

Amino Acid Replacement in a Mutant Lipoprotein of the *Escherichia coli* Outer Membrane

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The primary structure of a mutant lipoprotein of the outer membrane of *Escherichia coli* was investigated. This mutant was previously described as a mutant that forms a dimer of the lipoprotein by an S-S bridge (H. Suzuki et al., *J. Bacteriol.* 127:1494-1501, 1976). The amino acid analysis of the mutant lipoprotein revealed that the mutant lipoprotein had an extra cysteine residue, with concomitant loss of an arginine residue. From the analysis of tryptic peptides, it was found that the arginine residue at position 57 was replaced with a cysteine residue. The amino terminal structure of the mutant lipoprotein was found to be glycerylcysteine, as in the case of the wild-type lipoprotein. The present results show that the mutation that was previously determined to map at 36.5 min on the *E. coli* chromosome occurred in the structure gene (*lpp*) for the lipoprotein. This was further confirmed by the fact that a merodiploid carrying both *lpp*⁺ and *lpp* produces not only the wild-type lipoprotein but also the mutant lipoprotein.

The outer membrane of *Escherichia coli* contains a peculiar structural protein, a lipoprotein of molecular weight 7,200, which is one of the most extensively investigated membrane proteins (see reviews by Braun [2] and Inouye [5]). Recently, we isolated a mutant of the lipoprotein, which has a reactive sulfhydryl group (11). As a result, the mutant lipoprotein forms a dimer and also reacts with iodoacetic acid. The mutation was mapped at 36.5 min on the *E. coli* chromosome.

As described in the present paper, we purified the mutant lipoprotein and determined its primary structure. It was found that the arginine residue at the 57th position from the amino terminus changed into a cysteine residue in the mutant lipoprotein. The amino terminal glycerylcysteine was found to be intact in the mutant lipoprotein, contrary to our early speculation on the basis of the preliminary results (11). From these results, it was concluded that the mutation occurred in the structural gene for the lipoprotein. Thus, the gene was assigned as *lpp* instead of the previously proposed *lpm* (11).

MATERIALS AND METHODS

Bacterial strains and culture media. The following strains of *E. coli* were used: K63-1 (F⁺ *lpp-1* (previously assigned as *lpm* [11]) *his fadD gal str*); JE5512 Hfr Cavalli (*man pps*); JE5511 Hfr Cavalli

(*lpp-1 man pps*); JE5525 (*man lpp-1 pps trp gal str recA*); JE5525 (*man lpp-1 pps recA*)/F506 *man*⁺ *lpp*⁺ *pps*⁺ (R. Movva, E. Katz, P. L. Asdourian, Y. Hirota, and M. Inouye, manuscript in preparation). T broth was used for *E. coli* K63-1, and M9 glucose or Davis minimal medium was used for the other strain.

Purification of the lipoprotein. The mutant lipoprotein was purified from 200 g of *E. coli* K63-1 harvested from 100 liters of T broth culture. The purification was carried out according to the method of Inouye et al. (9).

Tryptic digestion. Seven milligrams of the purified mutant lipoprotein was suspended by sonic treatment in 2 ml of water. Two-tenths milliliter of a solution containing 1 mg of trypsin (Sigma)/ml was added to the suspension, and the pH of the mixture was adjusted to 8. After incubation for 3 hr at 37°C, the mixture was centrifuged for 20 min at 12,000 × *g*, and the supernatant fluid was lyophilized. The pellet was suspended in 2 ml of water and digested with trypsin as described above. The final pellet was washed twice with 0.5 ml of water. The tryptic peptides in the supernatant were lyophilized and oxidized with performic acid (4). The oxidized material was then dissolved in 0.1 ml of 50% acetic acid and applied as a 10-cm band to a cellulose:thin-layer plate (0.25-mm thickness; MN300; Macherey Nagel Co.). Peptides were then separated with a solvent of pyridine-acetic acid-water-1-butanol (24:6:20:30). The positions of the peptides on the plate were determined by spraying with 0.1% ninhydrin solution in acetone only at both edges of the peptide bands (the central part was covered by a

piece of aluminum foil). Then, each peptide band was scratched out from the plate, and the peptides were extracted from the cellulose with 50% acetic acid. In some cases, the peptide fractions were further purified on a second cellulose thin-layer plate with a solvent consisting of 1-butanol-pyridine-water (1:1:1).

Other methods. Edman degradation and carboxypeptidase digestion of peptides were carried out as described previously (8). Amino acid analysis was performed as described previously (8). Performic acid oxidation was carried out according to the procedure of Hirs (4).

Fatty acid analysis and circular dichroism measurements were carried out as described previously (9).

Gel electrophoresis. Sodium dodecyl sulfate-urea gel electrophoresis (12) and slab-gel electrophoresis (1) were used.

RESULTS

Amino acid composition of the mutant lipoprotein. Table 1 shows the amino acid analysis of the mutant lipoprotein. It appears that the mutant lipoprotein has only three arginine residues in contrast to four arginine residues of the wild-type lipoprotein. On the other hand, 0.7 mol of half-cystine was detected per mol of the mutant lipoprotein. In case of the wild-type lipoprotein, no half-cystine was detected, as shown previously (9). When the mutant lipoprotein, oxidized by performic acid, was analyzed, 1.0 mol of cysteic acid and 0.4 mol of an oxidized form of glycercylcysteine were detected. In case of amino acid analysis of the oxidized lipoprotein from the wild-type strain, 0.2 mol of cysteic acid and 0.4 mol of an oxidized form of glycercylcysteine were obtained per molecule of the lipoprotein (9). Therefore, it was concluded that the mutant lipoprotein has a cysteine residue besides the glycercylcysteine residue at the amino terminus. This extra cysteine residue is most likely derived from an arginine residue in the wild-type lipoprotein by the mutation.

Tryptic digestion of the mutant lipoprotein. To determine the position of the amino acid replacement, the mutant lipoprotein was digested with trypsin, and the resultant tryptic peptides were purified by thin-layer chromatography, except for the amino terminal peptide. The amino terminal peptide was isolated as insoluble material after extensive tryptic digestion of the original fraction. Table 2 shows the amino acid compositions of the tryptic peptides and their R_f values on the thin-layer plate. From their amino acid compositions, one can easily identify the peptides by comparison with those obtained from the wild-type lipoprotein. From the primary structure of the wild-type

TABLE 1. Amino acid analysis of the mutant lipoprotein

Amino acid	Contents ^a	Wild type ^b
Lysine	4.7 ± 0.1 (5)	5
Histidine	0 (0)	0
Arginine	2.9 ± 0.1 (3)	4
Aspartic acid	14.2 ± 0.1 (14)	14
Threonine	2.0 ± 0.1 (2)	2
Serine	5.6 ± 0.4 (6)	6
Glutamic acid	5.2 ± 0.1 (5)	5
Proline	0 (0)	0
Glycine	0 (0)	0
Alanine	9.0 (9)	9
Half-cystine	0.7 ± 0.1 (1)	0
Valine	4.1 ± 0.4 (4)	4
Methionine	2.0 ± 0.2 (2)	2
Isoleucine	1.0 ± 0.1 (1)	1
Leucine	4.1 ± 0.2 (4)	4
Tyrosine	1.0 ± 0.1 (1)	1
Phenylalanine	0 (0)	0
Glycercylcysteine ^c	0.4 (1)	1

^a All values are calculated assuming that there are nine alanine residues per molecule of the lipoprotein. Hydrolysis was carried out in 6 N HCl at 105°C for 24 h. All values are averages of three independent experiments except for glycercylcysteine. Values in parentheses represent integer values concluded from the data.

^b From the amino acid composition of the wild-type lipoprotein obtained previously (9).

^c Obtained as an oxidized form of glycercylcysteine that appeared at the relative mobility of 0.66 to aspartic acid in the amino acid analysis as described previously (9).

lipoprotein determined by Braun and Bosch (3), one can assign nine tryptic peptides (T1 to T9 from the amino terminus). All tryptic peptides corresponding to T1 through T7 were identified from their amino acid compositions (Table 2). Their amino acid compositions were exactly the same as those expected for the wild-type lipoprotein. Besides these peptides, T2C was recovered, which was the only peptide without a lysine or an arginine residue. From its amino acid composition, it was concluded that T2C resulted from the chymotryptic digestion of T2. Chymotryptic activity is known to be present in the trypsin preparation (8). It should be noticed that the final yield of T2C is about a third of the yield of T2 (Table 2).

The only peptides of the wild-type lipoprotein that could not be recovered from the mutant lipoprotein were T8 (Tyr-Arg) and T9 (Lys). Instead, peptides T8' and T8'C were isolated. Peptide T8' consisted of 1 Lys, 1 Cys, and 1 Tyr; and peptide T8'C had 1 Lys and 1 Cys. From the specificity of trypsin, the amino acid sequences of T8' is thought to be (Tyr, Cys) Lys; the sequence of T8'C is Cys-Lys. Since there is only

TABLE 2. Amino acid compositions and purification methods of tryptic peptides^a

Determination	T1	T2	T2C	T3	T4	T5	T6	T7	T8'	T8'C
Purification method ^b	Insoluble	1	1	1 and 2	1 and 2	1	1	1	1 and 2	1 and 2
<i>R_f</i>										
Solvent 1		0.57	0.63	0.50	0.31	0.21	0.26	0.41	0.38	0.50
Solvent 2				0.32	0.18				0.37	0.44
Final yield (%)	27	6.6	2.2	1.6	1.8	12.3	8.6	4.2	2.4	0.8
Lysine	0.8 (1) ^c	0.9 (1)			1.3 (1)			1.1 (1)	1.0 (1)	1.0 (1)
Arginine				1.0 (1)		1.2 (1)	1.0 (1)			
Glyceryl-cysteine ^d	0.4 (1)									
Cysteic acid									0.9 (1)	0.9 (1)
Aspartic acid	1.4 (1)	3.4 (3)	2.4 (2)	4.1 (4)	1.0 (1)	2.0 (2)	1.2 (1)	2.3 (2)		
Threonine		1.1 (1)	0.8 (1)					0.9 (1)		
Serine	1.8 (2)	1.7 (2)	1.5 (2)	0.7 (1)	0.6 (1)					
Glutamic acid	0.3	1.9 (2)	2.0 (2)	0.9 (1)	0.7 (1)		0.9 (1)			
Alanine	1.0 (1)	1.0 (1)		1.0 (1)	2.0 (2)	1.7 (2)	1.0 (1)	1.0 (1)		
Valine		1.0 (1)	1.0 (1)	1.9 (2)	0.6 (1)					
Methionine sulfone				1.0 (1)				0.9 (1)		
Isoleucine		0.8 (1)	0.9 (1)							
Leucine	0.2	2.0 (2)	2.0 (2)	1.0 (1)				0.9 (1)		
Tyrosine									0.6 (1)	

^a Obtained after extensive tryptic digestion as described in Materials and Methods. Tryptic peptides were assigned from the amino terminus as T1 to T7. T8' is the new tryptic peptide obtained from the mutant lipoprotein. T2C and T8'C indicate that these peptides are chymotryptic by-products of T2 and T8', respectively. According to the primary structure determined by Braun and Bosch (3), these peptides are as follows: T1, glyceryl-Cys-Ser-Ser-Asn-Ala-Lys⁹; T2, Ile-Asp-Glu-Leu-Ser-Ser-Asp-Val-Gln-Thr-Leu-Asn-Ala-Lys¹⁷; T2C, Ile-Asp-Glu-Leu-Ser-Ser-Asp-Val-Gln-Thr-Leu-Val-Asp-Glu-Leu-Ser-Asn-Asp-Val-Asn-Ala-Met-Arg³²; T3, Ser-Asp-Val-Gln-Ala-Ala-Lys³³; T4, Ser-Asp-Val-Gln-Ala-Ala-Lys³⁹; T5, Asp-Asp-Ala-Ala-Arg⁴⁰; T6, Ala-Asn-Glu-Arg⁴⁸; T7, Leu-Asp-Asn-Met-Ala-Thr-Lys⁵³; T8', Tyr-Cys-Lys⁵⁵; T8'C: Cys-Lys⁵⁵.

^b Solvent 1, pyridine-acetic acid-water-1-butanol (24:6:20:30); solvent 2, 1-butanol-pyridine-water (1:1:1).

^c Numbers in parentheses indicate integer values of amino acid compositions. Values less than 0.1 were not listed.

^d Obtained as a peak just before aspartic acid. When oxidized, 0.1 mol of cysteic acid and 0.3 mol of an oxidized form of glycerylcysteine were obtained.

one cysteine residue in the mutant lipoprotein (Table 1), T8'C is thought to be derived from the chymotryptic digestion of T8', as T2C is derived from T2. Therefore, the amino acid sequence of T8' is concluded to be Tyr-Cys-Lys. This sequence was confirmed by carboxypeptidase B digestion of T8', which released only lysine, and by the Edman degradation of the peptide. After the first step of the Edman degradation, the amino acid analysis of the remaining peptide yielded only cysteic acid (lysine was not examined), indicating that the amino terminus of the peptide is tyrosine. Thus, it was concluded that the amino acid sequence of peptide T8' is Tyr-Cys-Lys, which is derived from Tyr⁵⁶-Arg-Lys⁵⁸ as a result of the change of arginine residue to cysteine residue by the mutation.

Structural study of the amino terminus. The amino terminal tryptic peptide, T1, was recovered as insoluble material (Table 2). From the amino acid analysis of the peptide, it was found that the peptide contains glycerylcysteine. When the peptide was oxidized by per-

formic acid, only 0.1 mol of cysteic acid was recovered, together with an oxidized form of glycerylcysteine (Table 2). This small amount of cysteic acid is most likely a result of the decomposition of glycerylcysteine, as found in the case of the wild-type lipoprotein (9). Fatty acid compositions of peptide T1, as well as of the wild-type lipoprotein, are shown in Table 3. Total fatty acid content was estimated to be 2.0 mol per mol of the peptide. Although this value is lower than the value expected from the wild-type lipoprotein (3 mol per mol of the lipoprotein), the low values were reproducibly obtained. When peptide T1 was treated with 1 N NaOH at 23°C for 4 h, only about 50% of the fatty acid was released, suggesting that one of the two fatty acids ester-linked to glycerylcysteine is missing from the peptide. This would account for the low content of total fatty acid and the rather high content of C₁₆ fatty acid in peptide T1, since it is more abundant in amide-linked fatty acid than in those that are ester linked (9).

In the previous paper (11), we have suggested

TABLE 3. Fatty acid analysis of peptide T1

Fatty acid	Peptide T1 (%)	Wild type ^a (%)
C _{16:0}	79.3	58.6
C _{16:1}	12.0	3.6
C ₁₇ cyclo	0.4	13.5
C _{18:1}	6.4	12.8
C ₁₉ cyclo		8.5
Unknown	1.8	

^a From Inouye et al. (9).

that the SH group of the mutant lipoprotein may be derived from the amino terminal cysteine because of poor incorporation of [³H]glycerol. This was reexamined in the present paper by using not only [³H]glycerol but also [¹⁴C]arginine as an internal control to examine the extent of [³H]glycerol on the basis of the lipoprotein production (= [¹⁴C]arginine incorporation). Both the wild-type strain (JE 5512) and the mutant strain (JE 5511) were labeled with [³H]glycerol and [¹⁴C]arginine. The bound forms of the lipoproteins were prepared from these strains. The bound forms were then treated with lysozyme and examined by sodium dodecyl sulfate-urea gel electrophoresis. When the mutant lipoprotein was analyzed in sodium dodecyl sulfate-gel electrophoresis without β -mercaptoethanol treatment (Fig. 1C), a peak appeared at a position of much higher molecular weight than that of the bound form of the wild-type lipoprotein (Fig. 1A). This peak consists of dimers of the mutant lipoprotein, since the mutant lipoprotein migrates at exactly the same position as the bound form of the wild-type lipoprotein, when treated with β -mercaptoethanol (Fig. 1B). In this case, an extra small peak appears at the right-hand side of the bound-form position, as shown by an arrow in Fig. 1B. Since this position corresponds to the free form of the lipoprotein, it appears that the peak in Fig. 1C consists not only of dimers composed of the bound-form molecules by themselves, but also of dimers between the bound form and the free form of the mutant lipoprotein. It was found that [³H]glycerol was clearly incorporated into the mutant lipoprotein, and the ratios of [³H]glycerol to [¹⁴C]arginine are exactly the same in both the free and the bound forms of the mutant lipoprotein. In this experiment, the extent of [¹⁴C]arginine incorporation into the mutant lipoprotein was poorer than that into the wild-type lipoprotein. This was possibly caused by slight differences in cultural conditions.

Circular dichroism of the mutant lipoprotein. Circular dichroism spectra of the mutant lipoprotein and the wild-type lipoprotein in

0.05% sodium dodecyl sulfate were quite similar, suggesting that the conformation of the mutant lipoprotein is not significantly different from that of the wild-type lipoprotein.

Further evidence for the mutation in the structural gene. The present results indicate that the mutation occurred in the structural

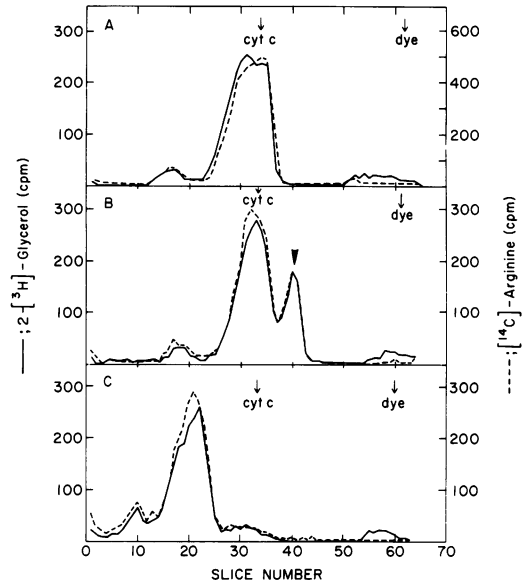


FIG. 1. Sodium dodecyl sulfate-urea gel electrophoresis of the bound forms of the wild-type and mutant lipoprotein labeled with [2-³H]glycerol and [¹⁴C]arginine. Both the wild-type strain (JE 5512) and the mutant strain (JE 5511) were labeled with 200 μ Ci of [2-³H]glycerol (New England Nuclear Corp.; specific activity, 200 Ci/mmol) in 25 ml of protease peptone beef extract broth supplemented with 20 μ g of glycerol per ml for two generations, and with 15 μ Ci of [¹⁴C]arginine (Schwarz/Mann; specific activity, 390 mCi/mmol) in 25 ml of M9 glucose medium supplemented with 10 μ g of arginine per ml for three generations. Envelope fractions were prepared as described previously (7). Phospholipids were extracted from [2-³H]glycerol-labeled envelope as described previously (10). The corresponding cell envelope fractions ([³H]glycerol and [¹⁴C]arginine of the same strain) were mixed, and the bound forms of the lipoprotein were prepared and digested with hen egg white lysozyme as described previously (6). They were then analyzed by sodium dodecyl sulfate-urea gel electrophoresis (12). (A) Bound form from the wild-type strain, JE 5512, solubilized in the presence of 1% mercaptoethanol; (B) bound form from the mutant strain, JE 5511, solubilized in the presence of 1% mercaptoethanol; (C) same as (B), except that the bound form was solubilized in the absence of mercaptoethanol. The positions of cytochrome c (cyt c) and pyronin (dye) are shown by arrows. A large arrow in (B) indicates the position of the free-form lipoprotein.

gene (*lpp*) for the lipoprotein. To confirm this conclusion, a merodiploid strain carrying both *lpp*⁺ and *lpp-1* (the present mutation; previously called *lpm* [11]) was constructed. The membrane fraction from this merodiploid strain was prepared and analyzed by sodium dodecyl sulfate-gel electrophoresis. When the membrane fraction, solubilized in the absence of mercaptoethanol, was analyzed, both the mutant and wild-type lipoproteins were detected, as shown in Fig. 2B. In this figure, the mutant lipoprotein migrated as a dimer at the position between hen egg white lysozyme and cytochrome *c* (shown by arrow d), whereas the wild-type lipoprotein moved as a monomer

(shown by arrow m; also see Fig. 2E). When this membrane fraction was treated with mercaptoethanol before gel electrophoresis, the dimer band disappeared, and only a dense band was observed at the monomer position (Fig. 2C). On the other hand, the membrane fraction from a haploid carrying *lpp-1* gave rise to only the dimer band in the absence of mercaptoethanol (Fig. 2D). This dimer band changed to the monomer band when the membrane fraction was treated with mercaptoethanol (Fig. 2E). These results clearly indicate that the gene carrying the present mutation is the structural gene for the lipoprotein.

DISCUSSION

The present results showed that in the mutant lipoprotein purified from a strain carrying *lpp-1*, the arginine residue at the 57th position of the lipoprotein is replaced with a cysteine residue. Thus, we can conclude that the mutation occurred in the structural gene for the lipoprotein, which we propose to designate as *lpp*. It was found that the amino terminal glycerylcysteine residue was not affected by the mutation (*lpp-1*), in contrast to our earlier interpretation based on preliminary results (11).

The mutant lipoprotein seems to have a conformation by circular dichroism similar to that of the wild-type lipoprotein. However, the mutant lipoprotein was found to be more susceptible to tryptic digestion. Furthermore, the mutant lipoprotein was extremely resistant to iodoacetic acid once it was purified. The addition of sodium dodecyl sulfate, urea, and guanidinehydrochloride to the reaction mixture (at 37°C for 2 h) did not enhance the modification of the purified mutant lipoprotein with iodoacetic acid (less than 5%). The reasons for these unusual properties are unknown at present.

The existence of both mutant and wild-type lipoproteins in a merodiploid carrying *lpp*⁺ and *lpp-1* suggests the gene dosage effect of *lpp* gene. This possibility has been currently investigated, and we have found that a merodiploid, *lpp*⁺/*lpp*⁺, contains twice as much the free-form lipoprotein as a haploid does (R. Movva et al., manuscript in preparation). The present mutant lipoprotein should prove to be an extremely useful tool when applied to many different areas of membrane biochemistry, since the mutant lipoprotein has a reactive sulfhydryl group. For instance, the lipoprotein can be modified with a specific probe for use in electron spin resonance or nuclear magnetic resonance studies. Currently we have successfully incorporated a spin label into the mutant lipoprotein in situ (N. Lee, C. Scandella, and M. Inouye, manuscript in preparation).

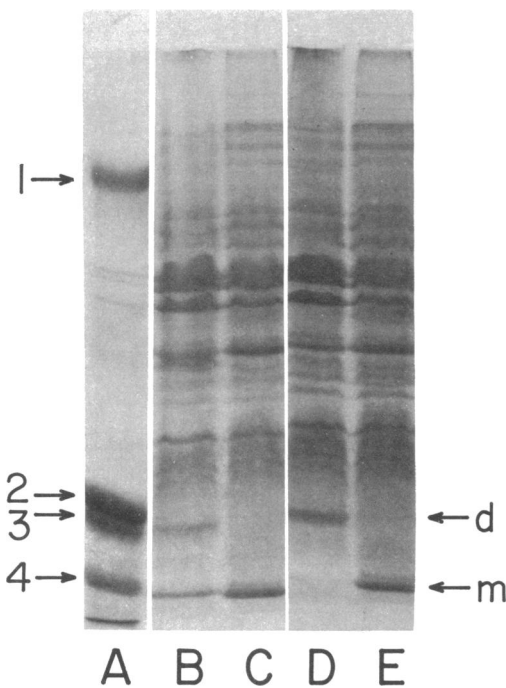


FIG. 2. Slab-gel electrophoresis of the membrane fractions from a merodiploid carrying both *lpp*⁺ and *lpp-1*. The membrane fractions were prepared from cells grown in Davis minimal medium with appropriate supplements. Slab-gel electrophoresis was carried out according to the method of Anderson et al. (1). (A) Protein standards: 1, bovine serum albumin; 2, hen egg white lysozyme; 3, cytochrome *c*; 4, purified wild-type lipoprotein. (B) Membrane fraction from a merodiploid strain, *E. coli* JE 5525 *man lpp-1 pps trp gal str recA/F'506 man⁺lpp⁺pps⁺*. (C) Same as (B), except that the membrane fraction was treated with mercaptoethanol before gel electrophoresis. (D) Membrane fraction from a haploid strain, *E. coli* JE 5525 *man lpp-1 pps trp gal str recA*. (E) Same as (D), except that the membrane fraction was treated with mercaptoethanol before gel electrophoresis. The gel was stained with Coomassie brilliant blue.

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