fig. 3 is most likely underestimated.

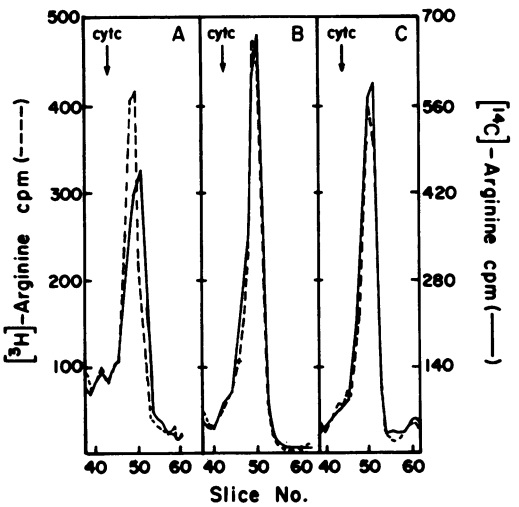

FIG. 5. *SDS-urea-polyacrylamide gel electrophoresis of lipoprotein immunoprecipitates of the mixed outer membranes from [^{3}H]arginine-labeled mutant and [^{14}C]arginine-labeled wild-type cells. (A) Control; (B) trypsin, 5 min; (C) trypsin, 30 min. Arrows indicate the positions of cytochrome c standard.*

TABLE 2. *Trypsin treatment of outer and cytoplasmic membranes from mutant cells (E614) labeled with [^{3}H]leucine or arginine and wild-type cells (E613) labeled with [^{14}C]leucine or arginine*

Protein	$^3H/^{14}C$ ratio					
	Leucine labeled			Arginine labeled		
	Control	5 min	30 min	Control	5 min	30 min
Outer Membrane						
I	2.53	2.59	2.60	1.32	1.34	1.33
H-2	2.47	2.52	2.44	1.16	1.16	1.11
II*	2.60			1.20		
II[a]		2.40	2.45		1.28	1.31
Lipoprotein[b]	2.25	1.83	1.62	0.73	0.66	0.65
Cytoplasmic membrane						
Lipoprotein[a]	5.01	3.97	3.54	2.48	2.05	1.84
All proteins[c]	2.58	2.63	2.59	1.41	1.40	1.42

[a] New protein peak is derived from protein II* after trypsin digestion.

[b] The $^3H/^{14}C$ ratio of lipoprotein was calculated from the peak in the SDS-urea-polyacrylamide gel profile of immunoprecipitate of each sample.

[c] The average ratio for total cytoplasmic membrane proteins.

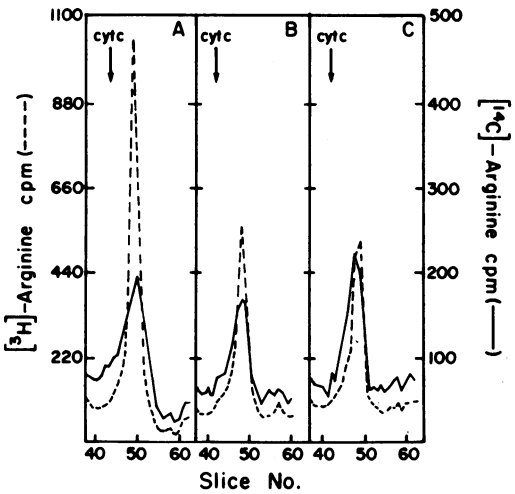

FIG. 6. *SDS-urea-polyacrylamide gel electrophoresis of lipoprotein immunoprecipitates of the mixed cytoplasmic membranes from [^{3}H]arginine-labeled mutant and [^{14}C]arginine-labeled wild-type cells. (A) Control; (B) trypsin, 5 min; (C) trypsin, 30 min. Arrows indicate the positions of cytochrome c standard.*

slowly than the wild-type lipoprotein. This is due to the higher molecular weight of the mutant prolipoprotein (7). After a 5-min treatment of trypsin, the ^{3}H/^{14}C ratio of arginine-labeled lipoprotein decreased (Fig. 5B). This decrease in the ^{3}H/^{14}C ratio can be quantitatively accounted for by the release of [^{14}C]arginine-labeled bound form of lipoprotein in the wild type from the murein layer by the trypsin treatment (6); the *mlpA* mutant cells are devoid of bound-form lipoprotein (13). After trypsin treatment, the total [^{14}C]arginine radioactivity increased substantially (Fig. 5B) due to the release of bound-form lipoprotein from the partially digested murein which did not readily enter the SDS-urea gel. However, the ^{3}H/^{14}C ratio remained constant after a 30-min digestion (Fig. 5C) even though the total counts of both [^{3}H]- and [^{14}C]arginine-labeled lipoprotein decreased, due to the release of one of the labeled arginine residues at the COOH terminus. It can be concluded that the bulk of mutant prolipoprotein is indeed assembled into the outer membrane in a way very similar to that in the wild-type cells. However, the slight shift in the mobility of the mutant prolipoprotein relative to that of the wild-type lipoprotein after the trypsin treatment of the outer membrane suggests that the NH_2 terminus of the mutant lipoprotein may be selectively sensitive to trypsin, especially at the second and fifth lysine residues of prolipoprotein. Since labeled arginine residues are located near the COOH terminus, the ^{3}H/^{14}C ratio of lipoprotein would not be drastically decreased. This conclusion is further supported by a similar experiment with leucine-labeled outer membranes (Fig. 7). Although the total counts of [^{14}C]leucine-labeled wild-type lipoprotein did not change between 5- and 30-min treatments of trypsin, the [^{3}H]leucine-labeled mutant lipoprotein decreases significantly with the longer time of trypsin treatment. This is consistent with the conclusion that the NH_2 terminus of the mutant lipoprotein in the outer membrane is more sensitive to trypsin digestion.

The gel patterns of the immunoprecipitable lipoprotein before and after the trypsin treatment of the arginine-labeled cytoplasmic membrane are shown in Fig. 6. It is obvious that the ^{3}H/^{14}C ratio of lipoprotein decreases significantly after the trypsin treatment. Furthermore, the longer the trypsin treatment, the faster the mobility of the [^{3}H]arginine-labeled mutant prolipoprotein. These results indicate that the mutant prolipoprotein accumulated in the cytoplasmic membrane fraction has a different conformation or environment from that in the outer membrane. It further suggests that the mutant lipoprotein recovered in the cytoplasmic membrane is not due to an excessive contamination with outer membrane material in this fraction.

Sarkosyl solubilization of outer membrane proteins of the mutant and wild-type cells. It has been shown previously that sodium lauryl sarcosinate (Sarkosyl) selectively solubi-

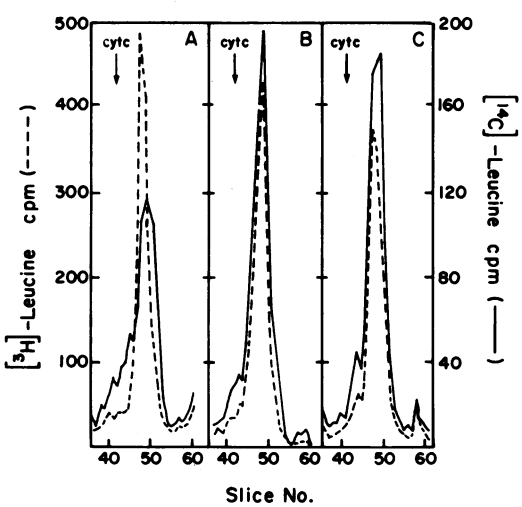

FIG. 7. *SDS-urea-polyacrylamide gel electrophoresis of lipoprotein immunoprecipitates of the mixed outer membranes from [^{3}H]leucine-labeled mutant and [^{14}C]leucine-labeled wild-type cells. (A) Control; (B) trypsin, 5 min; (C) trypsin, 30 min. Arrows indicate the positions of cytochrome c standard.*

lizes the cytoplasmic membrane proteins but not the outer membrane proteins (4). If the mutant lipoprotein is assembled into the outer membrane in the normal fashion as an integral membrane protein, it would not be solubilized by Sarkosyl treatment. On the other hand, it might be selectively released from the outer membrane by Sarkosyl treatment if it was simply adsorbed to the outer membrane like a peripheral membrane protein. We have mixed ^{3}H-labeled outer membrane of the mutant cells with ^{14}C-labeled outer membrane of the wild-type cells. This mixed outer membrane was treated with 0.5% Sarkosyl by the method of Filip et al. (4). The Sarkosyl-insoluble fraction was collected by centrifugation at 163,000 × *g* for 2 h at 4°C and analyzed by 10% SDS-polyacrylamide gel electrophoresis. The $^{3}H/^{14}C$ ratio of the individual outer membrane proteins was calculated from the gel profile. The results are shown in Table 3. Both the leucine- and arginine- labeled lipoproteins showed little changes in the $^{3}H/^{14}C$ ratio before and after Sarkosyl treatment. This result suggests that Sarkosyl did not selectively remove the mutant lipoprotein from the outer membrane. The recovery of each outer membrane protein after Sarkosyl treatment is about 70 to 80% for either the mutant or the wild-type cells. Therefore, we conclude that the majority of the mutant prolipoprotein is assembled into the outer membrane in a way indistinguishable from that of the wild-type lipoprotein. It indicates that the proteolytic cleavage of the prolipoprotein is not essential for the translocation and proper assembly of lipoprotein into the outer membrane of the cell envelope.

TABLE 3. *Sarkosyl treatment of the mixed outer membranes from [^{3}H]arginine- or [^{3}H]leucine-labeled mutant cells (E614) and [^{14}C]arginine- or [^{14}C]leucine-labeled wild-type cells (E613)*

Protein	$^{3}H/^{14}C$ ratio			
	Leucine labeled		Arginine labeled	
	Control[a]	Sarkosyl treated[b]	Control[a]	Sarkosyl treated[b]
I	2.53	2.45	1.32	1.43
H-2	2.47	2.53	1.16	1.31
II*	2.60	2.62	1.20	1.34
Lipoprotein	1.83	1.68	0.82	0.75

[a] The control sample was treated in the same way as the Sarkoyl-treated sample except 1 ml of 10 mM sodium phosphate buffer (pH 7.0) was used instead.

[b] The mixed outer membrane was treated with 1 ml of 0.5% Sarkosyl in 10 mM sodium phosphate buffer (pH 7.0) at the room temperature for 20 min. Outer membranes were recovered by ultracentrifugation and analyzed by 10% SDS-polyacrylamide gel electrophoresis.

DISCUSSION

In the present study, we have shown that the rate of assembly of the uncleaved but genetically altered prolipoprotein into the outer membrane is 20% slower than that of the wild-type mature lipoprotein. This reduction in the rate of assembly of the mutant prolipoprotein into the outer membrane cannot account for the accumulation of lipoprotein in the cytoplasmic membrane of the mutant cells. In contrast to the wild-type cells, pulse-labeled lipoprotein in the cytoplasmic membrane of the mutant cells cannot be efficiently chased into the outer membrane. Instead, the reduced rate of assembly of the lipoprotein in the mutant may be attributed to an anomalous conformation of the mutant prolipoprotein. Alternatively, the mutant lipoprotein may be more easily detached from the outer membrane, resulting in an apparent reduction in the rate of assembly. These two possibilities are not mutually exclusive.

Although the rate of assembly of the mutant prolipoprotein is slightly reduced as compared with that of the mature wild-type lipoprotein, the results presented in this paper provide the first direct evidence that proteolytic cleavage of the signal sequence of the prolipoprotein is not essential for the translocation and proper assembly of this outer membrane protein. The mutant prolipoprotein in the outer membrane is indistinguishable from the wild-type in its resistance to trypsin digestion and to extraction with Sarkosyl. The mutant prolipoprotein in the outer membrane does not appear to represent a simple insertion into the outer membrane by hydrophobic interaction between the lipid bilayer and the signal sequence.

The mutant prolipoprotein in the outer membrane is poorly assembled into the murein sacculus (13). It is possible that the conversion of the free-form lipoprotein to the murein-bound form requires the proteolytic cleavage of prolipoprotein and the attachment of fatty acids at the NH_2 terminus of the mature lipoprotein. Alternatively, the amino acid substitution has affected the conformation of the mutant prolipoprotein so that it cannot be covalently linked to the murein sacculus.

We have detected a transient intermediate of processed lipoprotein in the cytoplasmic membrane fraction during a 10-s pulse-labeling of the wild-type cells. The lipoprotein in the cytoplasmic membrane can be chased into the outer membrane (Fig. 3). In contrast, the pulse-labeled lipoprotein in the cytoplasmic membrane of the *mlpA* mutant does not decrease during the subsequent chase (Fig. 3). The reason for the difference in the exit of lipoprotein from the cytoplas-

mic membrane between the wild-type and the mutant cells remains unknown.

Whereas the rates of assembly of outer membrane protein I and H-2 are similar in the wild-type and mutant cells, the rate of assembly of protein II* is significantly reduced in the *mlpA* mutant. Protein II* has been shown to interact with lipoprotein either directly or physiologically. Protein II* is a transmembrane protein and can be cross-linked to both murein and free-form lipoprotein (3, 9). The presence of either protein II* or bound-form lipoprotein is essential for the structural integrity of the outer membrane. Double mutants lacking protein II* and bound-form lipoprotein have been shown to be greatly weakened in the anchorage of the outer membrane onto the rigid layer of the cell envelope (10, 15). These mutant cells also assume a spherical morphology, in contrast to the rod shape of the wild-type or single mutant cells (10, 15). It is tempting to speculate that the murein-bound form of lipoprotein provides a binding site for the assembly of protein II* into the outer membrane. The reduction in the murein-bound form of lipoprotein in the *mlpA* mutant may affect the rate of assembly of protein II* into the outer membrane due to a weaker secondary interaction between protein II* and the outer membrane-murein complex.

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