CHARACTERIZATION OF THE FREE FORM OF MUREIN-LIPOPROTEIN FROM THE OUTER MEMBRANE OF *ESCHERICHIA COLI* B/r

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1. Introduction

One of the major proteins in *E. coli* is a lipoprotein localized in the outer membrane [1] of the cell envelope. About one-third of the total amount of the lipoprotein is covalently linked to the murein net [1], while the other two-thirds exists in a free form [2]. The amino acid sequence, structure, lipid attachment site, and mode of binding to the murein in the case of the murein bound form are known [1], but due to difficulties in the isolation of the free form [3], no detailed structural information has been available to date.

In the course of studies on the proteins of the outer membrane [4–7], free lipoprotein has been isolated [6]. We show here that the free form of the lipoprotein most likely is identical with the murein bound form. These findings are of interest concerning the mode of biosynthesis of lipoprotein, its transfer into the outer membrane, and its attachment to the murein.

2. Materials and methods

Isolation of the free form of lipoprotein is described in detail elsewhere [6]. In brief, cell envelopes are extracted with SDS (4%) in the presence of 0.5 mM $MgCl_2$ and subsequently with SDS (2%) containing 5 mM EDTA. Free lipoprotein is in the latter extract. Its acetone precipitate is extracted with Triton X-100 (1.5%) containing 5 mM EDTA and the supernatant is again precipitated with acetone. Chromatography of this precipitate on Biogel P150 (in 2% SDS, 5 mM EDTA, and 10 mM Tris·HCl, pH 7.5) yields pure free form of lipoprotein.

The fatty acid composition was determined from 0.5 mg of dry lipoprotein, hydrolyzed for 2 h at 80°C in 0.5 g KOH in 0.5 ml H_2O plus 2 ml methanol. After acidification, the mixture was extracted with petrolether, the petrolether phase evaporated, and the remainder quantitatively converted to the fatty acid methyl esters by treatment with borotrifluoride in methanol [8]. The fatty acid methyl esters were determined by gas chromatography (Hewlett-Packard model 5750 G with a column filled with OV-17-H.P. on Chromosorb G AW-DMCS, 80–100 mesh) with added pentadecanoic acid methyl ester as standard.

For sequential cleavage of the C-terminal amino acids, 1 mg of lipoprotein was solubilized by sonication in water at pH 12 in an ice bath. After the pH was adjusted to 7.5 with sodium phosphate, 20 μg of carboxypeptidase B (Boehringer, Mannheim) were added. The reaction was stopped after 3 h (37°C) by addition of 0.1 ml 1 N HCl. Upon centrifugation, 0.3 ml of the supernatant were subjected to amino acid analysis. The remainder was readjusted to pH 7.5 and treated with 20 μg of carboxypeptidase A (Boehringer; dissolved in 10% LiCl) for 3 h at 37°C.

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Half of the acidified reaction mixture was analyzed again, and the remainder was treated once more with freshly added carboxypeptidase B as above.

The circular dichroism spectra were recorded at least twice at two different pathlengths using a Roussel-Jouan Dichrograph CD185 calibrated with a solution of epiandrosterone in dioxan for which an ellipticity at θ_{304} of 10925 degree cm^2/decimole was taken as standard. The mean residue ellipticity was calculated using an average residue mol. wt of 110.

3. Results and discussion

3.1. *Amino acid composition and C-terminal end of free lipoprotein*

The amino acid composition of the free lipoprotein was found to be the same as the amino acid composition calculated from the amino acid sequence of the murein-bound lipoprotein [8,9] except for the presence of 0.25 mol diaminopimelate and an excess of about 0.5 mol glutamic acid per mole protein. In confirmation of the data obtained by Hirashima et al. [3], there were no traces of muramic acid or glucosamine, and the diaminopimelate and the excess glutamate may be due to the action of an unknown protease during isolation.

The sequence analysis of the C-terminal end had been difficult because, depending on the protease used for degradation [9], various parts of the lipoprotein remain on the murein. The availability of the free form allowed the C-terminal end to be studied without this drawback. Sequential release of the C-terminal amino acids could be achieved with the use of carboxypeptidase A and B. The sequence is known to end with –Lys–Tyr–Arg–Lys [9]. Carboxypeptidase B released equimolar amounts of lysine and arginine. Carboxypeptidase A, added subsequently, released the same molar amount of tyrosine. The expected release of additional lysine by freshly added carboxypeptidase B, after tyrosine had been cleaved off, was, however, not obtained. It is possible that the peptide bond of this lysine residue is in a secondary structure causing resistance against cleavage by carboxypeptidase B (see below). No other amino acids were released, and the molar ratios of 1:1:1 for lysine, arginine, and tyrosine indicate the C-terminal sequence –Tyr–Arg–Lys. The actual values obtained (lysine, 24 nmol; arginine, 24.5 nmol; tyrosine, 23.5 nmol) correspond to 50% yield (mol. wt of lipoprotein: 7200). The lipoprotein preparation used contained 0.3 residues of diaminopimelate and an excess of about 0.5 residues of glutamate, and a dpm-Glu C-terminal sequence woud block this end against carboxypeptidase action. If, in addition, one takes into account the protein's water content (10–15% of its weight), the amounts of amino acids released correspond to more than 90% yield.

3.2. *Amount of lipid and fatty acid composition of free lipoprotein*

Lipoprotein bound to murein contains a covalently linked lipid composed of a fatty acid residue linked with the α-amino group and a diglyceride residue bound as a thioether to the mercapto group of the N-terminal cysteine residue [8]. The question arose as to whether the free lipoprotein contains the same fatty acids in the same amounts as the bound form. The result of the fatty acid analysis is shown in table 1 together with the previously determined composition of the murein bound form as reference. The fatty acid composition of both forms agree qualitatively and differences in the quantitative ratios of the individual fatty acids are within the range of fluctuation one observes with *E. coli* cells not grown under strictly identical conditions and not harvested at exactly the same growth phase. The amount of fatty acids (44.5 μg/0.5 mg dry lipoprotein) corresponds to a value calculated for 90% occupancy of the protein binding sites. Since the purity of the lipoprotein was based on

Table 1
Fatty acid composition of free lipoprotein

Fatty acid	Free form	Murein-bound form [8]
$C_{14:0}$	2.4	3.1
$C_{16:0}$	62.0	53.0
$C_{16:1}$	5.6	9.4
C_{17} cyclo	11.7	10.6
$C_{18:0}$	2.4	1
$C_{18:1}$	11.2	20.7
C_{19} cyclo	4.8	3.2

The amounts of individual fatty acids are given as percentage of the total. $C_{14:0}$, fatty acid with 14 carbon atoms without double bond; $C_{16:1}$, fatty acid with 16 carbon atoms with one double bond; cyclo = cyclopropane fatty acid.

the amino acid composition, which can not be determined with better than 90% accuracy, we do not take the 90% found as a significant deviation from 100% occupancy.

3.3. *Circular dichroism spectrum of free lipoprotein*

The conformation of free lipoprotein is of interest for several reasons. Having lipoprotein in the isolated state offers the possibility of determining the conformation of an integral membrane protein. We also are studying cross-reactions of antisera produced against free lipoprotein, murein-bound lipoprotein, and lipoprotein released from murein by trypsin in order to identify the antigenic determinants. These data are of importance for the serological determination of the lipoprotein's exposure at the surface of wildtype cells and outer membrane mutants, and such a determination certainly will yield information on the topology of free and murein-bound lipoprotein in the outer membrane (summarized in [10]). Finally, it has been found that lipoprotein released by lysozyme or trypsin treatment from the murein is a potent and specific B-cell mitogen [11], and we are in the process of identifying the structural requirements for stimulation.

The circular dichroism spectrum shown in fig.1 is typical for an α-helix. The α-helix content was calculated with the use of the 'isodichroitic' method of Myer [12] and the reference substances poly-L-lysine at pH 11.4 as α-helix, poly-L-lysine in 1% sodium dodecyl sulfate as β-structure, and poly-L-serine in 8 M LiCl as unordered structure, as recommended by Rosenkranz and Scholtan [13]. The resulting α-helix content is about 80%. This unusual high value is particularly interesting since lipoprotein was treated with hot 2% SDS during isolation. It is very unlikely that such a highly ordered structure represents an artifact. The conclusion that the in vivo structure as well is to a large extent α-helical is substantiated by the cross-reactivity of anti-whole cell rabbit antisera with the isolated lipoprotein [10].

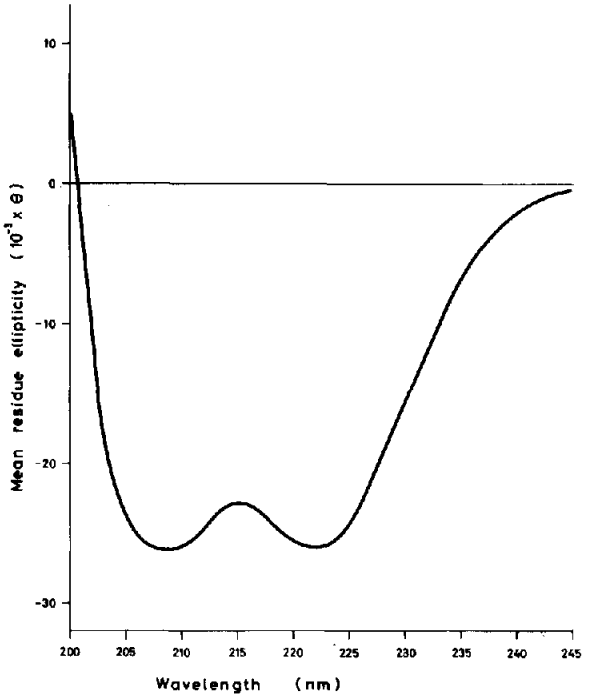

Fig.1. Circular dichroism spectrum of free lipoprotein at pH 7.0, 22°C.

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