DISTRIBUTION OF MUREIN–LIPOPROTEIN BETWEEN THE CYTOPLASMIC AND OUTER MEMBRANE OF *ESCHERICHIA COLI*

V. BOSCH and V. BRAUN

Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, Germany

Received 29 May 1973

1. Introduction

The envelope protein of *E. coli*, which is covalently attached at its C-terminal end to the murein sacculus and at its N-terminal end to fatty acids, is called murein–lipoprotein. Its structure, conformation and respective attachment sites have been studied in detail [1–3] and one could calculate that this murein-bound protein makes up about 10% of the cell wall in *E. coli* [4]. Later it has been shown that the same lipoprotein can also exist in a "free" form, i.e. not attached to the murein [5]. Until now no information concerning the localisation of this "free" form, which is present in double the amount of the bound form, has been available.

In this paper we have studied the distribution of lipoprotein between the cytoplasmic and outer membrane of *E. coli* separated according to Osborn [6]. Two approaches have been used: i) radioactive labeling of cells with the murein specific component, diaminopimelic acid, "chasing" with cold amino acid and determining the radioactivity in the separated cytoplasmic and outer membranes and ii) determination of the immunological crossreaction in separated membranes using anti-lipoprotein antiserum.

With these methods we were able to show conclusively that the lipoprotein attached to the murein extends *only* into the outer membrane where the "free" form could also be shown to be localised.

2. Materials and methods

2.1. *Strains*

E. coli W173-25/7 (abbreviated W7), a mutant which can neither synthesize diaminopimelate (Dpm) nor convert this to lysine (Dpm decarboxylase) [7], was used both for radioactive labeling and for immunological experiments.

2.2. *Labeling of the murein–lipoprotein complex with [^{3}H]diaminopimelic acid and preparation of cytoplasmic and outer membrane*

E. coli W7 was grown in Tryptone Broth (Difco), 0.5% NaCl supplemented with 0.33 μCi Dpm (3 μg) ml^{-1}. The labeled culture was then diluted with medium containing non-radioactive Dpm and grown further for several generations before separation of the outer and cytoplasmic membrane on sucrose density gradients according to Osborn [6]. 25 drop fractions were collected from the gradient and 50 μl aliquots were each diluted with 0.5 ml H_2O for radioactivity measurement. The membrane peaks were diluted with H_2O and collected by centrifugation at 140 000 *g* for 2 hr.

2.3. *Alkali-solubilisation of lipoprotein and membranes*

5 mg lipoprotein were taken up in 0.5 ml 0.1 M NaOH, incubated at 56°C for 1 hr and carefully neutralized with 0.5 ml 0.1 M HCl. Appropriate amounts of membranes were also "solubilized" in this way.

2.4. *Coating of erythrocytes with lipoprotein or "solubilized" membranes*

Human erythrocytes were washed three times with cold buffered saline (100 ml 0.9% NaCl, 23.9 ml 0.15 M KH_2PO_4, 76 ml 0.15 M Na_2HPO_4) and 0.1 ml of the viscous pellet was taken up in 10 ml of this buffer containing 400 μg alkali-treated lipoprotein or an appropriate amount of alkali-treated membranes. The suspension was incubated at 37°C for 1 hr which resulted in a coating of the erythrocytes with lipoprotein without the addition of a coupling agent. The coated erythrocytes were washed three times with phosphate buffered saline (for passive haemagglutination) and with isotonic Veronal pH 7.3 [8] (for immune haemolysis inhibition).

2.5. *Immune haemolysis inhibition*

Lipoprotein-coated erythrocytes were suspended in 0.15% bovine serum albumin in Veronal-buffered saline such that 0.1 ml of the cell suspension in 1.4 ml H_2O gave an absorbance of 1.3 at 405 nm. Guinea pig complement (Serva) was also diluted in Veronal-NaCl plus albumin such that, under the assay conditions, 5 times diluted antiserum caused approx. 90% haemolysis. The complement solution was stored at −20°C for no longer than 1 month. Endogenous complement present in the anti-lipoprotein antiserum was destroyed by heating at 56°C for 1 hr.

The assay was carried out as described in [9, 10]. In short, a doubling dilution series of antiserum, with each sample having a volume of 0.2 ml containing 2 μg muropeptide, is set up. 0.1 ml of an appropriate dilution of alkali-treated lipoprotein or membrane is added and incubated at 37°C for 15 min with gentle shaking. Then 0.1 ml erythrocyte suspension followed by 0.1 ml complement solution is added and incubation at 37°C is continued for 30 min with vigorous agitation. Each sample is then diluted with 1 ml cold Veronal-NaCl, centrifuged at 6000 g for 10 min and the absorbance of the supernatant at 405 nm measured.

3. Results and discussion

3.1. *Distribution of the "bound" form*

Since *E. coli* W7 can neither synthesize diaminopimelate (Dpm) nor decarboxylate it to lysine, radioactive Dpm in the medium can only be used for murein synthesis. When such labeled cells are "chased" with non-radioactive Dpm, the radioactivity is localised exclusively in the murein and is no longer present in the cytoplasm or cytoplasmic membrane in the form of free Dpm or murein precursors. Digestion of such chased cells with lysozyme followed by washing and separation of membranes according to Osborn [6] results in removal of about 80% radioactivity in the form of muropeptides. The remaining 20% is not washed away because it is lipoprotein bound: i.e. the "bound" form of the lipoprotein has been specifically labeled. When we look at the distribution of the remaining radioactivity between the cytoplasmic and outer membrane, it is seen that only 5–10% is in the cytoplasmic membrane, an amount which can be accounted for by contamination from the outer membrane. This distribution of the radioactivity indicates conclusively that the murein–lipoprotein projects from the murein towards the outer membrane and not towards the cytoplasmic membrane or in both directions as could previously have been imagined, i.e. possibility (a) in fig. 1 is confirmed to be correct.

3.2. *Distribution of "total" lipoprotein*

Lipoprotein, prepared from a lysozyme digestion of purified murein–lipoprotein complex [11], was used to prepare antiserum in rabbits. The immunization method for lipoprotein was adopted from the preparation of anti-lipopolysaccharide antisera [12] by H. Mayer. The anti-lipoprotein antisera prepared in this way from pure lipoprotein exhibited rather low titres which were, however, sufficient for our purposes. Since the lipoprotein antigen contains a muropeptide dimer and we are concerned only with the detection of the protein portion excess muropeptides are present in all experiments to inhibit anti-muropeptide antibodies present (20%).

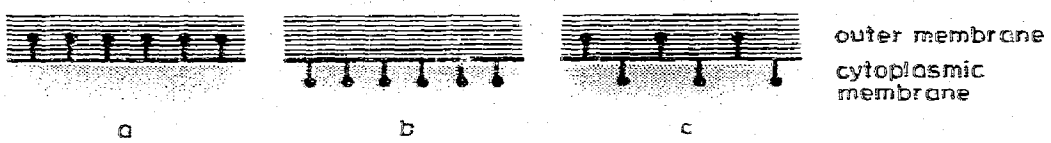

Fig. 1. Schematic representation of the possibilities concerning the direction of projection of lipoprotein from the murein. The spotted area represents cytoplasmic membrane, the lined area outer membrane, and the heavy line between them represents the murein. The three possibilities a), b), and c) show possible directions in which the murein-bound lipoprotein could project.

Two methods were used to assay the extent of immunological cross-reaction in the cytoplasmic and outer membrane of *E. coli* W7 cells. The first, passive haemagglutination, was carried out as described in [13] and the results are shown in table 1. Considering the direct determination of lipoprotein in membranes coated on erythrocytes (table 1a), it can be seen that the outer membrane (H) gives rise to higher titres than the cytoplasmic membrane ($L_1 + L_2$). Comparison of the amounts of membranes used reveals that the difference in titre is of the order of 8–16. Since, in this type of experiment, the titre does not increase linearly with the amount of *E. coli* membrane on the erythrocytes, it is rather difficult to be certain that this difference in titre reflects the relative amounts of lipoprotein in the outer and cytoplasmic membranes. However, further experiments have shown that this is, in fact, the case. Table 1b gives the results of inhibition experiments in which the antiserum is incubated with different amounts of alkali-treated membranes before determination of the anti-lipoprotein activity by passive haemagglutination. The activity remaining, expressed by the titre, reflects the amount of lipoprotein present in the membrane used for inhibition. Again, on comparison of the amounts used, it can be seen that the outer membrane reduced the

Table 1

Determination of lipoprotein in the cytoplasmic and outer membrane of *E. coli* W7 by passive haemagglutination.

a) Direct determination of lipoprotein in *E. coli* membranes (H = outer membrane, L = $L_1 + L_2$ = cytoplasmic membrane) coated on the surface of human erythrocytes.

Membranes	Relative amounts	*E. coli* membrane µg protein in each hole	Reciprocal titre
H	4	2	128
H	2	1	128
H	1	0.5	64
L	4	0.8	8
L	2	0.4	8
L	1	0.2	8

b) Determination of lipoprotein by previous absorption of the antiserum with *E. coli* membranes (competition experiments).

Membranes	Relative amount	*E. coli* membrane µg protein present in 50 µl As	Reciprocal titre
H	4	100	2
H	2	50	4
H	1	25	8
H	0.2	5	16
L	8	70	32
L	4	35	32

a) Aliquots of erythrocytes coated with alkali-treated membranes were added to each sample of a doubling dilution series of muropeptide-treated (5 mg/ml) anti-lipoprotein antiserum. The figures concerning the amounts are such that we have assumed complete adsorption of *E. coli* membrane protein to the erythrocyte surface. In the table they are given in relative amounts, that is aliquots of the outer and inner membrane, and in absolute amounts expressed in µg *E. coli* membrane protein present in the erythrocyte suspension added to each fraction of the antiserum dilution series. The titre, measured after incubation at 37°C for 1 hr, is that final dilution of antiserum which still causes agglutination of the erythrocytes.

b) Appropriate amounts of alkali-treated *E. coli* membranes were added to 0.1 ml samples of antiserum pre-absorbed with 5 mg/ml muropeptides. After incubation at 0°C for 1 hr, a doubling dilution series was set up and tested for remaining anti-lipoprotein activity with alkali-treated lipoprotein fixed to erythrocytes. In the table the amounts of membranes used are given in relative amounts and in absolute amounts expressed in µg membrane protein present in that volume of antiserum (50 µl) used to start the dilution series.

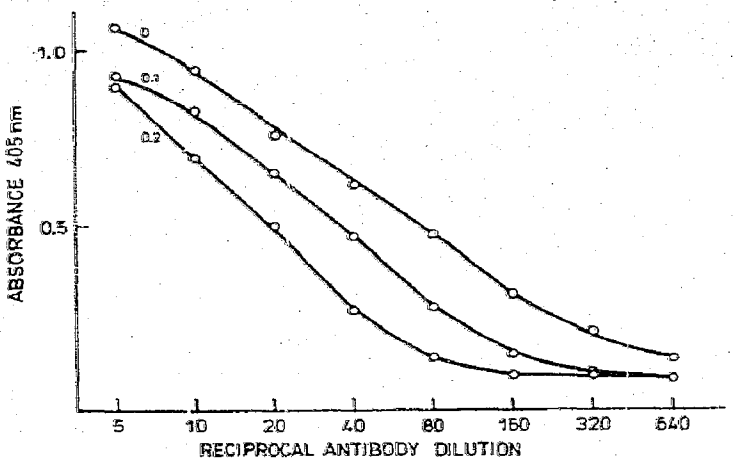

Fig. 2. Immune haemolysis inhibition. The assay was carried out as described in Methods. Each sample was set up in duplicate together with controls lacking complement or antiserum or both. The difference in area between the 100% curve (no lipoprotein present) and the curves obtained when the antiserum dilutions are previously incubated with 0.1 μg and 0.2 μg lipoprotein is proportional to the amount of antibody bound.

Table 2
Determination of lipoprotein (LP) in separated cytoplasmic (L) and outer (H) membranes by immune haemolysis inhibition.

Added substance	(μg)	Relative curve area	Anti-lipoprotein bound (%)	Amount of LP present (μg)
LP	0.05	80.5	19.5	
LP	0.1	70	30	
LP	0.2	49	51	
L	1	95	5	
L	2	87	13	> 0.05
H	0.5	69	31	0.1
H	1	49	51	0.2

Immune haemolysis inhibition was performed as described in Methods and the % anti-lipoprotein antibody bound was calculated. By comparison to standard curves obtained with known amounts of lipoprotein, the amount of lipoprotein present in the added membranes was deduced.

titre 16 times more than the cytoplasmic membrane. This can cautiously be interpreted as meaning that it contains an order of 16 times more lipoprotein.

The second immunological method which we used was immune haemolysis inhibition which, in our hands, was a more sensitive assay giving more quantitative results. Fig. 2 shows the haemolysis inhibition caused by different amounts of lipoprotein and table 2 summarises the results obtained using alkali-treated cytoplasmic and outer membranes as inhibitors. It can be seen that 2 μg cytoplasmic membrane contains less than 0.05 μg lipoprotein, i.e. below 0.025 μg LP/μg membrane protein. Outer membrane, on the other hand, contains 0.2 μg LP/μg membrane protein. One has to consider that the cytoplasmic membrane makes up only 30–40% of the total envelope protein and one can therefore calculate that below 8% of the total lipoprotein is in the cytoplasmic membrane. Again this is an amount which can be accounted for by contamination from the outer membrane.

In summary, from the experiments described here, one can conclude that the lipoprotein attached to the murein extends only into the outer membrane and that the "free form" is also localised in the outer membrane.

Acknowledgements

We thank Dr. H. Mayer, Max-Planck-Institut für Immunbiologie, Freiburg, for his introduction into immunological methods. Part of this work was supported by the Deutsche Forschungsgemeinschaft.

References

[1] Braun, V. and Bosch, V. (1972) Proc. Natl. Acad. Sci. U.S. 69, 970.
[2] Braun, V. (1973) J. Infect. Diseases, Supplement. Vol, July issue.
[3] Hantke, K. and Braun, V. (1973) European J. Biochem. 34, 284.
[4] Braun, V., Rehn, K. and Wolff, H. (1970) Biochemistry 9, 5041.
[5] Inouye, M., Shaw, J. and Shen, C. (1972) J. Biol. Chem. 247, 8154.
[6] Osborn, M.J., Gander, J.E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962.
[7] Hartmann, R., Höltje, J.-V. and Schwarz, U. (1972) Nature 235, 426.
[8] Kabat, E.A. and Mayer, M.M. (1961) in: Experimental Immunochemistry, 2nd ed., p. 149 C.C. Thomas, Springfield, Ill.
[9] Arquilla, E.R., Bromer, W.W. and Meriola, D. (1969) Diabetes 18, 193.
[10] Arquilla, E.R., Hamlin, J., Hamashige, S. and Miller, A. (1964) J. Exptl. Med. 120, 841.
[11] Braun, V. and Wolff, H. (1970) European J. Biochem. 14, 387.
[12] Beckman, L., Lüderitz, O. and Westphal, O. (1964) Biochem. Z. 339, 401.
[13] Kabat, E.A. and Mayer, M.M. (1961) in: Experimental Immunochemistry, 2nd ed., p. 119 C.C. Thomas, Springfield, Ill.