IN VIVO BIOSYNTHESIS OF MUREIN–LIPOPROTEIN OF THE OUTER MEMBRANE OF *E. COLI*

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1. Introduction

About 2×10^5 lipoprotein molecules [1] are covalently bound to the murein [2] (peptidoglycan) of the outer membrane of *E. coli* and related enterobacteriaceae [3, 4]. Twice this amount of lipoprotein was found free in the cell envelope [5] without specification whether the free-form occurs in the outer membrane, the cytoplasmic membrane or in both. We found by immunological means that more than 90% of the total lipoprotein is localized in the outer membrane [6]. The lipoprotein most probably is synthesized in the cytoplasm or the cytoplasmic side of the cytoplasmic membrane and the question arises, how it is transferred through the cytoplasmic membrane into the outer membrane and how it is built into the murein? The two simplest alternatives are: a murein-repeating unit is already fixed to the lipoprotein in the cytoplasmic membrane and incorporated as such into the murein or the lipoprotein is transferred to the preformed murein in the outer membrane. *In vitro* studies with soluble and particulate enzyme systems revealed the pathway of murein synthesis up to the stage where the repeating unit, a disaccharide–pentapeptide, is bound to a lipid carrier, a C_{55}-polyisoprenoid alcohol, by a pyrophosphate linkage. Polymerisation of the lipid-linked repeating unit to peptidoglycan chains and cross-linkage between peptidoglycan chains, which is inhibited by penicillin, was also achieved *in vitro* (see review [7]).

Our *in vivo* studies described here were concerned with the questions whether lipoprotein is already linked to a murein-repeating unit in the cytoplasmic membrane, whether muropeptides are polymerized- or cross-linked in the cytoplasmic membrane and then transferred as oligomer to the outer membrane and whether lipoprotein is incorporated randomly or at specific sites into the murein of the outer membrane.

2. Methods

2.1. *Strains*

E. coli W7 (dap^-, lys^-) is a derivate of *E. coli* W173-25 (dap^-) [8] and was obtained from Dr. U. Schwarz, Tubingen [9]. Strain W7-2 is a spontaneous revertant of W7 with a functional Dap-decarboxylase.

2.2. *Labeling of cytoplasmic and outer membrane of E. coli* W7 *or E. coli* W7-2 *with* [^{3}H] *diaminopimelate*

500 ml Cultures of *E. coli* W7-2 were grown at 37°C in minimal citrate [10], 0.1% casamino acids, 0.5% glucose supplemented with 10 μg diaminopimelate. At A_{578} = 0.4–0.44, cells were harvested, taken up in 1/15 of the original volume medium and shaken for 5 min. 5 μCi [^{3}H] diaminopimelate (5.5 μg) were added per ml and incorporation stopped after 45 sec by pouring onto ice + 0.1 M KCN (pulse). [^{3}H] diaminopimelate was chased by diluting a sample of the 45 sec pulse mixture 4-fold into medium with 1.5 mg/ml of cold diaminopimelate. Membranes were prepared according to Osborn [14]. Treatment of cells with lysozyme was in some cases replaced by treatment of envelopes with trypsin at pH 8. Treatment with ultra sound, sucrose density centrifugation etc. followed otherwise the lysozyme method of Osborn. The trypsin version was first developed by R. Hartmann, R. Hakenbeck and U. Schwarz (personal communication).

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2.3. *Separation of* [^{3}H] *diaminopimelate and* [^{3}H] *lysine*

Aliquots of the cytoplasmic and outer membrane were hydrolyzed with 2 ml 6 N HCl for 20 hr at 105°C. They were then evaporated to dryness and applied to a 4 × 0.7 cm Dowex 50 × 2 column which had been equilibrated with 0.1 M pyridine formate, pH 3.0 and washed twice with 1 ml water. After application of the sample the column was rinsed twice with 1 ml 0.1 M pyridine formate pH 3.0, 4 times with 1 ml of 0.3 M pyridine formate pH 3.5 and 4 times with 1 ml of 2 M pyridine formate pH 5.0. 1 ml fractions were collected separately in counting vials and counted after addition of 10 ml Aquasol. Diaminopimelate and lysine appear mainly in fractions 5 and 9 respectively and are completely separated. 10 columns can easily be handled at once and the separation is finished within 1.5 hr.

2.4. *Miscellaneous procedures*

Preparation of [^{3}H] diaminopimelate labeled murein–lipoprotein complex [11] and degradation by lysozyme and pronase have been described [12, 13]. Incubation of the cytoplasmic membrane with lysozyme and pronase followed these procedures.

3. Results and discussion

The murein of the diaminopimelate auxotroph which also lacks diaminopimelate decarboxylase can be specifically labeled with [^{3}H] diaminopimelate. *E. coli* W7 harbors a large cytoplasmic pool of diaminopimelate and therefore a revertant which has no pool since it decarboxylates diaminopimelate to lysine was used for pulse and chase experiments. The amount of labeled lysine was determined in acid hydrolysates of envelopes after separation from diaminopimelate on a small Dowex-50 column. It amounted to only 5% of the radioactivity in the cytoplasmic membrane after a 45 sec pulse of logarithmically growing cells. Incubation with lysozyme released about 80% of the diaminopimelate label from the envelope in the form of muropeptides. The lysozyme-treated envelope was separated into cytoplasmic and outer membrane by sucrose density centrifugation. 70% of the remaining 20% label were found in the cytoplasmic membrane (fig. 1, $L_1 + L_2$ bands),

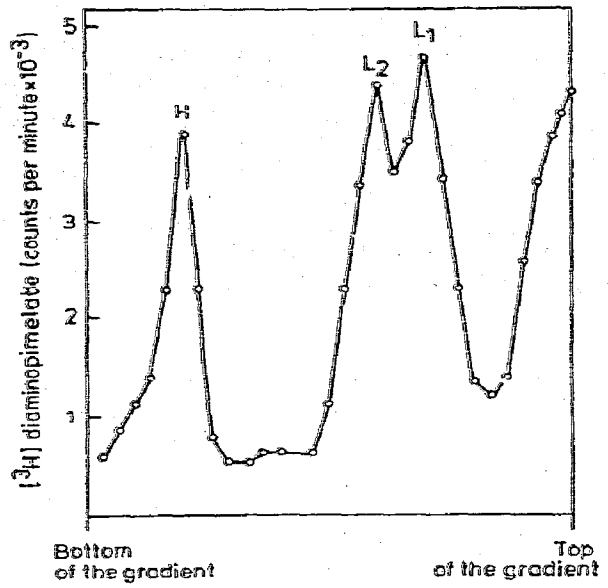

Fig. 1. Pulse labeling of cytoplasmic and outer membrane of *E. coli* W7-2 with [^{3}H] diaminopimelate. Logarithmically growing cells were harvested, taken up in 1/15 of the original volume of medium, then shaken for 5 min at 37°C before addition of 5 μCi diaminopimelate/ml. After 45 sec shaking they were poured into ice + 0.1 M KCN. Cells were digested with lysozyme, treated with ultra sound and separated on a sucrose density gradient as described by Osborn [14]. The gradient was collected in 25 drop fractions, 50 μl aliquots from each fraction were diluted with 0.5 ml H_2O and radioactivity measured in the Nuclear Chicago Isocap/300 with 5 ml Aquasol scintillation fluid (New England Nuclear Corp.). Peak fractions were collected, diluted with water and spun down at 140 000 *g* for 2 hr.

the rest was in the outer membrane (fig. 1, H-band). Muropeptides sometimes remaining after washing of the envelope stayed at the top of the gradient. When a sample of the cytoplasmic membrane bands L_1 or L_2 (fig. 1) was chromatographed on paper (fig. 2), only one component was observed which had an R_f-value identical to the lipid carrier-linked muropeptide [7]. But surprisingly lipoprotein-linked muropeptide dimer had the same R_f-value. This was found by lysozyme digestion of isolated [^{3}H] diaminopimelate labeled murein–lipoprotein of *E. coli* W7. When the products were chromatographed in the same system, they moved to positions as indicated in fig. 2. We therefore digested the cytoplasmic membrane with pronase to degrade lipoprotein. This, however, had no effect on the R_f-value of the fast running [^{3}H] diaminopimelate-labeled component. Thus a lipoprotein-

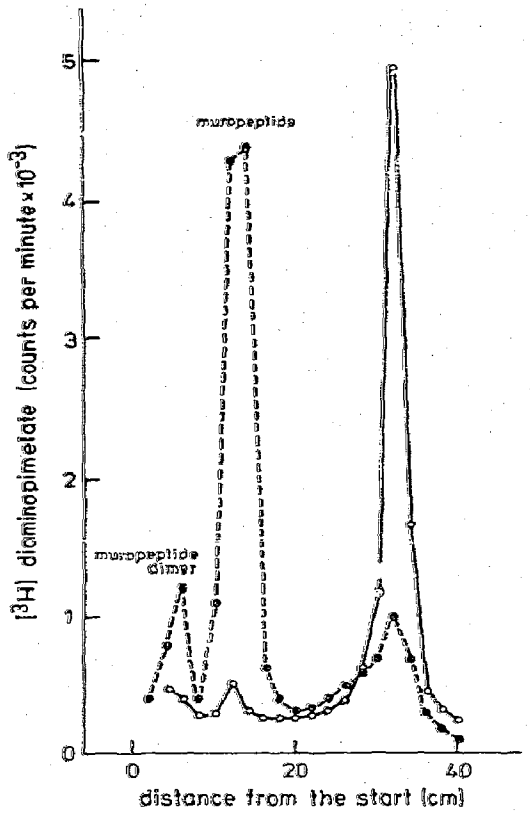

Fig. 2. Paper chromatography of cytoplasmic membrane pulse labeled for 45 sec with [^{3}H] diaminopimelate. Cytoplasmic membrane (L_1 or L_2 of fig. 1) was chromatographed on Whatman No. 3 paper with the system isobutyric acid – 1 M ammonia (5 : 3) [7] for 18 hr. The paper was dried, pieces 2 cm square were cut out and the radioactivity measured with Aquasol scintillation fluid. Cytoplasmic membrane untreated (o—o). Cytoplasmic membrane treated for 15 min with 0.1 N HCl at 105°C (•– –•).

linked muropeptide was excluded. Weak acid hydrolysis (0.1 N HCl, 15 min, 105°C) known to cleave the pyrophosphate bond between the carrier lipid and the muropeptide [7] gave rise to a new component which chromatographed at the position of a muropeptide subunit (fig. 2, (•– –•)) (R_f 0.27). This shows that the only diaminopimelate-labeled compound in the cytoplasmic membrane is the lipid carrier-linked murein-repeating unit. Cross-linked muropeptides run at the position of muropeptide dimer in fig. 2 (R_f 0.125). The small amount detectable at this position is due to incomplete hydrolysis of the pyrophosphate bond, probably resulting in a noncross-linked muropeptide-phosphate. This is concluded from a set of hydrolysis experiments with 10% acetic acid instead of 0.1 N HCl. After 15 min at 105°C mainly the component with an R_f-value of 0.125 beside the lipid carrier-linked muropeptide (50%) is obtained. The latter disappears by longer incubation as does the R_f 0.125 component, and at the end (60 min) only the muropeptide is left. The cross-linking peptide bond between murein-repeating units is not hydrolysed under these conditions. The results so far show that after lysozyme digestion of the envelope only the lipid carried-linked muropeptide is detectable in the cytoplasmic membrane and no lipoprotein-linked or cross-linked muropeptide.

Lysozyme treatment of the envelope, a necessary step in the Osborn procedure for separating the membranes, would prevent detection of polymerized murein in the cytoplasmic membrane. We therefore used trypsin instead of lysozyme. This approach is based on our observation that the cytoplasmic membrane detaches from the outer membrane when the envelope is treated with trypsin for a short period [1]. The lipoprotein molecules are released from the murein since the C-terminal arginyl-lysine bond is cleaved [4, 12]. A pulse cytoplasmic membrane was obtained separated from the outer membrane. The yield was about 70% of that obtained with lysozyme. After paper chromatography of the cytoplasmic membrane, two [^{3}H] diaminopimelate-labeled components were obtained. One remained at the start (30–50%), the other moved like the lipid carrier-linked muropeptide. The latter disappeared by weak acid hydrolysis and gave rise to only one component at the chromatographic position of the murein-repeating unit. The material remaining at the origin was unaffected by weak acid hydrolysis. It was converted by treatment with lysozyme into two components which chromatographed at the positions of the uncross-linked and the cross-linked muropeptides. No lipid carrier-linked muropeptide was observed which would have been expected if the polymer at the start was lipid-linked. Instead contamination from the outer membrane accounts for this amount of murein polymer in the cytoplasmic membrane. About 80% of the [^{3}H] diaminopimelate label was in the outer membrane when cells were pulsed for 45 sec with [^{3}H] diaminopilemate. One has to stress that these membranes were not treated with lysozyme. Cross-

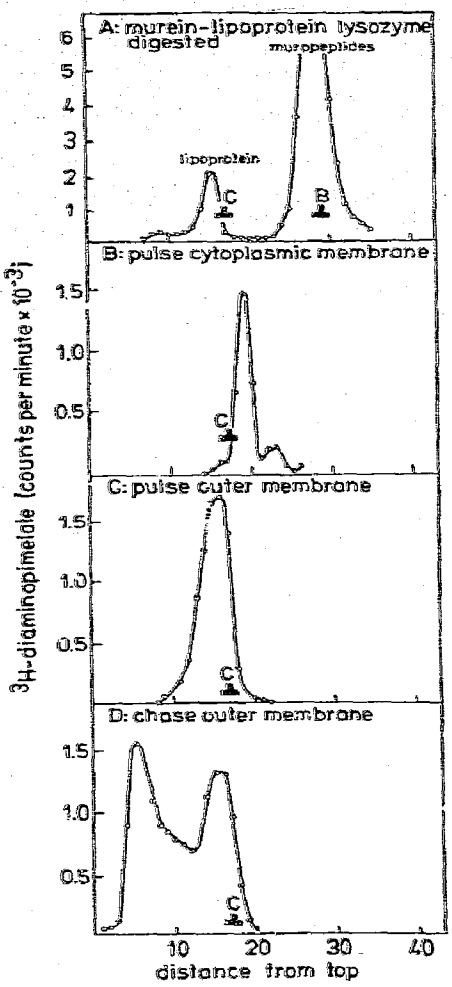

Fig. 3. SDS-polyacrylamide gel electrophoresis of membranes: A) [^{3}H] diaminopimelate labeled murein–lipoprotein digested with lysozyme; B) cytoplasmic membrane; C) outer membrane after a 45 sec incorporation of [^{3}H] diaminopimelate and D) outer membrane after a 2.5 min chase was heated in 1% SDS (sodium dodecylsulfate), 0.1 M Tris–HCl.pH 7 for 5 min at 100°C. They were then applied to 15% polyacrylamide gels which contained 0.01 M Tris–HCl, 0.01 M EDTA, 0.1% SDS, pH 7.9. The same buffer was filled into the electrode chambers. Electrophoresis was performed at a constant current of 3 mA/gel until the tracking dye bromophenol blue (B) had moved to 1 cm from the end of the gel. Otherwise the method followed that described by Weber and Osborn [15]. The gels were cut into 35 equal pieces, extracted overnight at 60°C with 0.5 ml 0.1% SDS in counting vials and counted after addition of 10 ml Aquasol.

contamination of the cytoplasmic and outer membrane of about 10% was found by us and others [14] and this accounts for the murein polymer found in the cytoplasmic membrane of trypsin-treated envelopes.

Lipoprotein in new and old murein: Lysozyme cleaves the glycan chains of murein. Depending on the degree of cross-linkage between the glycan chains, cleavage products of different size are formed. Around the lipoprotein attachment sites lysozyme does not cleave, probably for steric reasons, so that 2–3 murein-repeating units remain bound to the lipoprotein [1, 4, 13]. The pretreatment of cells before the membranes are separated involves lysozyme digestion between 0–4°C. From this it can be expected that degradation of murein with lysozyme is not complete. When the murein surrounding the lipoprotein is studied in [^{3}H] diaminopimelate pulse-labeled and chased membranes, striking differences have been observed. Such membranes were treated for 5 min in 1% SDS and then separated by SDS-polyacrylamide gel electrophoresis. In fig. 3 A the electrophoretic mobility of muropeptides and lipoprotein-linked muropeptides as they result from a 15 hr digestion of isolated [^{3}H] diaminopimelate-labeled murein-lipoprotein complex at 37°C is shown. In fig. 3 B the electrophoretic pattern of a 45 sec pulsed cytoplasmic membrane is shown. The position of added cyt. *c* and the tracking dye bromophenol blue are marked as C resp. B. Only the lipid-linked muropeptide is seen which runs in this system slightly faster than cyt. *c*. In the pulsed outer membrane a somewhat broad peak at the position of liporpotein-linked muropeptides is observed (fig. 3 C). A very different pattern is seen when the outer membrane of a 2.5 min chase experiment is run on SDS-gels (fig. 3 D). An even more striking difference can be seen after paper chromatography as described in fig. 2. The pulse outer membrane forms one peak with an R_f-value of 0.5. The chase outer membrane, however, remained to 50% on the origin. These results are taken as evidence that the lipoprotein is not randomly incorporated into pre-existing murein but rather in newly made, less cross-linked murein. As the degree of cross-linkage between glycan chains increases and as probably the glycan chains grow longer and more lipoprotein is added, the enzymatic attack by lysozyme is rendered more difficult. This leads to larger polymers with a lower electrophoretic mobility in SDS-gels and a low R_f-value on paper. These tentative interpretations have to be sub-

stantiated by further experiments. They open the possibility to study the mode of growth of the murein macromolecule which spans around the whole cell by investigating the murein surrounding the lipoprotein of pulsed and chased cells.

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