LIPID DEFICIENCY IN A LIPOPROTEIN MUTANT OF *ESCHERICHIA COLI*

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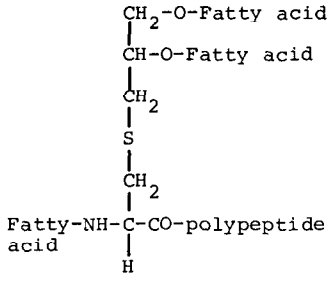
1. Introduction

The most abundant protein in *Escherichia coli,* in terms of number of molecules per cell, is a lipoprotein in the outer membrane [1]. It contains at the amino-terminal end of the polypeptide chain a covalently-linked lipid whose structure is as follows:



Two fatty acids form esters with the glycerol residue of glycerylcysteine; one fatty acid residue is bound to the α-amino group of the cysteine moiety [2]. A mutant has been described which appeared to have lost the diglyceride residue [3]. This conclusion was based on the finding of a free thiol group (there being no other cysteine residue in the wild-type protein) and upon a very low incorporation of glycerol into the mutant lipoprotein. We initially considered that the presumed failure to transfer the diglyceride residue to the thiol group of cysteine might give rise to an accumulation of lipid in the cell membrane which in sum could point to the biosynthetic precursor of the diglyceride residue. During the course of the studies it was found that the mutant does, in fact, contain the glycerol residue and that it only lacks the fatty acids attached by ester linkages. One fatty acid residue per polypeptide chain remains, presumably the one attached via an amide linkage. The free thiol group in this mutant is due to an amino acid exchange arginine/cysteine at the penultimate carboxylterminal residue of the lipoprotein.

2. Materials and methods

Cells of *E. coli* K12 and *E. coli* K 12 *lpm* (the latter was obtained from Y. Hirota, National Institute of Genetics, Japan) were grown on M9 minimal medium to the late logarithmic growth phase and kept frozen at −60°C until used. For labeling with [2-^{3}H]glycerol, glucose was replaced by 0.2% glycerol in the growth medium [2].

Isolation of murein–lipoprotein complex from *E. coli* by treatment of cell envelopes with boiling 4% sodium dodecylsulfate has been described [4]. To isolate pure murein–lipoprotein complex from *E. coli* K 12 *lpm,* 1% mercaptoethanol was added to the 4% SDS solution. Free lipoprotein was isolated from the 4% SDS, 1% mercaptoethanol supernatant according to Inouye et al. [5].

The fatty acid composition was determined on 1–3 mg lipoprotein/sample plus added pentadecanoic acid methyl ester as internal standard by hydrolysis for 18 h at 105°C in double distilled 6 N HCl under nitrogen. The hydrolysates were extracted with petrol/ether, the petrol/ether phase was evaporated, and the remainder quantitatively converted to the fatty acid methyl esters by treatment with *N,N*-dimethylformamide-dimethylacetal (Machery Nagel and Co.). The concentrations of the fatty acid methyl esters were determined by gas chromatography (Varian Aerograph 1400 with a 180 cm long 2 mm diameter column filled with OV 1 on Gas Chrom Q, 100–120 mesh, Serva

Heidelberg, at 170°C) with pentadecanoic methyl ester as standard. Lipoprotein concentration was determined by amino acid analysis of the extracted hydrolysate. The procedure of amino acid analysis [4], and the details of infrared spectroscopy and circular dichroic measurements were previously reported [6]. Murein–lipoprotein complex was degraded with trypsin as described [7].

3. Results

3.1. *Lipid composition of the mutant lipoprotein*

The mutant lipoprotein was shown to contain a free thiol group which upon oxidation formed disulfide bridges between lipoprotein molecules [3]. We noticed that the addition of mercaptoethanol especially during the heating step in 4% SDS was a prerequisite for obtaining pure murein sacculi with covalently linked lipoprotein. In the absence of mercaptoethanol, amino acids not present in the lipoprotein such as glycine, histidine, proline, phenylalanine, were revealed upon amino acid analysis, indicating the presence of contaminating proteins. The fatty acid composition of the murein-bound and of the free form of lipoprotein is listed in table 1. Compared with the wild type lipoprotein [2,8] the percentage of hexadecanoic acid ($C_{16:0}$) is unusually high and comparable with the amide linked portion in the wild-type lipoprotein (65% [2]). Consequently the percentage of the unsaturated fatty acids and especially the amount of the cyclopropane fatty acids is very low (wild type 11%). The latter are in wild type lipoprotein mainly attached by ester linkages to the glyceryl-residue.

Table 1
Fatty acid analysis of free lipoprotein and murein-lipoprotein complex of *E. coli* K 12 *lpm*

Fatty acid	Free lipoprotein	Murein–lipoprotein complex
$C_{14:0}$	11%	14%
$C_{16:0}$	71%	63%
C_{16} unsat	8%	9%
C_{17} cyclo	< 1%	< 1%
$C_{18:0}$	6%	8%
C_{18} unsat	4%	6%
Mol fatty acid/mol lipoprotein[a]	0.71	0.76

[a] The figures are the average of 5 determinations with a standard deviation of 0.2 made with 2 independently isolated samples. For wild type free lipoprotein we obtained 2.61 mol fatty acids/mol lipoprotein

To determine whether the mutant lipoprotein contained an unusual fatty acid composition or only fatty acids bound by an amide linkage to the N-terminal cysteine residue, the absolute amount of total fatty acids/lipoprotein molecule was determined. Only 0.71 and 0.76 moles fatty acids per mole of lipoprotein were found in the mutant compared with a value of 2.61 in the wild type. Whereas the wild type lipoprotein contains three fatty acid residues the mutant lipoprotein apparently carries only one. To substantiate the assumption that the single fatty acid is attached at the α-amino group, infrared spectra were recorded using a special technique with a high resolution [9] described earlier for lipoprotein analysis [6]. The distinct band in the wild type lipoprotein (fig.1) at 1740 cm^{-1}, due to C = O stretching vibration of the fatty acid esters, has disappeared in the mutant lipoprotein. It is therefore concluded that in the mutant the single fatty acid residue is predominantly attached to the α-amino group of the polypeptide.

The question then arose as to whether the glyceryl residue was absent in the mutant lipoprotein. As done earlier when the structure of the lipid was elucidated [2], cells were labeled with [2-^{3}H]glycerol and glycerylcysteine was determined on the amino acid analyser. Studies on wild type lipoprotein were done concurrently. Results for mutant cells showed a peak which eluted from the column shortly before aspartic acid and which moved to the elution position of cysteic acid upon performic acid oxidation. To determine the elution positions of the radioactively labeled compounds, the column's outlet was disconnected from the ninhydrine detection system. The radioactive peak obtained before aspartic acid and close to cysteic acid agreed quantitatively with those of the wild type. The mutant lipoprotein therefore contains glycerylcysteine, as does the wild type.

Amino acid analysis of the mutant lipoprotein showed that after performic acid oxidation, a peak more than twice as high as the wild type appeared at

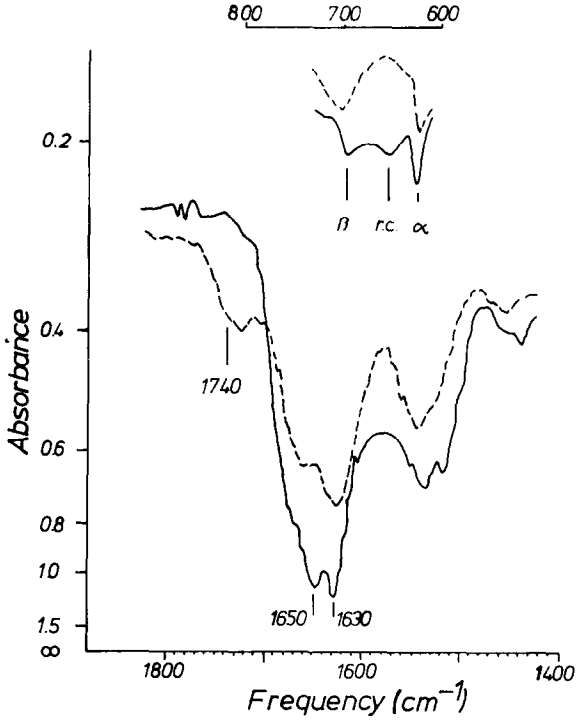


Fig.1. Infrared spectra of dried films of the wild type free lipoprotein (- - - -) and the mutant free lipoprotein (——). The spectra are identical in an atmosphere 90% saturated with H_2O. Both protein samples were identically isolated and were free of phospholipids. β denotes the band at 692 cm^{-1} indicating β-structure, r.c. denotes the band at 650 cm^{-1} indicating random coil and α denotes the band at 620 cm^{-1} indicating α-helix conformation.

the position of cysteic acid. It was therefore possible that the mutant contained additional cysteine which would show up as cysteic acid at the position of glyceryl–cysteine sulfone in the amino acid analysis after performic acid oxidation.

3.2. *Amino acid sequence of the mutant lipoprotein*

The amino acid analysis of the mutant lipoprotein revealed only three arginine residues instead of four (table 2) and one additional cysteine residue. The numbers of the other amino acids were the same as in the wild type: five lysine, fourteen aspartic acid and four valine residues are listed as examples. After trypsin digestion of the wild type murein–lipoprotein complex, only lysine remained attached to the murein [4]. In contrast tyrosine, cysteine and lysine remained

Table 2
Selected amino acids bound to the mutant murein before and after treatment with trypsin

Amino acids	Mol/mol lipoprotein	
	Before trypsin treatment	After trypsin treatment
Tyrosine	0.87	1.02
Cysteine	1.24	1.12
Lysine	4.89	1.26
Arginine	3.11	0.34
Aspartic acid	13.69	1.59
Valine	4.00	0.47

The amounts of the individual amino acids were calculated on the basis that 1 lipoprotein molecule is bound to every eleventh diaminopimelate residue of the murein [2]. The mutant contains as much murein-bound lipoprotein as the wild type

with the murein of the trypsin-treated mutant (table 2). Cleavage was about 90% complete as can be seen from the rest of the other amino acids still bound to murein. The known sequence of the lipoprotein [7] ends with the residues –Tyr–Arg–Lys [8] and the C-terminal lysine is attached to the murein. By analogy we conclude that the mutant lipoprotein has the C-terminal sequence –Tyr–Cys–Lys.

The lack of two fatty acids leads to a reduced hydrophobicity of the lipoprotein which results in an increased solubility of the mutant lipoprotein in buffer and a more complete degradation by trypsin as compared to the wild type lipoprotein isolated in an identical way. One also has to consider an altered conformation due to the changes in the lipid structure and in the amino acid sequence. Hence the infrared spectrum (fig.1) of the mutant lipoprotein shows in the amide I region a band at 1630 cm^{-1} and in the amide V region a band at 692 cm^{-1} (inset of fig.1), indicating β-structure, bands at 1650 cm^{-1}, 650 cm^{-1} and 620 cm^{-1} indicating random coil and α-helix regions. The random coil region is not detected in the wild type free lipoprotein. The differences in the conformations between mutant and wild-type lipoprotein are also revealed in the circular dichroism spectra (not shown). For the free lipoprotein, estimates showed 60% β-structure for the wild type and 40% for the mutant lipoprotein in buffer solution. In deoxycholate above

the critical micelle concentration (c.m.c.) the helical content of the wild type lipoprotein is 86% and for the mutant lipoprotein only 54%. In SDS solution both proteins behave the same: the α-helical content below the c.m.c. is 75%, above the c.m.c. 54%.

4. Discussion

The lipoprotein mutant was fortuitously discovered in a thermosensitive mutant of cell division but genetic analysis showed it unrelated to this phenotype [3]. The lipoprotein mutation maps at 36.5 min between *uid*A and *aro*D on the *E. coli* chromosome map. The mutant lipoprotein has two defects and it is unknown whether they are related. The lack of two fatty acids could indicate that the glycerol residue is attached to the thiol group of cysteine before the fatty acids are transferred to the glycerol. It is also possible that the fatty acids are cleaved after the diglyceride moiety has been bound to the cysteine. The lipoprotein is synthesized with an additional hydrophobic peptide 20 amino acids long at the N-terminal end [10]. In glycerol starved cells the apoprotein is synthesized but at a reduced rate [11]. In a mutant lacking the lipid completely the prolipoprotein is not processed normally so that the size remains larger and it is poorly bound to the murein sacculus [12]. Processing of the prolipoprotein may also be affected by the amino acid exchange in the *lpm* mutant which leads to an altered lipid structure. Alternatively both events may be independent of each other. Indirect evidence suggests that the diglyceride moiety of murein lipoprotein may be derived from phosphatidylglycerol or cardiolipin (or both) [13]. Further evidence may suggest that the *lpm* phenotype most likely results from two independent mutations, one leading to the amino acid exchange, the other to the cleavage of the fatty acids from the diglyceride moiety.

Acknowledgements

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