Characterization of a Novel Lipoprotein Mutant in *Escherichia coli*\(^*\)

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Mutants altered in the structural gene for murein lipoprotein in *Escherichia coli* can be isolated by globomycin selection. We have isolated a unique globomycin-resistant mutant, strain 6-23, which synthesizes a structurally altered, albeit modified and processed, lipoprotein. DNA sequence analysis of the mutant *ipp* allele and determination of the amino acid composition of the mutant lipoprotein revealed a single amino acid substitution of cysteine for arginine at the 68th amino acid residue of prolipoprotein. Pulse-chase experiments revealed that the kinetics of lipoprotein maturation was affected by this alteration in the structure of lipoprotein.

Murein lipoprotein, a major outer membrane protein in *Escherichia coli*, is first synthesized as a prolipoprotein and then modified and processed to become the mature form of lipoprotein (1). The biochemical mechanisms for the assembly of lipoprotein into the outer membrane have been extensively studied. In vivo and in vitro studies have established the sequence of events shown in Fig. 1 (2). The proteolytic cleavage of the modified prolipoprotein by prolipoprotein signal peptidase is inhibited by a cyclic peptide antibiotic, globomycin (3). The accumulation of modified prolipoprotein in the cytoplasmic membrane of globomycin-treated cells results in spheroplast formation and eventual cell lysis (4). An *E. coli* mutant containing no murein lipoprotein due to a deletion in the lipoprotein structural gene (*ipp*) is resistant to globomycin. Likewise, an *E. coli* mutant containing unmoldified prolipoprotein due to a mutation in the signal sequence of prolipoprotein is also more resistant to globomycin than the parental strain (5). Globomycin has therefore become an invaluable tool in the biochemical and genetic studies of the biosynthesis of lipoprotein. Globomycin-resistant mutants of *E. coli* have been isolated; a large fraction of these mutants either synthesize a reduced or undetectable level of lipoprotein or they contain structurally altered prolipoproteins which are defective as substrates in the first step of the modification reactions, i.e. the attachment of a glyceryl moiety to the 21st amino acid residue (cysteine) of the prolipoprotein (6). Inasmuch as a large portion of the unmoldified prolipoprotein can be translocated into the outer membrane in a signal sequence mutant of the *ipp* gene (7), *ipp* mutants defective in the modification of prolipoprotein would be resistant to globomycin. In the course of this study, we have isolated a unique globomycin-resistant mutant, strain 6-23, which synthesizes a structurally altered, albeit modified and processed, lipoprotein. This paper reports the cloning and DNA sequence analysis of the mutant *ipp* allele as well as the purification and characterization of the mutant lipoprotein. In addition, we report the effect of the amino acid alteration in the mutant lipoprotein, as deduced from the DNA sequence analysis, on the kinetics of lipoprotein maturation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Phage, and Culture Medium**—Bacterial strains used in this study include *E. coli* E609, E610, JE5511, JE5505, and E6081, which have been previously described (8-11). A derivative of 

**Labeling Experiments**—For labeling experiments with radioactive amino acids, M9-minimal medium supplemented with 0.4% glucose, 20 μg/ml of required amino acids, and 5% PPBE was used. Unlabeled amino acids (10 μg/ml final concentration) were added to the culture before labeling. PPBE medium was used for labeling experiments with [2-3H]glycerol or [3H]palmitate. The labeling procedures were described in detail previously (13).

**Determination of Glycerolcysteine and Cysteine Contents in Lipoprotein**—Both mutant 6-23 and parental strain E609 were doubly labeled with [35S]sulfuric acid and [2-3H]glycerol. The membrane fraction was isolated and extracted with chloroform/methanol (2:1, v/v) in order to remove phospholipids and then solubilized in 1% SDS (in 10 mM Na phosphate buffer, pH 7.0). Immunoprecipitation of the solubilized lipoprotein was carried out as previously described (13). The lipoprotein doubly labeled with [2-3H]glycerol and [35S]sulfate was further purified by preparative SDS-urea gel electrophoresis (14), followed by Sephadex G-100 column chromatography. The purified lipoprotein was oxidized with performic acid overnight at 4 °C (15), and then hydrolyzed in 6 N HCl at 110 °C for 20 h in vacuo. The acid hydrolysates were analyzed by high voltage paper electrophoresis according to the procedure described previously (16).

**Bacterial Genetic and Recombinant DNA Techniques**—The nutrient agar selection for JE5505 (Aipp6-23) clones was the same as previously described (12) for the cloning of the wild type *ipp* allele with slight modifications. High titer phage stock was prepared according to Ref. 17 using strain E609 as the host. Phage DNA was prepared according to Ref. 12. Restriction enzymes (HindIII and XbaI) and T4 DNA ligase were purchased from Bethesda Research Laboratory; AMV reverse transcriptase was purchased from Life Sciences Inc., St. Petersburg, FL. The conditions for restriction enzyme digestion and DNA ligation are described in Ref. 12. Plasmid DNA was prepared according to Ref. 18. The 3'-end labeling reaction was carried out with reverse transcriptase using DNA with staggered ends and [α-32P]dCTP. The reaction conditions are described in Ref. 12.

**Purification of Free Form Lipoprotein from Mutant 6-23**—The free form of lipoprotein from mutant 6-23 was purified by the procedure described by Lin et al. (20). The last step of purification following the Sephadex G-100 column chromatography was preparative SDS-15%polyacrylamide gel electrophoresis (21). The 18% Laemmli slab gel system ensured the complete separation of lipoprotein from contaminating proteins.

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The abbreviations used are: PPBE, proteose peptone beef extract; SDS, sodium dodecyl sulfate; PLP, prolipoprotein; kb, kilobase.
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Isolation of Globomycin-resistant E. coli Mutant Strain 6-29—Wild type E. coli strain E609 was mutagenized with nitrosoguanidine (50 μg/ml). Following phenotypic expression and segregation, the mutagenized cells were treated with globomycin (30 μg/ml) in PPBE broth overnight. Single colonies were obtained and individual colonies which survived globomycin treatment were tested for the presence of murein lipoprotein by the Ouchterlony double diffusion test using antisera against lipoprotein. Globomycin-resistant mutants containing normal levels of lipoprotein based on the Ouchterlony test were further studied by biochemical analysis of mutant lipoproteins using SDS-polyacrylamide gel electrophoresis. Mutant 6-23 was chosen for further studies based on the anomalous mobility of mutant 6-23 lipoprotein in SDS gels (see below).

Globomycin Sensitivity of the Mutant Strain—The minimal inhibitory concentrations of globomycin were 80 and 20 μg/ml for mutant strain 6-23 and the parental wild type strain E609, respectively. In vivo labeling experiments revealed that the globomycin concentrations required for an accumulation of prolipoprotein as 50% of lipoprotein synthesized during a 5-min labeling were 80 and 40 μg/ml for mutant strain 6-23 and wild type strain E609, respectively.

Mutant Lipoprotein Forms Dimer in the Absence of Sulfhydryl-reducing Agent—One of the unusual features of the lipoprotein present in mutant strain 6-23 is the formation of a lipoprotein dimer in the absence of 2-mercaptoethanol (Table I). The dimer form of the mutant lipoprotein was converted into monomeric form when treated with 2-mercaptoethanol. The mutant lipoprotein in its reduced form migrated more slowly than that of the wild type lipoprotein in an SDS-urea-polyacrylamide gel (Table I). The mobility of the mutant

<table>
<thead>
<tr>
<th>Characterization of free form lipoprotein in mutants of E. coli</th>
<th>Mobility in SDS-polyacrylamide gel electrophoresis</th>
<th>Presence of glyceride</th>
<th>Dimer formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Re valuea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (E609)</td>
<td>0.56</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>lpp 77-1 (lpm)</td>
<td>0.56</td>
<td>0.4</td>
<td>+</td>
</tr>
<tr>
<td>lpp 14-1 (milA)</td>
<td>0.52</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>lpp 68-1 (6-23)</td>
<td>0.54</td>
<td>0.34</td>
<td>+</td>
</tr>
</tbody>
</table>

a Re values were determined by normalizing the mobility of lipoproteins against that of the tracking dye in the SDS-urea-polyacrylamide gel electrophoresis.

The presence of glyceride in lipoprotein was determined by the following method. E. coli cells were labeled at steady state with either [2-3H]glycerol or [14C]arginine or [3H]palmitate and [14C]arginine. The cells were then washed and disrupted by sonication. The membrane fraction was collected by centrifugation at 200,000 X g for 1.5 h and SDS-solubilized membrane fraction was immunoprecipitated with antisera against purified lipoprotein. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The incorporation of [2-3H]glycerol or [3H]palmitate into lipoprotein was measured by scintillation counting of sliced gel fractions.

The dimer formation of lipoprotein was determined by SDS-polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol.
lipoprotein was compared further with two other dimer-forming mutant lipoproteins. Mutant JE5511 contains the lip-1 allele and synthesizes a modified and processed lipoprotein with a single amino acid substitution of cysteine for arginine at the 57th residue of the mature lipoprotein (9). Mutant E610 contains the mlpA allele and synthesizes an unmodified and unprocessed prolipoprotein with an unmodified cysteine at the 21st position (23). The presence of the extra cysteine in lip-1 lipoprotein and the unmodified cysteine in mlpA prolipoprotein accounts for the dimer formation of both mutant proteins (Table I). It is interesting to note that both the dimeric form and monomeric form of the 6-23 lipoprotein exhibited an intermediate mobility in an SDS-urea gel as compared to the lip-1 lipoprotein and the mlpA prolipoprotein (Table I). Similar results were obtained using SDS-12.5% polyacrylamide slab gel electrophoresis without urea (data not shown).

Mutant Lipoprotein Is Modified with Glyceride—To determine whether the cysteine at the 21st position of prolipoprotein is engaged in disulfide linkage to form a lipoprotein dimer, we labeled the mutant cells with either [2-3H]glycerol plus [3H]palmitate (Table I). These results strongly suggest that in addition to the glyceride-modified cysteine at the 21st position, the mutant lipoprotein contains an extra cysteine. This speculation was confirmed by the analysis of [35S]sulfate-labeled mutant lipoprotein. Whereas the wild type lipoprotein contained 1 mol of glycerylcysteine sulfone/mol of performic acid-oxidized lipoprotein, mutant 6-23 lipoprotein contained 1 mol of cysteic acid and 1 mol of glycerylcysteine sulfone/mol of performic acid oxidized mutant lipoprotein (Table II).

Purification and Characterization of Free Form Lipoprotein from Mutant 6-23—The free form lipoprotein of mutant strain 6-23 was purified as described under "Experimental Procedures." The amino acid composition of the mutant lipoprotein is almost identical with that of wild type lipoprotein except for the substitution of a cysteine residue for arginine (Table II). Five hundred nmol of purified mutant lipoprotein were subjected to sequence analysis by automated Edman degradation. As expected, the NH2 terminus of the mutant lipoprotein is blocked.

Cloning of lip A Allele from the Globomycin-resistant E. coli Mutant 6-23—in order to determine the structural alteration that occurred within the 6-23 mutant lipoprotein, the 6-23 lip A allele was cloned by in vivo homogenization and complementation. Using a λ phage (λd2) which carries the lip A-flanking sequences while having the lip A region deleted, Fig. 2 illustrates the strategy used in the cloning and subcloning of the mutant 6-23 lip A allele. Phages capable of transducing the 6-23 lip A allele could be isolated after λd2 had been grown lytically on mutant 6-23. The transducing phages carrying the 6-23 lip A allele could complement the lip A deletion strain JE5505 when present as prophages, since the mutant 6-23 lip A allele transduced by the phage (λd2-23) encoded a functional lipoprotein. The 6-23 lip A-transducing phages allowed the lysogen (JE5505/λd-23) to grow as large colonies on nutrient agar plates at 42 °C and phages could be readily induced from the large colonies (JE5505/λd-23) by UV irradiation. The X6-23 phage DNA contains a single XbaI site residing between the promoter and structural gene of the lip A allele, as would be expected from a phage containing the full complement of the 10-kb HindIII fragment spanning the lip A region (24). The separation of the lip A promoter from the structural gene allows easy cloning of the lip A gene region on a multiple copy plasmid.

**TABLE II**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mutant lipoprotein</th>
<th>Normalized value</th>
<th>Wild type lipoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>4.7 ± 0.3</td>
<td>5 (−1)</td>
<td>5</td>
</tr>
<tr>
<td>His</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Arg</td>
<td>5.2 ± 0.3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Asx</td>
<td>3.2 ± 0.4</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Thr</td>
<td>1.9 ± 0.2</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Ser</td>
<td>5.5 ± 0.4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Gln</td>
<td>5.0 ± 0.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Pro</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Gly</td>
<td>0.3 ± 0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ala</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Val</td>
<td>4.4 ± 0.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Met</td>
<td>1.6 ± 0.2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>1.0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Leu</td>
<td>4.3 ± 0.3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.1 ± 0.3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phe</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cys</td>
<td>0.97 ± 0.1</td>
<td>1 (+1)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Glycerylcysteine was detected by the procedure described in the text.

**FIG. 2.** A schematic diagram of the procedure used in cloning and subcloning the 6-23 mutant lip A allele. The cloned DNA fragment and vector DNA are illustrated by open bars and continuous lines, respectively. The X6-23 phage DNA contains a single XbaI site which lies within the ribosomal binding site of the lip A gene. The single HindIII site in PACYC184 DNA is within the tet' gene, and is about 200 base pairs away from the XbaI site. The size of the XbaI-HindIII fragment carrying the lip A allele is approximately 6.5 kb.
vector such as pACYC 184 without its expression, thus avoiding the overproduction of a major outer membrane protein which may be lethal for the host cells. The proximity of the XbaI site to the lpp-coding sequence also makes possible the rapid DNA sequence analysis of various lpp alleles in a single sequencing run. The plasmid containing the 6.5-kb XbaI-HindIII fragment from the lpp region of mutant 6-23 is designated as pJG03.

DNA Sequence Analysis of Mutant 6-23 Lipoprotein Structural Gene—Plasmid pJG03, which contained the lipoprotein structural gene of mutant 6-23, was digested with XbaI restriction enzyme and end-labeled at the 3'-position with reverse transcriptase as described under "Experimental Procedures." The end-labeled DNA was then digested with HindIII enzyme to generate uniquely end-labeled DNA fragments. The fragment containing the lpp allele was purified as described (12), subjected to Maxam and Gilbert sequencing reactions, and analyzed on 6% polyacrylamide urea gels (19). Fig. 3 shows the amino acid sequence of the 6-23 mutant lpp prolipoprotein as deduced from the DNA sequence. The mutant 6-23 lpp allele was found to contain a single point mutation which caused the 68th amino acid residue of the prolipoprotein to change from an arginine to a cysteine. The sense strand of the nucleotide sequence surrounding the point mutation is shown. The G to A transition in the boxes occurs in the 5604

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Fig. 3. Amino acid sequence of 6-23 mutant prolipoprotein deduced from the nucleotide sequence of the mutant 6-23 lpp allele. The nucleotide sequence surrounding the site of the point mutation is shown. The G to A transition in the boxes occurs in the sense strand of the lpp gene and results in a change of the 68th amino acid in the prolipoprotein from arginine to cysteine.

The nucleotide sequence of mutant 6-23 is

\[
\begin{align*}
\text{AAC-CAG-} & \text{ C GT-CTG-GAC} \\
\text{TGT-GTC-} & \text{CA-GAC-CTG} \\
\text{Asp-Ala-A} & \text{Arg-Ala-Asn-Gln-(Arg)-Leu-Asp-Asn-Met-Ala-Thr-Lys-Tyr-Arg-Lys} \\
\end{align*}
\]

\[
\begin{align*}
\text{A CAC-} & \text{AG-TGT-CTG-GAC} \\
\text{TGT-GTC-} & \text{ACA-GAC-CTG} \\
\end{align*}
\]

The nucleotide sequence of the lpp gene and results

48th residue of the mature protein. In contrast, the mobility of the lpp-77-1 mutant lipoprotein in SDS gels either in the presence or absence of urea, was the same as that of the wild type lipoprotein. Thus, the substitution of Arg by Cys at the 48th residue of the mature protein could still be detected. These results suggest that the rate of maturation of the mutant lipoprotein is reduced as a result of the substitution of cysteine for arginine at the 68th amino acid of the prolipoprotein.

Pulse-Chase Studies—In order to examine the effect of the arginine to cysteine change on the maturation of 6-23 mutant lipoprotein, pulse-chase experiments were carried out by the methods described under "Experimental Procedures." After obtaining the autoradiograms, bands containing the lipoprotein and prolipoprotein were sliced from the gel and counted. Fig. 4 shows a comparison of the kinetics of prolipoprotein processing in the mutant 6-23 and the wild type strain. Approximately 24% of the total lipoprotein was present as prolipoprotein during the pulse in the mutant 6-23, while only 4% of the total lipoprotein was found as prolipoprotein for the wild type strain. During the chase period, the prolipoprotein in the wild type strain rapidly disappeared, while the mutant lipoprotein could still be detected. These results suggest that the rate of maturation of the mutant lipoprotein is reduced as a result of the substitution of cysteine for arginine at the 68th amino acid of the prolipoprotein.

**DISCUSSION**

We have isolated globomycin-resistant mutants from *E. coli* which can be classified into several groups based on the amount or structure of the mature lipoprotein which they possess. The first group is represented by a deletion mutant lacking the lpp gene (lpp-0) which has been isolated by Hirota et al. (10). The second group is comprised of mutants with a reduced amount of lipoprotein. The third group, which is more frequently encountered than the previous two, is represented by the lpp-14-1 allele of lpp gene; these mutants contain unmodified mutant prolipoprotein. SDS gel electrophoresis in a phosphate buffer gel system indicated the mutants comprising the latter group all contain structurally altered prolipoproteins, since their mobilities differed from the wild type unmodified prolipoprotein previously identified in a strain containing a malE-lacZ hybrid protein (2). The fourth group contains two novel structural gene mutants: the lpp-77-1 allele with an Arg to Cys alteration at the 57th position of the mature lipoprotein (9), and the lpp-68-1 allele with an Arg to Cys substitution at the 48th position of the mature protein. Finally, the majority of globomycin-resistant mutants belong to an uncharacterized group which appears to contain a normal level of structurally unaltered lipoprotein.

The mutant lipoprotein (lpp-68-1 allele) showed anomalous mobility in SDS gels, especially in the presence of urea. This may be indicative of an abnormal conformation of the mutant lipoprotein caused by the substitution of Arg by Cys at the 48th residue of the mature protein. In contrast, the mobility of the lpp-77-1 mutant lipoprotein in SDS gels either in the presence or absence of urea, was the same as that of the wild type lipoprotein. Thus, the substitution of Arg by Cys at the 57th residue of the mature protein in the lpp-77-1 mutant does not seem to significantly affect the conformation of mutant lipoprotein. A slower migration in SDS-polyacrylamide gels of a mutant protein with Arg to Cys substitution has been previously reported for the histidine-binding protein (hisd) in *Salmonella typhimurium* (25).

\[^{2}\text{S. Hayashi, J. S. Lai, and H. C. Wu, unpublished data.}\]
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The mutant lpp-68-1 prolipoprotein, unlike prolipoprotein from other globomycin-resistant mutants such as lpp-14-1 (23), can be modified and processed into the mature form, albeit at a slower rate (Fig. 4). A three-dimensional model for the structure of lipoprotein previously proposed by Braun (26) shows that lipoprotein is composed of two very long stretches of \(\alpha\)-helix (residues 5–24 and 30–47), three segments of \(\beta\)-turn residues (1–4, 25–29, and 48–51), one \(\beta\)-pleated sheet (residues 52–56), and one random coil region (residues 57 and 58). The lpp-68-1 mutation introduces a charge difference into the \(\beta\)-turn region in residues 48–51 which hinges between the second stretch of \(\alpha\)-helix (residues 30–47) and the long non-helical structure (residues 52–58). It is particularly worth noting that the nonhelical segments in residues 1–4 and residues 51–58 serve as the lipid and murein attachment sites, respectively. Since the lpp-68-1 mutation lies in a nonhelical region, it is possible that this portion of the lipoprotein molecule also protrudes out of the \(\alpha\)-helical structure and functions as part of the recognition site for either the lipid modification enzymes or the PLP signal peptidase. The reduction in the rate of processing of the lpp-68-1 mutant prolipoprotein and the processing of the mutant prolipoprotein by PLP signal peptidase in the presence of globomycin are most suggestive of the 48–51 \(\beta\)-turn region being involved in the recognition of prolipoprotein by PLP signal peptidase. The Arg \(\rightarrow\) Cys mutation may result in partial restoration of the interaction between the globomycin-inhibited PLP signal peptidase and the lipid-modified prolipoprotein, thereby allowing cleavage of the signal peptide in the presence of globomycin. Alternatively, the mutation may alter the recognition site for the fatty acyl transferase(s) and give rise to a deficiency in one of the ester-linked fatty acids which in turn alters processing of the prolipoprotein by globomycin-treated PLP signal peptidase. Whether the lpp-68-1 mutant lipoprotein is lipid-deficient remains to be determined. The proximity of the amino acid alteration to the COOH terminus of the lpp-68-1 mutant lipoprotein which affects the kinetics of lipoprotein maturation, also suggests that the modification and processing of lipoprotein probably occur after its synthesis. This post-translational modification and processing are not too surprising, considering the small size of lipoprotein.

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