Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4891–4895, October 1978
Biochemistry

An *Escherichia coli* mutant with an amino acid alteration within the signal sequence of outer membrane prolipoprotein

(membrane assembly/precursor/signal hypothesis)

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Communicated by M. J. Osborn, August 9, 1978

ABSTRACT **Lipoprotein has been purified from an *Escherichia coli* strain carrying a mutation in the structural gene for murein lipoprotein (*mlpA*). Amino acid analysis of the purified mutant lipoprotein indicates that the mutant lipoprotein corresponds to the uncleaved prolipoprotein with a single amino acid replacement of glycine with aspartic acid. Automated Edman degradation has established the precise location of this amino acid substitution to be at the 14th residue of the prolipoprotein. This alteration in the signal sequence of prolipoprotein results in a failure of the mutated prolipoprotein to be processed. Furthermore, the structural alteration in the mutant lipoprotein appears also to have affected its topological localization in the mutant cell. Whereas lipoprotein in the wild-type strain is exclusively located in the outer membrane of the cell envelope, the membrane-bound lipoprotein in this mutant is recovered in both the inner and outer membranes of the cell envelope. The data suggest, however, that proteolytic cleavage of prolipoprotein to form mature lipoprotein is not essential for the translocation and assembly of lipoprotein into the outer membrane.**

One of the major outer membrane proteins in Gram-negative enteric bacteria is the murein lipoprotein discovered by Braun and his coworkers (1, 2). More recently, Inouye and his coworkers (3) have characterized a precursor form of lipoprotein, the prolipoprotein, which contains 20 extra amino acids at the amino terminus. It has been suggested that these extra amino acids at the amino terminus of prolipoprotein may constitute the so-called signal sequence, postulated to play an important role in the biogenesis of this outer membrane protein.

We have previously described the isolation and characterization of an *Escherichia coli* mutant altered in the structure of murein lipoprotein (4). The biochemical phenotype of this mutant lipoprotein includes a deficiency in covalently linked diglyceride, a defect in the assembly of the free form of mutant lipoprotein into the murein sacculus, and an apparent increase in the size of the mutant lipoprotein as compared to that of wild type (5). In this paper, we present evidence showing that the mutant lipoprotein corresponds to the uncleaved prolipoprotein with a single amino acid replacement within the signal sequence of prolipoprotein, a glycyl residue at position 14 being substituted by aspartate. We also find that while the majority of the mutant lipoprotein is assembled into the outer membrane of the cell envelope, there is a significant amount of lipoprotein in the inner membrane.

MATERIALS AND METHODS

Bacterial Strains and Media. The *E. coli* strains used in the present study were wild-type E600 (*mlpA*$^+$) and mutant E602 (*mlpA*$^-$) as described (4). Isogenic transductant strains E613 (*mlpA*$^+$) and E614 (*mplA*$^-$) (6) were used in the study of lipoprotein localization in the cell envelope. Media used in the present study included M9 minimal medium and proteose peptone beef extract broth (6)

Purification of Murein Lipoprotein from Wild-Type Strain E600 and Mutant Strain E602. The free form of murein lipoprotein was purified by a modification of the procedure described by Inouye *et al.* (7). The details of the modified procedure for purification will be published in a separate communication. The last step of purification was a preparative polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) and urea, which ensures complete separation of murein lipoprotein from a low molecular weight contaminating protein. The purity of the murein lipoprotein used for structural studies was confirmed by polyacrylamide gel electrophoresis and amino acid analysis. There was a single band both in $NaDodSO_4$ gel electrophoresis (8) and in $NaDodSO_4$/urea gel electrophoresis (9). Purified mutant lipoprotein contained no histidine, proline, or phenylalanine, but a significant amount of glycine was present (see Table 1).

Amino Acid Analysis. Lipoprotein was hydrolyzed in sealed evacuated ampoules containing 1 ml of 6 M HCl at 105° for 24, 48, and 72 hr. The hydrolysates were evaporated to dryness and dissolved in sample buffer at pH 2.2 for amino acid analysis, which was carried out in a Beckman model 121 analyzer. The contents of glycerylcysteine, cysteine, and methionine were determined in lipoprotein by performic acid oxidation of the free form of lipoprotein according to Hirs (11). Glycerylcysteine was estimated as described (12).

Sequence Determination. Purified free form of mutant lipoprotein (168 nmol) was dissolved in 0.4 ml of 88% formic acid and subjected to automated Edman degradation with a Beckman model 890C sequencer using a peptide program 102974 supplied by the manufacturer (13). The phenylthiohydantoin derivatives of the amino acids (PTH-amino acids) were identified both by high-pressure liquid chromatography (Waters Associates System) and by amino acid analysis after back conversion of derivatives to the corresponding amino acids by hydrolysis in hydriodic acid vapor as described (13).

Isolation of Labeled Lipoprotein. Mutant prolipoprotein labeled with [^{3}H]isoleucine was prepared from toluene-treated cells according to Halegoua *et al.* (14). Labeled prolipoprotein was purified by immunoprecipitation and preparative $NaDodSO_4$ gel electrophoresis (3). For preparation of mutant lipoprotein labeled *in vivo*, intact cells were labeled with [^{3}H]arginine, [^{14}C]arginine, or [^{3}H]lysine. Envelope proteins were isolated and immunoprecipitated with rabbit antiserum raised against lipoprotein as described (4). The immunoprecipitate was solubilized in 1% $NaDodSO_4$/50 mM ammonium

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

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acetate (pH 7.0) and applied to a Sephadex G-100 column (1.8 × 50 cm) that had been equilibrated with the solubilizing buffer. The radioactive lipoprotein peak was pooled and washed with acetone to remove $NaDodSO_4$.

Preparation of Inner and Outer Membranes. *E. coli* wild-type strain E613 and *mlpA* mutant strain E614 were cultured in 1000 ml of proteose peptone beef extract broth at 37° and harvested at logarithmic phase of growth. Inner and outer membranes were prepared by the method of Osborn *et al.* (15). NADH oxidase, phospholipase A, and 2-keto-3-deoxyoctonate were assayed by the method described by Osborn *et al.* (15). Lipoprotein content was estimated both by Ouchterlony double-diffusion (4) and by radial immunodiffusion, with purified wild-type lipoprotein as standard. Radial immunodiffusion was performed in 1% agarose plate containing 50 mM sodium barbital buffer (pH 8.6), 0.05% $NaDodSO_4$, and 5% rabbit antiserum against purified lipoprotein. After incubation at room temperature for more than 70 hr, the diameter of the precipitate ring was measured and compared to that with a known amount of lipoprotein standards.

Other Biochemical Techniques. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out according to Inouye and Guthrie (8). $NaDodSO_4$/urea/polyacrylamide gel electrophoresis was performed according to Swank and Munkres (9). Cyanogen bromide cleavage was carried out according to Hirashima *et al.* (16). The product was dissolved in 10% acetic acid/8 M urea. The soluble fraction was subjected to Biogel P-6 column (1.8 × 85 cm) chromatography. Protein was determined by the method of Lowry *et al.* (17).

Chemicals and Radiochemicals. All chemicals were of reagent grade and were purchased from commercial sources. Radioactive chemicals used in the present study included L-[*U*-^{14}C]arginine (309 Ci/mol), L-[3-^{3}H(N)]arginine (27 Ci/mmol), L-[^{3}H]isoleucine (40 Ci/mmol), and L-[^{3}H]lysine (3.3 Ci/mmol) purchased from New England Nuclear Corp. or Schwarz/Mann.

RESULTS

Size of mutant lipoprotein synthesized *in vivo* is same as that of prolipoprotein synthesized in toluene-treated cells

Inouye *et al.* have shown that both the *in vitro* translational product of lipoprotein mRNA (3) and the lipoprotein precursor synthesized in toluene-treated cells (3, 18) contain 20 extra amino acid residues at their amino termini. In a previous report, we demonstrated that lipoprotein from *mlpA* mutant strain E602 was of higher molecular weight than that of the wild type (5). We have now found that the electrophoretic mobilities in $NaDodSO_4$ gels of both mutant lipoprotein isolated from intact cells and mutant lipoprotein precursor synthesized in toluene-treated cells were identical (data not shown). This result suggested that mutant lipoprotein might, in fact, represent the uncleaved form of prolipoprotein. To test this possibility, we purified [^{3}H]isoleucine-labeled prolipoprotein from toluene-treated mutant cells and [^{3}H]lysine-labeled lipoprotein from intact mutant cells. Both samples were analyzed by automated Edman degradation and the radioactivity released at each cycle of sequencer run was measured. The mutant prolipoprotein contained isoleucine at the positions 12 and 27 (Fig. 1*A*). The mutant lipoprotein labeled *in vivo* contained lysine at positions 2, 5, 26, and 40 (Fig. 1*B*). Both of these results were consistent with the sequence of prolipoprotein determined by Inouye *et al.* (3). We concluded that the mutant lipoprotein synthesized *in vivo* contained 20 extra amino acids at its amino terminus and therefore represented uncleaved prolipoprotein.

Mutant lipoprotein contains an amino acid substitution in signal sequence of prolipoprotein

Amino Acid Composition of Mutant Lipoprotein. Amino acid analysis (Table 1) of the free form of lipoprotein purified from wild-type strain E600 and mutant strain E602 showed that there were multiple differences in their amino acid compositions, which would be consistent with the possibility that mutant lipoprotein represented uncleaved prolipoprotein. The mutant lipoprotein is almost identical to wild-type prolipoprotein except for a possible replacement of one glycine by aspartic acid. Since glycine is found only in the signal sequence of prolipoprotein (3), this raises the interesting possibility that mutant lipoprotein may correspond to the uncleaved form of prolipoprotein with an amino acid substitution in the signal sequence which prevents the subsequent processing of the mutant prolipoprotein by a putative proteolytic enzyme.

Amino Acid Sequence of Amino Terminus of Mutant Lipoprotein. To verify the working hypothesis that the mutant lipoprotein corresponds to uncleaved prolipoprotein, we carried out 40 cycles of Edman degradation. The PTH-amino acid released during each cycle of Edman degradation was identified by high-pressure liquid chromatography. The yield of each amino acid was determined by amino acid analysis, except for methionine released during the first cycle. Since PTH-methionine was totally destroyed after HI hydrolysis, the first residue PTH-methionine was determined from high-pressure liquid chromatography by comparison with PTH-methionine standard. The results for the first 30 cycles are shown in Fig. 2. Based on the results obtained from both amino acid analysis and high-pressure liquid chromatography of PTH-amino acids, the sequence of the first 40 residues from the amino terminus of mutant lipoprotein was found to be identical to that of the wild-type prolipoprotein except for the residue at position 14. Aspartic acid was identified as residue 14 in the mutant prolipoprotein by high-pressure liquid chromatography and by amino acid analysis. No ammonia was released during the back conversion of PTH-derivative by HI hydrolysis. The sequence of the first 30 residues is shown in Fig. 3*B* along with the sequence of the amino terminus of prolipoprotein reported by Inouye *et al.* (3) (Fig. 3*A*). It clearly showed that the mutant

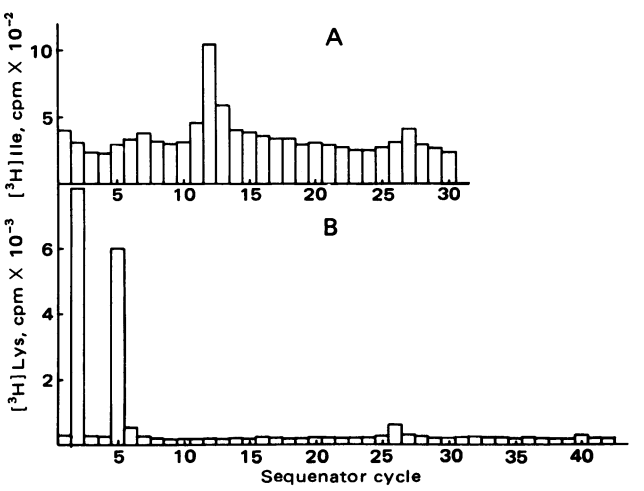

FIG. 1. Automated Edman degradation of [^{3}H]Ile-labeled prolipoprotein synthesized in the toluene-treated mutant cells (*A*) and [^{3}H]Lys-labeled lipoprotein isolated from intact mutant cells (*B*). Total radioactivity applied to the sequencer was (*A*) 3.1 × 10^4 and (*B*) 7.0 × 10^4 cpm.

Table 1. Amino acid compositions of free form of lipoprotein purified from wild-type strain E600 and mutant strain E602 cells

Amino acid	E600	Theoretical LP*	E602	Theoretical PLP†	E602 CNBr fragments CN-I	Theoretical CN-I*	CN-II	Theoretical CN-II*	CN-(III+IV)	Theoretical CN-(III+IV)*
Lys	4.8	5	7.2	7	3.3	3	1.3	1	2.4	2
His	0	0	0	0						
Arg	4.3	4	3.6	4	3.8	4	2.6	3	1.3	1
Asp	14.2	14	14.7	14	6.4	6	6.2	6		
Thr‡	2.0	2	3.0	4	0.9	1			1.2	1
Ser‡	6.4	6	6.9	7	0.6	1	0.8	1		
Glu	5.0	5	5.0	5	3.0	2	2.4	2		
Pro	0	0	0	0						
Gly	0	0	2.1	3			0.9	0		
Ala	9.3	9	11.6	12	6.0	6	5.0	5	1.0	1
Val	4.4	4	5.9	6	1.0	1	0.9	1		
Met§	1.7	2	3.0	3						
Ile	0.9	1	1.8	2						
Leu	4.0	4	8.9	9	0.8	1	0.6	1		
Tyr	0.7	1	0.8	1	0.7	1			0.8	1
Phe	0	0	0	0						
Cys§	0	0	0.6	1						
Glyceryl-Cys§	0.6	1	0	0						

* Theoretic value calculated from known sequence data of lipoprotein from ref. 10. LP, lipoprotein.
† Theoretic value calculated from known sequence data of prolipoprotein from ref. 3. PLP, prolipoprotein.
‡ Values obtained from extrapolation to zero time hydrolysis.
§ Methionine, cysteine, and glycerylcysteine were analyzed as oxidized forms.

lipoprotein contained aspartic acid at position 14 instead of glycine present in the wild-type prolipoprotein.

Cyanogen Bromide Cleavage of Mutant Lipoprotein. Biogel P-6 chromatographic profile of CNBr fragments of wild-type ([^{14}C]Arg-labeled) and mutant ([^{3}H]Arg-labeled) lipoproteins is shown in Fig. 4. Judging from the amino acid composition of CNBr fragments of wild-type lipoprotein (data not shown), fragment CN-III appears to be the same peptide as CN-IV except that the tyrosine residue is modified. Both CN-III and CN-IV corresponded to the peptide from residues 73–78 of prolipoprotein. CN-II represented the peptide from residues 52–72 of prolipoprotein, whereas CN-I was the partial cleaved peptide from residues 52–78 of prolipoprotein (3). CN-0 region represented the void volume of the column, which contained a mixture of uncleaved lipoprotein and the large fragment containing residues at the amino terminus of both wild-type and mutant lipoproteins. As can be seen in Table 1, there was a good agreement of amino acid composition of CN-II and CN-(III+IV) of mutant lipoprotein with the theoretical values. In addition, the Biogel P-6 profiles of CNBr fragments of wild-type and mutant lipoproteins showed no difference in the apparent sizes of peptide CN-II and CN-(III+IV). These data strongly suggest that the amino acid sequence of the carboxyl-terminal portion of the mutant lipoprotein (residues 52–78) is identical with that of the wild-type prolipoprotein. Taken together with data described above, we concluded that mutant lipoprotein corresponds to uncleaved prolipoprotein with a single amino acid replacement of aspartic acid for glycine at residue 14.

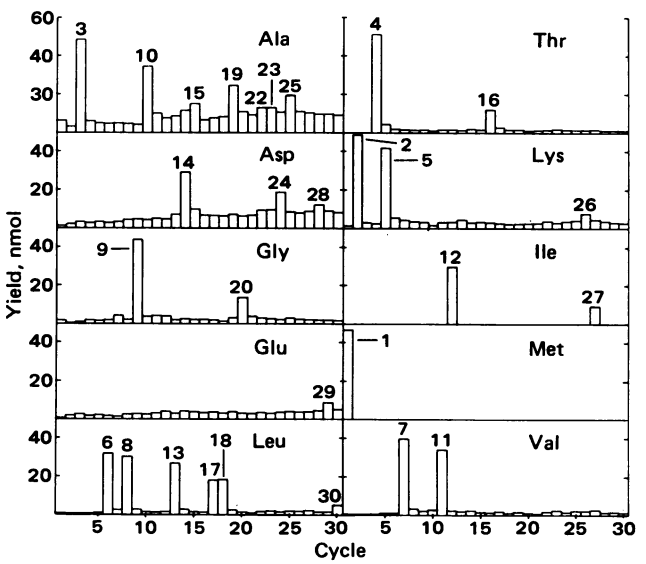

FIG. 2. Automated Edman degradation of free form of lipoprotein purified from mutant E602. Threonine residues at positions 4 and 16 were regenerated by HI hydrolysis as α-aminobutyric acid. Serine residues at positions 15, 22, and 23 were regenerated by HI hydrolysis as alanine. Isoleucine residues at positions 12 and 27 were regenerated as alloisoleucine and isoleucine. Cysteine at position 21 was recovered as alanine. Alanine, serine, and cysteine can be distinguished by high-pressure liquid chromatography. Asparagine at position 24 was regenerated as aspartic acid and ammonia.

Distribution of mutant lipoprotein in submembrane fraction

The amino acid substitution of prolipoprotein found in mutant lipoprotein results in the failure of post-translational modifications, including proteolytic processing of prolipoprotein. This

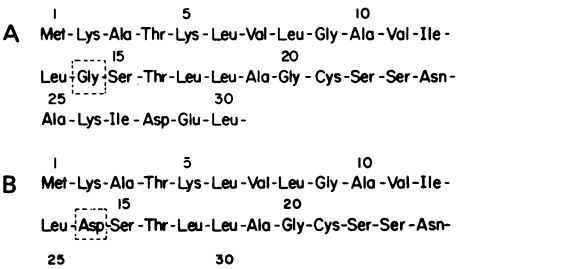

FIG. 3. Amino-terminal sequences of the wild-type prolipoprotein (*A*) and mutant E602 lipoprotein (*B*).

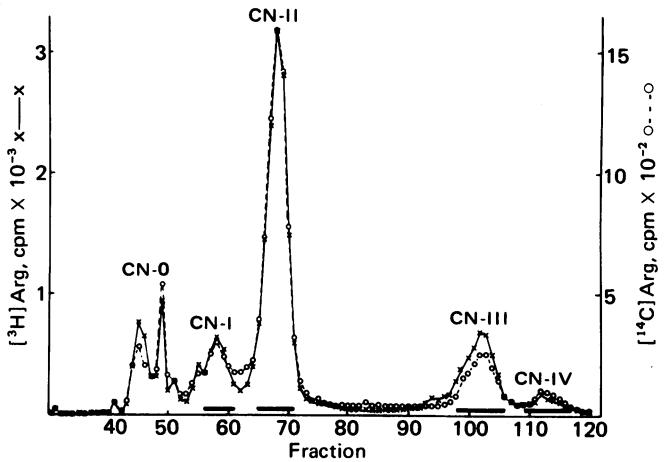

FIG. 4. Biogel P-6 chromatography of CNBr fragments of [^{14}C]Arg-labeled lipoprotein from wild-type E600 and [^{3}H]Arg-labeled lipoprotein from mutant E602. Fractions of 0.7 ml were collected and monitored for radioactivity. The black bar under each peak indicates the fractions pooled for the determination of amino acid composition in the nonradioactive sample.

raises an interesting question as to whether the altered prolipoprotein is translocated and integrated into outer membrane in a normal way. In a previous report, we found about 20% of mutant lipoprotein in 275,000 × *g* cell supernatant fraction (5). The distribution of lipoprotein in the submembrane fraction was determined. Outer and inner membranes of both wild-type and mutant cells were obtained by the method of Osborn *et al.* (15). 2-Keto-3-deoxyoctonate and phospholipase A and NADH oxidase activities were used as outer and inner membrane markers, respectively (15). The amounts of lipoprotein in submembrane fractions were measured by the two immunological assays described in *Materials and Methods*. While the wild-type lipoprotein was nearly exclusively located in the outer membrane fraction, 20.1% of the total membrane-bound mutant lipoprotein was found in the inner membrane fraction (Table 2). Since contamination of outer membrane material in the inner membrane fraction was minimal, as judged by the relative specific activities of phospholipase A and by keto-deoxyoctonate content, we conclude that there is a selective enrichment of lipoprotein in the inner membrane of the mutant cell envelope. This was borne out by the NaDodSO$_4$ gel electrophoretic profile of inner and outer membrane proteins (data not shown). The presence of mutant lipoprotein in the inner membrane is distinct and unique, without concomitant increase of other major outer membrane proteins. In addition to the accumulation of lipoprotein in the inner membrane fraction of the mutant cell envelope, there was also significant and selective enrichment of lipoprotein in the mixed fraction of the cell envelope which could not be attributed to the amount of outer membrane components present in this fraction. This abnormal distribution of mutant lipoprotein was also found in one other *mlpA* transductant (strain E610, ref. 6), but not in the *lpp-1* mutant (data not shown). The lipoprotein of *lpp-1* mutant lacks both *O*-acyl fatty acids at its amino terminus (19) in addition to the replacement of arginine with cysteine at position 57 (20). These results would suggest that the abnormal distribution of *mlpA* mutant lipoprotein was not due to the deficiency in the covalently linked lipid moiety. On the other hand, no accumulation of lipoprotein was found in the inner membrane and mixed fractions of the cell envelope of two spontaneous *mlpA*$^+$ revertants (data not shown). The latter strains have been shown to contain lipoprotein of apparently normal structures (6). These data strongly suggest that the aberrant subcellular distribution of lipoprotein in *mlpA* mutant cells is directly related to the unique structural alteration in this mutant lipoprotein.

DISCUSSION

Based on both biochemical and genetic evidence, we have previously concluded that the alteration in the structure of murein lipoprotein in *mlpA* mutant was due to a single mutation in the structural gene for this protein (6, 21). In this paper, we have demonstrated that this mutation results in a single amino acid replacement of glycine by aspartic acid at residue 14 of prolipoprotein. The mRNA codon for glycine residue at position 14 has been shown to be GGU (22). The observation of this particular amino acid substitution is consistent with a single base change in the sequence of lipoprotein gene from CCA to CTA at this position. As a consequence of this single amino acid substitution, a number of post-translational modifications of prolipoprotein are aborted. The introduction of a negatively charged residue into the signal segment is likely to affect the conformation of prolipoprotein. Alternatively, the glycine at position 14 may constitute part of the binding site recognized by the putative protease in view of the structural homology among the signal sequences of immunoglobulins (23). Further analysis of the amino acid sequence of prolipoprotein from spontaneous revertants of this *mlpA* mutant may provide further insight concerning the structural feature important for post-translational modifications of prolipoprotein.

It has been suggested that the extra amino acids at the amino terminus of prolipoprotein, being enriched in hydrophobic amino acids, are likely to play a role in the assembly mechanism of lipoprotein in the outer membrane (3). Hydrophobic sequences, also found in many precursor forms of secretory proteins, are considered to be important in the transport of these proteins across the membranes, as suggested by the signal hy-

Table 2. Submembrane distribution of lipoprotein of wild-type E613 and *mlpA* mutant E614

	Total protein (mg)	NADH oxidase μmol/min per mg	%	Phospholipase A pmol/min per mg	%	Ketodeoxyoctonate μmol/mg	%	Lipoprotein μg/mg	%
E613 (*mlpA*$^+$)									
Total membrane	15	0.36		0.95		0.19		166	
H (outer)	4.8	0.01	1.7	2.70	75.5	0.44	77.0	306	78.0
M (mixed)	3.75	0.17	21.7	1.05	23.0	0.15	20.4	104	20.7
L (inner)	2.46	0.92	76.6	0.11	1.5	0.03	2.6	10	1.3
E614 (*mlpA*$^-$)									
Total membrane	26.6	0.39		0.73		0.23		96	
H (outer)	6.98	0.01	1.2	2.08	86.0	0.51	82.4	144	57.7
M (mixed)	5.93	0.09	9.0	0.20	7.1	0.08	10.9	65	22.2
L (inner)	5.85	0.91	89.8	0.20	6.9	0.05	6.7	60	20.1

pothesis (24). Two functions can be visualized for these signal sequences: (*i*) the hydrophobic segment is needed for insertion of the nascent chains of membrane proteins or secretory proteins into the lipid bilayer of the cellular membrane during or immediately following its synthesis on membrane-bound polyribosomes; and (*ii*) the conversion of precursor into its mature form by proteolytic cleavage is coupled either with its secretion or, for some membrane proteins, with their translocation from the inner surface of the membrane to its outer surface. These two possibilities are not mutually exclusive.

The present study on a mutant lipoprotein with an uncleaved, genetically modified signal sequence at its amino terminus provides an opportunity to ascertain whether removal of the signal sequence plays an obligatory role in the assembly of lipoprotein into the outer membrane. While a significant amount of mutant lipoprotein was found in the supernatant fraction after centrifugation at 275,000 × *g* for 2 hr (5), the membrane-bound lipoprotein in this mutant is recovered in both the inner and outer membranes of the cell envelope. At the present time, no evidence exists which suggests microheterogeneity in the structure of mutant lipoprotein, nor are we able to distinguish among lipoproteins in the soluble, inner membrane, and outer membrane fractions. The fact that 57.7% of membrane-bound mutant lipoprotein was located in the outer membrane of the cell envelope strongly suggests that the conversion of prolipoprotein into the mature form of lipoprotein is not absolutely essential for the translocation and assembly of this outer membrane protein. A simple model envisioning an obligatory coupling between proteolytic cleavage of prolipoprotein and its assembly into outer membrane can be ruled out. However, a significant fraction of the mutant lipoprotein was found in the inner membrane fraction. This suggests that the aberrant conformation of the mutant lipoprotein may have affected its translocation into outer membrane by interfering with the proper attachment of the nascent chain of mutant prolipoprotein to the cytoplasmic membrane. More severely affected than translocation and assembly into outer membrane are the post-translational modifications of prolipoprotein, including proteolytic cleavage, transfer of glycerol from phosphatidylglycerol to the sulfhydryl group of (pro)lipoprotein (25), and the joining of lipoprotein to the murein sacculus. While the last reaction, the conversion of the free form of lipoprotein to the murein-bound form, probably follows the former two reactions, the present study does not allow determination of the ordering of the first two reactions. The data do suggest that proteolytic cleavage of prolipoprotein to lipoprotein probably precedes the covalent attachment of lipid to the free form of lipoprotein.

We are grateful to Drs. M. J. Osborn, L. Rothfield, and Nicholas Jones for critical reading of the manuscript. This investigation was supported by U.S. Public Health Service Grant CA-11371 from the National Cancer Institute and by Grant 75-632 from the American Heart Association.

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