JOURNAL OF BACTERIOLOGY, July 1976, p. 555-563

Vol. 127, No. 1
Printed in U.S.A.

Lipoprotein from the Outer Membrane of *Escherichia coli*: Purification, Paracrystallization, and Some Properties of Its Free Form

SUMIKO INOUYE, KEIICHI TAKEISHI,[1] NANCY LEE, MARC DEMARTINI, AKIKAZU HIRASHIMA,[2] AND MASAYORI INOUYE*

Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

Received for publication 23 March 1976

In the envelope of *Escherichia coli*, is a lipoprotein of molecular weight 7,200 as a major envelope protein. This lipoprotein was previously shown to exist in two different forms in the outer membrane of *E. coli*: the free form and the bound form, which is covalently linked to the peptidoglycan. The free form of the lipoprotein has been purified and paracrystallized by adding acetone to a sodium dodecyl sulfate solution in the presence of magnesium ion. The paracrystals were needle shaped. An electron micrograph of the negatively stained paracrystals showed a highly ordered ultrastructure. The chemical structure of the free form was compared with that of the bound form by (i) the amino acid composition, (ii) the fatty acid composition, and (iii) the peptide analysis after cyanogen bromide cleavage. The α-helical content of the free form of the lipoprotein was measured from the circular dichroism spectrum of the lipoprotein in 0.01% sodium dodecyl sulfate and found to be 87%. Using the purified lipoprotein as antigen, antiserum against the free form of the lipoprotein was obtained. Immunoprecipitation of the lipoprotein with the antiserum was found to be very specific, since only the free form of the lipoprotein was found as a major peak when the antiserum was reacted with the whole envelope proteins solubilized in 0.2% sodium dodecyl sulfate, and the immunoprecipitate thus formed was analyzed by polyacryamide gel electrophoresis.

A large quantity of a peculiar lipoprotein exists in the outer membrane of *Escherichia coli* (1, 23). The complete amino acid sequence of this lipoprotein has been determined. The lipoprotein consists of 58 amino acid residues with an unusual amino acid, glycerylcysteine, *S*-(propane-2',3'-diol)-3-thio-2-aminopropionic acid, at the aminoterminal position (3, 4, 13). The lipoprotein exists in two different forms in the outer membrane (17, 22): one-third as a bound form covalently linked to the peptidoglycan and the remaining two-thirds as a free form unbound to the peptidoglycan (6, 7). The number of lipoprotein molecules per cell has been estimated to be 5×10^5 and 2.5×10^5 for the free and the bound forms, respectively (5, 22). Thus, it appears to be the most abundant protein in *E. coli*. The function of the lipoprotein has not been determined. However, we have proposed a three-dimensional molecular assembly model of the lipoprotein, which provides a passive diffusion pore through the outer membrane (20). The mode of the biosynthesis and assembly of the lipoprotein has been investigated (1, 14, 15, 22), and a system for the cell-free synthesis of the lipoprotein directed by the purified messenger ribonucleic acid has been established (16).

In the present paper, we have purified the free form of the lipoprotein and paracrystallized it. The free form has exactly the same chemical structure as the bound form, except for the covalent linkage between the amino group of the carboxyl terminal lysine residue of the bound form and the diaminopimelic acid of the peptidoglycan. The α-helical contents of the lipoprotein in different concentrations of sodium dodecyl sulfate and the immunological properties of the lipoprotein have also been investigated.

(This work was presented in part at the 59th Annual Meeting of American Societies for Experimental Biology [Fed. Proc. 34:669, 1975].)

MATERIALS AND METHODS

Bacteria. For the purification of the lipoprotein, *E. coli* B (late exponential phase; Grain Processing Corp., Muscatine, Iowa) was used. For the radioac-

[1] Present address: Department of Virology, Saitama Institute for Cancer Research, Saitama, Japan.

[2] Present address: Department of Molecular Biology, School of Medicine, Keio University, Shinjuku, Tokyo, Japan.

tive membrane preparations, *E. coli* MX74T2 (Thy , Thi , Lac) was used.

Protein chemistry. Hydrolysis of the lipoprotein or peptides was carried out at 105 to 106 C in 6 N HCl for 24 and 72 (only for the lipoprotein) h in sealed, evacuated tubes. Amino acid analysis was performed with a Beckman-Spinco model 120B automatic amino acid analyzer.

Performic acid oxidation of the lipoprotein was carried out according to Hirs (18). Cyanogen bromide cleavage of the lipoprotein was performed as described previously (16). The insoluble material after cyanogen bromide cleavage was removed by centrifugation, and the soluble peptides were purified by two-dimensional peptide mapping as previously described (16). Carboxypeptidase digestion was carried out in 0.2 M sodium bicarbonate buffer, pH 8.0, containing 0.1 M NaCl at 37 C with carboxypeptidases A and B (Worthington Biochemical Corp.). Tryptophan content was estimated spectrophotometrically by the method of Goodwin and Morton (10).

Fatty acid analysis. The fatty acids were determined as methyl esters with the gas chromatograph (JEOL JGC-20K) using a 2-m-long column, 4 mm in diameter. The column was filled with 12% diethyl glycol succinate on GasChrom P (80 to 100 mesh). The temperature was continuously changed from 160 to 190 C at the rate of 2 C/min. Ester-linked fatty acids in the lipoprotein were methylated by incubating 2 mg of the lipoprotein at 80 C for 20 min in 0.5 ml of 0.5 N sodium methylate in a sealed tube. After the reaction, 1 ml of water was added, and the methyl esters were extracted three times with 1 ml of petroleum ether. Amide-linked fatty acids were methylated as follows. The residue remaining after removing the ester-linked fatty acids was hydrolyzed at 105 C for 24 h in 6 N HCl in a vacuum-sealed tube. After hydrolysis, the hydrolysate was dried. To the dried hydrolysate 2 ml of 3% HCl in methanol and 0.25 ml of benzene were added, and the total mixture was refluxed for 2 h. After the reaction, 1 ml of water was added to the mixture, and the methyl esters were extracted as described previously. The total fatty acid analysis was also carried out (as the analysis of amide-linked fatty acids) as described above. The molar ratios of fatty acids to lipoprotein were estimated by adding known amounts of stearic acid to the sample as an internal standard prior to hydrolysis. The molar ratios of fatty acids to the lipoprotein were calculated from the molar ratio of stearic acid to the lipoprotein and to the other fatty acid components.

Organic phosphate determination. The content of organic phosphates in the lipoprotein preparation was determined by the method of Fiske and Subbarow (9).

Immunoassay of the lipoprotein. Antiserum against the lipoprotein was prepared by injecting 2 mg of the purified lipoprotein into each of two male rabbits. The lipoprotein was suspended in Freund complete adjuvant (Difco) and injected into rabbits intradermally at multiple sites on the back. One month after the first injection, another 1 mg of the lipoprotein was injected into each of the rabbits as described above. Antiserum was then collected from the rabbits 10 days after the second injection.

The immunoprecipitation was carried out as follows: 80 μl of 0.25 M sodium phosphate buffer, pH 7.0, 80 μl of 0.25% sodium dodecyl sulfate containing various amounts of the purified lipoprotein, and 40 μl of serum were mixed, and the mixture was incubated at room temperature for 30 min. After the incubation, the mixture was kept at 4 C overnight. The precipitate was collected by centrifugation at 5,000 $\times$ *g* for 10 min and was washed three times with 1.5 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.9% NaCl, 0.1 mM ethylenediaminetetraacetate (EDTA), and 0.1% sodium dodecyl sulfate. The final pellet was dissolved in 0.4 ml of 5 mM NaOH, and the protein content was estimated by the method of Lowry et al. (24). The antigenic activity remaining in the supernatant fraction after the immunoprecipitation was examined by the method of Ouchterlony (25).

The treatment of *E. coli* envelope proteins with antilipoprotein serum was carried out as follows. A 45-ml culture of *E. coli* MX74T2 growing at 37 C in M9 medium supplemented with thiamine (2 μg/ml) and thymidine (4 μg/ml) was labeled with 75 μCi of [^{3}H]arginine in the presence of 10 μg of non-radioactive arginine per ml for one generation (from 2 $\times$ 10^8 cells/ml). The envelope fraction was prepared as described previously (21). The final envelope fraction was solubilized in 0.9 ml of 1% sodium dodecyl sulfate in 0.01 M sodium phosphate buffer, pH 7.0, containing mercaptoethanol and 10% glycerol (solubilizing solution; 21). The reaction mixture for immunoassay consisted of 1.25 ml of 0.12% sodium dodecyl sulfate in 0.12 M sodium phosphate buffer, pH 7.0, 0.15 ml of the envelope protein solution prepared above, and 0.1 ml of antilipoprotein serum or nonimmunized serum. The mixture was incubated at room temperature (21 to 23 C) for 30 min and at 4 C overnight. The immunoprecipitate thus formed was collected by centrifugation at 5,000 $\times$ *g* for 10 min and was washed three times with 1.0 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 0.9% NaCl, 0.1 mM EDTA, and 0.01% sodium dodecyl sulfate. The final precipitate then was solubilized in 0.25 ml of the solubilizing solution (21) at 70 C for 20 min. The solution (0.2 ml) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the use of a 7.5% gel as described previously (21). Gel electrophoresis was carried out with the use of internal molecular weight standards (19).

Circular dichroism. Circular dichroism measurements were determined with a JASCO 20A spectropolarimeter with use of a cylindrical quartz cells (light path, 1 cm).

RESULTS

Purification procedure. The free form of the lipoprotein was purified by the following procedure.

(i) Homogenization of cells. *E. coli* B (180 g) was suspended in 180 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.01 M EDTA (S buffer), and 540 g of glass beads (di-

ameter, 0.02 mm) was added to the cell suspension. The cells were homogenized in a Waring blender for 7.5 min in a cold room (5 C). The cell homogenate was chilled to 10 C in an ice bath, and the homogenization procedure was repeated eight times in total.

(ii) Preparation of membrane fraction. The final cell homogenate was centrifuged at 3,000 × *g* for 10 min to remove glass beads. The precipitated glass beads were washed two times with 100 ml of S buffer. The glass bead washes were combined with the original supernatant fluid (390 ml) and centrifuged at 34,000 × *g* for 20 min to precipitate the membrane fraction. The membrane fraction was washed three times with 100 ml of S buffer.

(iii) Solubilization of membrane fraction. The final membrane fraction was suspended in 400 ml of S buffer by sonication, and 16 g of sodium dodecyl sulfate was added to the suspension. The mixture was then incubated at 95 C for 10 min. The solubilized membrane fraction was adjusted to 500 ml by adding 4% sodium dodecyl sulfate in S buffer.

(iv) Acetate treatment. The solubilized membrane fraction was treated with acetate as described for the purification of phospholipase from the *E. coli* membrane (26). The solubilized membrane fraction was supplemented with 250 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.5, and 250 ml of 0.02 M EDTA, and the pH was adjusted to 8. To the mixture was added 150 ml of *n*-butanol, and the final mixture was chilled in an ice bath. The addition of *n*-butanol prevents sodium dodecyl sulfate from precipitating at a low temperature. After cooling, 25 ml of 4 N sodium acetate buffer, pH 5.5, was added to the mixture, and the pH was adjusted to 5.2. After 20 min of incubation in an ice bath, the precipitate thus formed was removed by centrifugation at 13,000 × *g* for 10 min.

(v) Acetone fractionation. To the supernatant fluid (1,050 ml) was added 20 ml of 1 M $MgSO_4$ and 54 ml (0.05 volume) of acetone. After 20 min of incubation in an ice bath, the mixture was centrifuged at 13,000 × *g* for 10 min. The precipitate was discarded, and 260 ml (0.3 final volume) of acetone was added to the supernatant fraction. The mixture was incubated in an ice bath for another 20 min and centrifuged at 13,000 × *g* for 10 min.

(vi) Phenol extraction. The precipitate obtained above was solubilized in 100 ml of S buffer containing 1% sodium dodecyl sulfate. Then 100 ml of 90% phenol was added to the solution, and the mixture was shaken vigorously to extract the lipoprotein into the phenol layer. The lipopolysaccharide as the main contaminant at this purification step remained in the water layer. The two layers were separated by centrifugation at 13,000 × *g* for 10 min, and the resultant water layer was once more extracted with 100 ml of 90% phenol. The combined phenol fraction was back-washed with 100 ml of water, and the two layers were separated by centrifugation at 13,000 × *g* for 10 min. The phenol layer was back-washed with 50 ml of water. When the separation of the two layers was difficult even by centrifugation, about 50 mg of NaCl was added to the mixture before centrifugation. To the final phenol fraction (220 ml) was added 440 ml of acetone and 440 ml of ether. The resulting precipitate was collected by centrifugation and dissolved in 50 ml of 1% sodium dodecyl sulfate. The purity of the lipoprotein at this step can be examined by measuring the molar ratios of glycine and alanine in the preparation, because the lipoprotein has nine alanine residues but no glycine residues. A ratio of 0.6 was determined, indicating that the preparation at this step still contained a large amount of contaminating proteins. Furthermore, the preparation contained as much phenylalanine as tyrosine, whereas the pure lipoprotein has one tyrosine residue but no phenylalanine residues.

(vii) Second acetone fractionation. The protein solution was supplemented with 0.5 ml of 1 M $MgSO_4$ and precipitated with 5 ml of acetone at room temperature. Twenty minutes after the addition of acetone, the resultant precipitate was removed by centrifugation at 12,000 × *g* for 10 min. To the supernatant fluid was added 5 ml of acetone, and the mixture was incubated for 20 min at room temperature. The lipoprotein was precipitated by this treatment and collected by centrifugation, and the precipitate was again dissolved in 20 ml of 1% sodium dodecyl sulfate. The molar ratio of glycine to alanine decreased in the preparation to about 0.1. However, it still contained appreciable amounts of phenylalanine and proline. Assuming that all the alanine in the preparation was derived from the lipoprotein, the yield of the lipoprotein was calculated to be 98 mg.

(viii) Third acetone fractionation. To the solution was added 0.2 ml of 1 M $MgSO_4$ followed by 2 ml of acetone. After 20 min of incubation at room temperature, the precipitate was removed by centrifugation at 12,000 × *g* for 10 min. To the supernatant fraction was added an additional 2 ml of acetone to precipitate the lipoprotein. The molar ratio of glycine to alanine contents came to less than 0.01, indicating that the preparation was highly pure. Furthermore, proline and phenylalanine were not detected in the preparation. The total yield of the

lipoprotein was calculated to be 90 mg from the alanine content, and the preparation was also shown to be homogeneous and highly pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The amino acid analysis of the preparation will be shown later (Table 1). Most of the experiments used this preparation. However, this preparation still contained a small amount of phospholipids (about 0.5 to 1.4 mol/mol of the lipoprotein on the basis of the phosphate analysis).

(ix) Dephospholipidation. Purified lipoprotein (8 mg) was dissolved in 2 ml of 1% sodium dodecyl sulfate and mixed with 2 ml of chloroform, and the mixture was shaken vigorously. Then, 4.4 ml of methanol was added to the mixture to make it a one-phase solution. The resultant precipitate was collected by centrifugation at 12,000 × *g* for 10 min. The precipitate was dissolved in 2 ml of sodium dodecyl sulfate, and the chloroform-methanol treatment was repeated four times. The whole procedure was carried out at room temperature. The final precipitate was washed with 3 ml of 50% acetone twice and with 4 ml of water twice to remove the sodium dodecyl sulfate. The recovery of the lipoprotein at this step was 74%. The washed precipitate was suspended in water and stored in a freezer. This preparation contained less than 0.02 mol of phosphate per mol of the lipoprotein, indicating that the lipoprotein was essentially free of phospholipids and lipopolysaccharides.

Paracrystallization. Ten milligrams of purified lipoprotein at step viii or ix was dissolved in 5 ml of 1% sodium dodecyl sulfate, and $MgSO_4$ was added to a final concentration of 0.01 M. The paracrystals were formed by adding acetone gradually to the mixture to a final concentration of 20% at room temperature. When observed under a phase microscope, the paracrystals were needle shaped (Fig. 1). The lipoprotein was paracrystallized from the Triton X-100 solution as follows. The lipoprotein solubilized in 1% sodium dodecyl sulfate was precipitated by adding acetone (final concentration, 50%). The resultant precipitate was washed with water several times and solubilized in 0.1% Triton X-100 to a final concentration of about 5 mg/ml. $MgSO_4$ was then added

TABLE 1. *Amino acid analysis of the free form of the lipoprotein*

Amino acid	Hydrolysis						Total avg	Integer value	From the sequence[b]
	24 h[a]			72 h[a]					
	1	2	Avg	1	2	Avg			
Lysine	5.1	4.7	4.9	4.8	5.0	4.9	4.9	5	5
Histidine	0	0	0	0	0	0	0	0	0
Arginine	4.4	4.2	4.3	3.9	3.9	3.9	4.1	4	4
Aspartic acid	13.7	13.2	13.5	13.0	14.3	13.7	13.6	14	14
Threonine	1.7	2.0	1.9	1.7	1.7	1.7	2.0[c]	2	2
Serine	5.7	5.4	5.6	4.5	5.2	4.9	5.9[c]	6	6
Glutamic acid	5.2	5.1	5.2	5.0	5.4	5.2	5.2	5	5
Proline	0	0	0	0	0	0	0	0	0
Glycine	0	0	0	0	0	0	0	0	0
Alanine	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9	9
Half-cystine	0	0	0	0	0	0	0	0	0
Valine	3.9	4.1	4.0	3.8	4.2	4.0	4.0	4	4
Methionine	1.7	1.7	1.7	1.8	1.9	1.9	1.8	2	2
Isoleucine	1.1	1.1	1.1	1.0	1.0	1.0	1.1	1	1
Leucine	4.1	4.2	4.2	3.9	4.0	4.0	4.1	4	4
Tyrosine	0.9	0.9	0.9	0.7	0.8	0.8	0.9	1	1
Phenylalanine	0	0	0	0	0	0	0	0	0
Glycerylcysteine[d]	0.6		0.6					1	1
Tryptophan[e]								0	0

[a] All values are calculated assuming that there are nine alanine residues per molecule of the lipoprotein.

[b] From the complete amino acid sequence of the bound form of the lipoprotein determined by Braun and Bosch (3, 4).

[c] The value is estimated by extrapolation to zero time of hydrolysis.

[d] Obtained from amino acid analysis after performic acid oxidation of the lipoprotein. Two peaks which appeared at the relative mobilities of 0.43 and 0.66 to aspartic acid, respectively, were taken as oxidized forms of glycerylcysteine. Their recoveries were 0.2 and 0.4 for the first and second peaks, respectively, assuming that their ninhydrin color yields are the same as that of aspartic acid. These values were calculated on the basis of nine alanine residues per one molecule of the lipoprotein.

[e] Determined by the spectrophotometric technique of Goodwin and Morton (10).

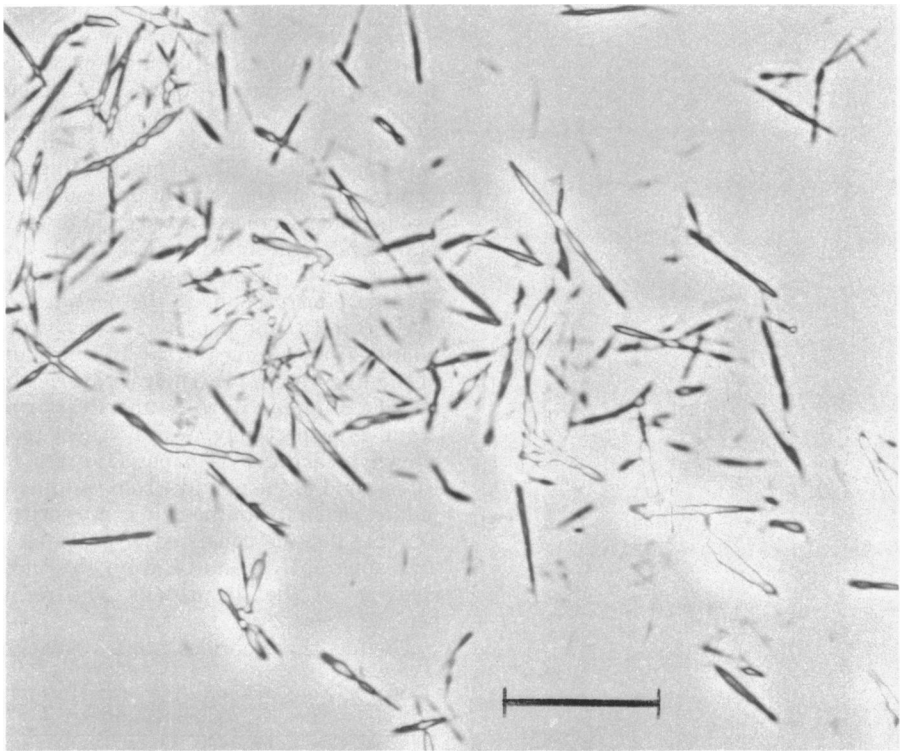

FIG. 1. *Paracrystals of the lipoprotein formed from the sodium dodecyl sulfate solution. The picture was taken with a phase-contrast microscope. Bar, 10 μm.*

to 0.01 M. Paracrystals were formed by adding acetone gradually. Figure 2 shows a paracrystal thus formed and observed under a light microscope with Nomarski interference optics. The light and dark patterns seen in the paracrystal probably represent changes in its thickness. Ultrastructural characteristics of these paracrystals have been studied and are discussed in the accompanying paper (8).

Amino acid analysis. The amino acid analysis of the purified lipoprotein is shown in Table 1. The amount of glycerylcysteine was estimated from the amino acid analysis of the performic acid-oxidized protein. When oxidized, two peaks, a minor and a major peak, appeared, and their positions relative to aspartic acid in the amino acid analysis were found to be 0.43 and 0.66, respectively. Their recoveries were 0.2 and 0.4 mol/mol of the lipoprotein for the minor and the major peak, respectively (Table 1). Both peaks were assumed to be derived from the oxidation of glycerylcysteine, as shown for the bound form of the lipoprotein (13). The minor peak in the present paper was identified as cysteic acid from its position in amino acid analysis.

The amino acid composition of the free form of the lipoprotein was identical to that obtained for the complete amino acid sequence of the bound form (13; see Table 1). As shown previously (17), diaminopimelic acid was not detected in the free form.

Fatty acid analysis. The fatty acid composition of the free form of the lipoprotein obtained from step ix is shown in Table 2. Total fatty acid content was estimated at 2.5 mol/mol of the lipoprotein. This value agrees with the value expected from the structure of the bound form (3 mol of fatty acids per mol of the lipoprotein) (13). In Table 2, ester-linked fatty acid and amide-linked fatty acid compositions are shown. We have confirmed the data of Hantke and Braun (13), in which the content of amide-linked palmitic acid in the lipoprotein is much higher than that of ester-linked palmitic acid.

Cyanogen bromide cleavage. To compare the structure of the free form, the purified lipoprotein was treated with cyanogen bromide and the resultant peptides were separated by two-dimensional separation on a thin-layer plate as described previously (16). Cyanogen bromide cleavage was chosen because the lipoprotein

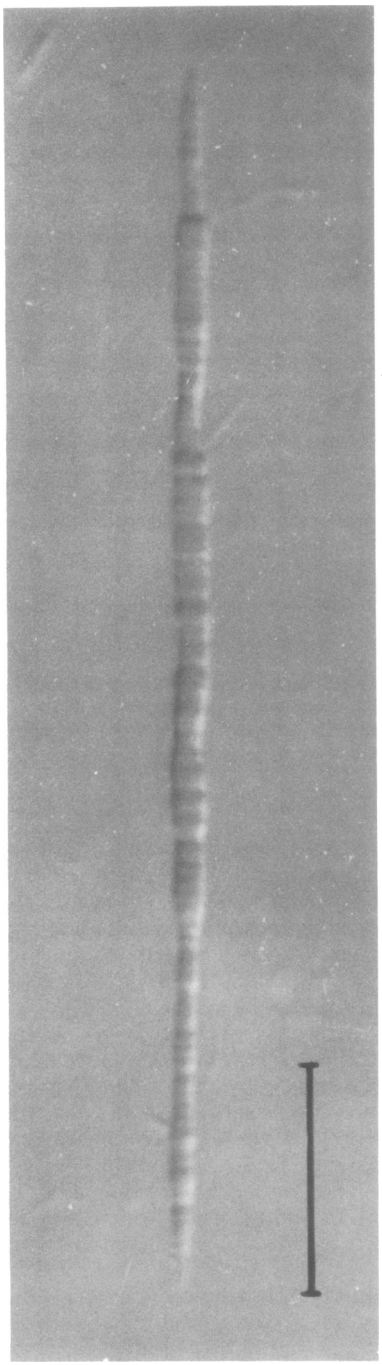

FIG. 2. *Nomarski optical micrograph of a paracrystal of the lipoprotein prepared from Triton X-100 solution. Bar, 10 μm.*

has been shown to be very resistant to proteases (4). After cyanogen bromide cleavage, insoluble material was isolated by centrifugation and washed with water three times. The amino acid analysis of this insoluble material showed the same amino acid composition as that of the amino terminal peptide from the first glycerylcysteine to the 31st methionine residue of the bound form (3, 4). The recovery of this peptide was 42%. The soluble fraction was separated into two major components by peptide mapping as shown previously (16). The two peptides were extracted from the thin-layer plate and hydrolyzed in 6 N HCl, and their amino acid compositions were determined. Peptide 1 corresponds to the peptide from residues 53 to 58, and peptide 2 corresponds to the peptide from residues 32 to 52 of the bound form of the lipoprotein (3). Their recoveries from the peptide mapping were 18 and 19% for peptides 1 and 2, respectively. A minor spot (peptide 3) shown in a previous paper (16) was found to correspond to the peptide from residues 32 to 58 judging from its amino acid composition (Table 3). This peptide consisted of peptides 1 and 2 and apparently resulted from the incomplete cleavage of the lipoprotein. The recovery of peptide 3 was 2%.

The fact that peptides 1 and 3 have no homo-

TABLE 2. *Fatty acid analysis of the free form of the lipoproteins*

Fatty acid[a]	Ester linked (%)	Amide linked (%)	Total (%)
$C_{14:0}$	1.0		2.1
$C_{16:0}$	44.9	68.5	58.6
$C_{16:1}$	13.6	5.8	3.6
$C_{17\ cyclo}$	14.9	8.5	13.5
$C_{18:1}$	18.7	15.0	12.8
$C_{19\ cyclo}$	7.0	2.7	8.5

[a] Fatty acid composition was analyzed as described in Materials and Methods. Those fatty acids of which amounts are less than 1% are not listed.

TABLE 3. *Amino acid composition of the peptides obtained by cyanogen bromide cleavage of the free form of the lipoprotein*

Amino acid	Insoluble	Spot 1	Spot 2	Spot 3
Lysine	2.0 (2)[a]	2.1 (2)	1.0 (1)	3.1 (3)
Arginine		1.1 (1)	2.7 (3)	3.8 (4)
Aspartic acid	7.5 (8)		6.0 (6)	5.1 (6)
Threonine	1.0 (1)	0.9 (1)		1.3 (1)
Serine	4.2 (5)		1.0 (1)	1.2 (1)
Glutamic acid	3.0 (3)		2.2 (2)	2.4 (2)
Alanine	3.0 (3)	1.0 (1)	5.0 (5)	6.0 (6)
Valine	2.6 (3)		1.0 (1)	0.8 (1)
Methionine				1.0 (1)
Isoleucine	0.8 (1)			
Leucine	2.8 (3)		0.9 (1)	0.9 (1)
Tyrosine		0.2 (1)		0.3 (1)
Glycerylcysteine[b]	0.4 (1)			
Homoserine	0.7 (1)		0.4 (1)	

[a] The amino acid compositions are expressed as relative ratios. Numbers in parentheses are the amino acid compositions from the sequence of the bound form of the lipoprotein (13).

[b] Determined as described in Table 1.

serine residues means that they were derived from the carboxyl terminal part of the protein. Peptide 1 was digested with carboxypeptidases A and B for 5 min at 37 C. The molar ratios of the amino acids released were: Lys, 0.4; Arg, 0.2; Thr, 0; Ala, 0; and Tyr, 0. When digested for 15 min under the same conditions, the following molar ratios were released: Lys, 0.5; Arg, 0.4; Thr, 0; Ala, 0; and Tyr, 0.1. These data, as well as the amino acid composition, indicate that peptide 1 is the same peptide as the carboxyl terminal peptide of the bound form, Ala-Thr-Lys-Thr-Arg-Lys. From these data we can conclude that the primary structure of the free form is identical to that of the bound form.

Circular dichroism spectra. Figure 3 shows the circular dichroism spectrum of the purified lipoprotein in 0.01% sodium dodecyl sulfate. Using the formula, percentage of the α helix = ($[\theta]$ 208 nm − 4,000)/(33,000 − 4,000) (reference 11), the α-helical content was estimated to be 87%. More detailed analysis of circular dichroism spectra under different conditions will be published elsewhere.

Immunoassay of the lipoprotein. Figure 4 shows the specific immunoprecipitation of the lipoprotein by antiserum against the lipoprotein. Since the lipoprotein is completely water insoluble, the immunoprecipitate was carried out in 0.1% sodium dodecyl sulfate. Under these conditions, the amounts of the immunoprecipitate increased as the amount of the lipoprotein in the reaction mixture increased up to 30 μg. The lipoprotein seemed to be precipitated completely when the amounts of the lipoprotein in the reaction mixture were 7.5 and 15 μg, since the lipoprotein was not detected in the supernatant fractions (Fig. 4). The immunoprecipitate appears to be caused by specific antibodies, since no formation of the immunoprecipitate was observed between nonimmunized serum and the lipoprotein (Fig. 4).

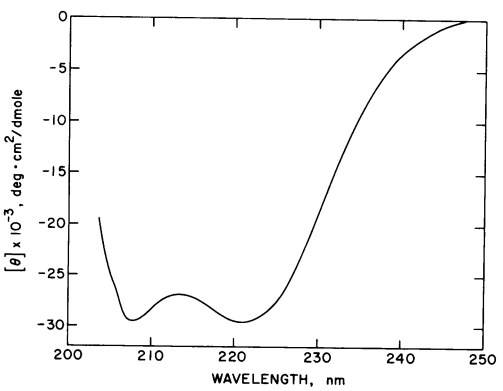

FIG. 3. *Circular dichroism spectrum of the free form of the lipoprotein in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.01% sodium dodecyl sulfate.*

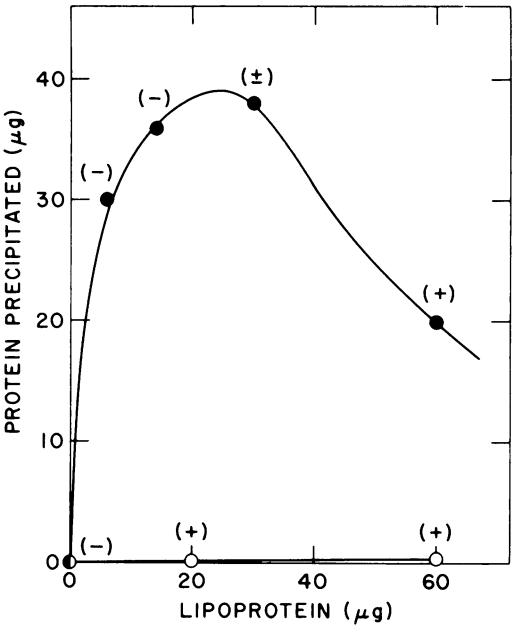

FIG. 4. *Immunoprecipitation of the lipoprotein by antilipoprotein serum. Immunoprecipitation was carried out as described in Materials and Methods. Symbols: (●) The reaction was carried out with antilipoprotein serum; (○) with nonimmunized serum. The supernatant of each immunoassay mixture was tested for the remaining antigen by the immunodiffusion test (25). (+) and (−) at each point represent the presence and the absence of the lipoprotein in the supernatant, respectively.*

The specificity of the immunoprecipitation is further shown in Fig. 5. Total envelope proteins labeled with [^{3}H]arginine were solubilized in sodium dodecyl sulfate and reacted with antilipoprotein serum in a final concentration of 0.2% sodium dodecyl sulfate. The resultant precipitate was washed with 0.01% sodium dodecyl sulfate instead of 0.1% sodium dodecyl sulfate. The final immunoprecipitate was solubilized in 1% sodium dodecyl sulfate for 20 min at 70 C and subjected to polyacrylamide gel electrophoresis. The lipoprotein peak located between the internal standards e (cytochrome *c*) and f (5-dimethylaminonaphthalene-1-sulfonyl [Dns]-insulin) is the only major protein recovered in the immunoprecipitate except a small broad peak near the internal standard b (Dns-bovine serum albumin) (Fig. 5B). Because no protein was precipitated with nonimmunized serum, the immunoprecipitation reaction with antilipoprotein serum in 0.2% sodium dodecyl sulfate appears to be very specific for the lipoprotein.

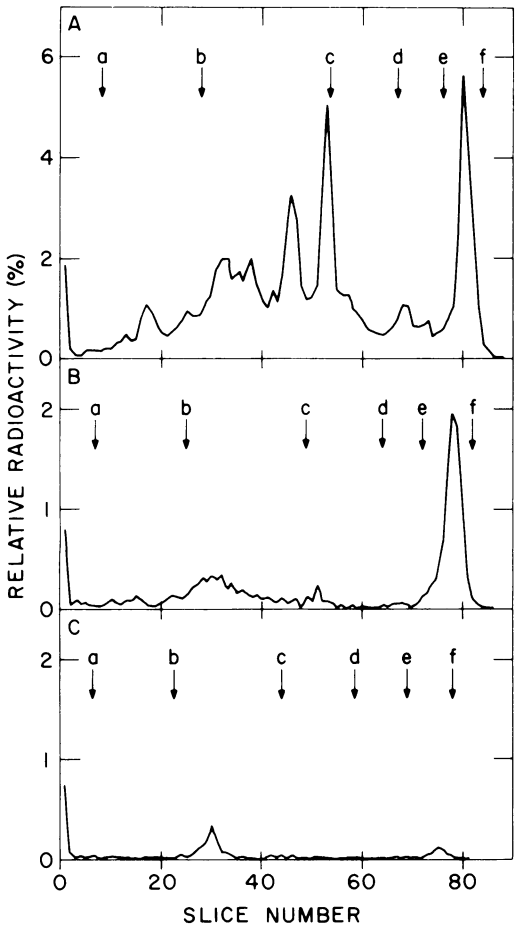

FIG. 5. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the E. coli envelope proteins before and after treatment with antilipoprotein serum. The envelope proteins were labeled with [^{3}H]-arginine and treated with antilipoprotein serum or nonimmunized serum as described in Materials and Methods. Gel electrophoresis was carried out as described previously (21). (A) Total envelope proteins without treatment with antilipoprotein serum; (B) immunoprecipitate with antilipoprotein serum; (C) immunoprecipitate with nonimmunized serum. Arrows with letters indicate positions of the internal molecular weight standards (19): a, dimer; b, monomer of Dns-bovine serum albumin; c, dimer; d, monomer of Dns-egg white lysozyme; e, cytochrome c; f, Dns-insulin.*

DISCUSSION

In previous papers (17, 22) we have shown that the lipoprotein in the *E. coli* outer membrane exists as a bound form covalently linked to the peptidoglycan and as a free form unlinked to the peptidoglycan. In the present paper, we have achieved the purification of the free form of the lipoprotein on a large scale. The final preparation was judged to be homogeneous from the following criteria. (i) The preparation had less than 0.02 mol of phosphate per mol of the lipoprotein. This indicates that the preparation was essentially free of phospholipids as well as lipopolysaccharides. (ii) The amino acid composition was identical to that of the bound form. (iii) The preparation did not contain diaminopimelic acid and glucosamine, indicating that it did not contain the bound form as a contaminant. (iv) The preparation had the same fatty acid composition as the bound form. The fatty acid composition also supports the data that the preparation is free of the lipopolysaccharides, since it did not contain β-hydroxymyristic acid, which is specific for the lipopolysaccharides. (v) Cyanogen bromide cleavage of the preparation gave rise to the same peptides as those expected from cyanogen bromide cleavage of the bound form. (vi) The preparation was homogeneous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and formed paracrystals showing highly ordered ultrastructural characteristics. (vii) The preparation had a very high α-helical content (87%), as shown for the bound form (12). (viii) Antiserum prepared against the present preparation specifically precipitated the free form of the lipoprotein from the *E. coli* envelope. The specificity of the antilipoprotein serum has been demonstrated previously for several different gram-negative bacteria (12).

In the course of developing the purification procedures, we attempted gel filtration on the basis of the low molecular weight (7,200) of the free form of the lipoprotein. However, it was unsuccessful because the lipoprotein tended to aggregate into forms having extremely high molecular weights. The purification method with the use of trichloroacetic acid in 1% sodium dodecyl sulfate was suggested in a previous paper (17), but a small amount of contaminating proteins could not be removed by this method. One of the most critical steps in the present purification method is step iv, which was developed by Scandella and Kornberg (26) for the purification of phospholipase A1. Another important step is step vi, in which the lipoprotein is extracted into a phenol layer and separated from the lipopolysaccharides and other proteins. Apparently, the lipoprotein was not denatured irreversibly by these procedures, since the final preparation retained a high α-helical content. Amino acid analysis was used throughout the purification procedures to examine the purity as well as the recovery of the lipoprotein at each step. Amino acid analysis was particularly useful because of the unique

amino acid composition of the lipoprotein, which lacks proline, glycine, phenylalanine, tryptophan, and histidine.

Highly ordered ultrastructural characteristics were observed in the negatively stained lipoprotein paracrystals (8). Elucidation of the assembly mechanism of the lipoprotein molecules in the paracrystals is thought to be important for understanding the structure and function of the lipoprotein. We have observed some variation in ultrastructural characteristics of paracrystals, depending upon slight differences in paracrystallization methods. We have proposed models of the assembly mechanisms of the lipoprotein in the paracrystals (8).

ACKNOWLEDGMENTS

This research was supported by grants from U.S. Public Health Service (BM 19043, Bacteriology and Mycology group), the American Cancer Society (BC-67), and the National Science Foundation (BO 42237).

LITERATURE CITED

1. **Bosch, V., and V. Braun.** 1973. Distribution of murein lipoprotein between the cytoplasmic and outer membrane of *Escherichia coli*. FEBS Lett. 34:307-310.
2. **Braun, V.** 1973. Molecular organization of the rigid layer and the cell wall of *Escherichia coli*. J. Infect. Dis. 128:S9-S16.
3. **Braun, V., and V. Bosch.** 1972. Repetitive sequences in the murein-lipoprotein of the cell wall of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 69:970-974.
4. **Braun, V., and V. Bosch.** 1972. Sequence of the murein-lipoprotein and the attachment site of the lipid. Eur. J. Biochem. 28:51-69.
5. **Braun, V., K. Kehn, and H. Wolff.** 1970. Supramolecular structure of the rigid layer of the cell wall of *Salmonella, Serratia, Proteus,* and *Pseudomonas fluorescens*. Number of lipoprotein molecules in a membrane layer. Biochemistry 9:5041-5049.
6. **Braun, V., and J. Sieglin.** 1970. The covalent murein-lipoprotein structure of the *Escherichia coli* cell wall. The attachment site of the lipoprotein on the murein. Eur. J. Biochem. 13:336-346.
7. **Braun, V., and H. Wolff.** 1970. The murein-lipoprotein linkage in the cell wall of *Escherichia coli*. Eur. J. Biochem. 14:387-391.
8. **DeMartini, M., S. Inouye, and M. Inouye.** 1976. Ultrastructure of paracrystals of a lipoprotein from the outer membrane of *Escherichia coli*. J. Bacteriol. 127:564-571.
9. **Fiske, C. H., and Y. Subbarow.** 1925. The colorimetric determination of phosphorous. J. Biol. Chem. 66:375-400.
10. **Goodwin, T. W., and R. A. Morton.** 1946. The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. 40:628-632.
11. **Greenfield, N., and G. D. Fasman.** 1969. Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 8:4108-4116.
12. **Halegoua, S., A. Hirashima, and M. Inouye.** 1974. Existence of a free form of a specific membrane lipoprotein in gram-negative bacteria. J. Bacteriol. 120:1204-1208.
13. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the *Escherichia coli* outer membrane. Eur. J. Biochem. 34:284-296.
14. **Hirashima, A., G. Childs, and M. Inouye.** 1973. Differential inhibitory effects of antibiotics on the biosynthesis of envelope proteins of *Escherichia coli*. J. Mol. Biol. 79:373-389.
15. **Hirashima, A., and M. Inouye.** 1973. Specific biosynthesis of an envelope protein of *Escherichia coli*. Nature (London) 242:405-407.
16. **Hirashima, A., S. Wang, and M. Inouye.** 1974. Cell-free synthesis of a specific lipoprotein of the *Escherichia coli* outer membrane directed by purified messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 71:4149-4153.
17. **Hirashima, A., H. C. Wu, P. C. Venkateswaran, and M. Inouye.** 1973. Two forms of a structural lipoprotein in the envelope of *Escherichia coli*. Further characterization of the free form. J. Biol. Chem. 248:5654-5659.
18. **Hirs, C. H. W.** 1956. The oxidation of ribonuclease with performic acid. J. Biol. Chem. 219:611-621.
19. **Inouye, M.** 1971. Internal standards for molecular weight determinations of proteins by polyacrylamide gel electrophoresis. J. Biol. Chem. 246:4834-4838.
20. **Inouye, M.** 1974. A three-dimensional molecular assembly model of a lipoprotein from the *Escherichia coli* outer membrane. Proc. Natl. Acad. Sci. U.S.A. 71:2396-2400.
21. **Inouye, M., and A. B. Pardee.** 1970. Changes of membrane proteins and their relation to DNA synthesis and cell division of *Escherichia coli*. J. Biol. Chem. 245:5813-6819.
22. **Inouye, M., J. Shaw, and C. Shen.** 1972. The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. 247:8154-8159.
23. **Lee, N., and M. Inouye.** 1974. Outer membrane proteins of *Escherichia coli*: biosynthesis and assembly. FEBS Lett. 39:167-170.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
25. **Ouchterlony, O.** 1968. Handbook of immunodiffusion and immunoelectrophoresis, p. 21-23. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.
26. **Scandella, C. J., and A. Kornberg.** 1971. A membrane bound phospholipase A1 purified from *Escherichia coli*. Biochemistry 10:4447-4456.