MAJOR OUTER MEMBRANE PROTEINS OF *ESCHERICHIA COLI* K-12: EVIDENCE FOR PROTEIN II* BEING A TRANSMEMBRANE PROTEIN

Rainer ENDERMANN, Cornelia KRÄMER and Ulf HENNING
Max-Planck-Institut für Biologie, Tübingen, FRG

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1. Introduction

The outer cell envelope membrane of *Escherichia coli* contains a set of a few major or abundant proteins. For one pair of these proteins, polypeptides Ia and Ib ([1,2]; identical with proteins 1a/b [3,4], b/c [5], 0–9/0–8 [6,7], A_1/A_2 [8], and Rosenbusch's matrix protein [9]) there is good evidence that it represents a transmembrane protein [9–11]. This arrangement very well fits the evidence that it can serve as a transmembrane hydrophilic pore [12–14].

In this communication we present data strongly suggesting that another major outer membrane protein, polypeptide II* [1], (identical with 3a [15], B [8], d [5], 0–10 [6,7]) also is a transmembrane polypeptide.

2. Experimental

The mutant missing the lipoprotein (*lpo*, [16]) was kindly donated by Y. Hirota. The *lpo* character was transferred by phage P1-mediated transduction into an *aro*D strain by selection for *aro*D$^+$ [16]. The isogenic pair *aro*D *lpo*$^+$, *aro*D$^+$ *lpo* was used in this study. Cells were grown aerobically in a complete medium (antibiotic No. 3, Difco) at 30°C. Preparation of cell envelopes and dodecylsulfate-polyacrylamide gel electrophoreses were done as described repeatedly (e.g. [2]). Cross-linking was performed as outlined [8] and the dithiobis (succinimidyl propionate) was always allowed to act for 1 min at room temperature. For experiments with radioactive diaminopimelate (^{3}H-labeled, Amersham Code TRA.76, spec. radioact. 300 Ci/M) the *lys–dap* auxotroph W945 T3282 [17] was grown in complete medium supplemented with non-radioactive diaminopimelate (3.75 μg/ml) and radioactive diaminopimelate (0.5 μCi/ml).

3. Results

3.1. *Crosslinking of protein II* to the murein layer*

Cell envelopes from the *lpo*$^+$ strain were crosslinked with dithiobis (succinimidyl proprionate) at a ratio (w/w) of crosslinker to total protein of 1:1. Material non-solubilized by two times boiling (3 min) in 2% dodecylsulfate containing 5 mM EDTA and 10 mM Tris·HCl, pH 7.5, and recovered by centrifugation together with the murein (1 h at 45 000 × *g*) was used for further analyses. As expected, electrophoresis in the absence of mercaptoethanol did not allow any protein to migrate into the gel. The profile obtained upon reduction is shown in fig.1b. Aside from several minor bands, lipoprotein, large amounts of protein II*, probably some protein I, and another protein in the 70 000–80 000 dalton range were recovered. That the protein designated in fig.1b in fact represents this protein could easily be verified by using a mutant in the *aro*D background missing the protein (obtained by selection for resistance to phage TuII*, [18]): no protein of this electrophoretic position could be linked to the murein of the mutant.

The result in essence is a confirmation of the interpretation [8] that protein II* (protein B [8]) can be crosslinked to the lipoprotein and that both proteins

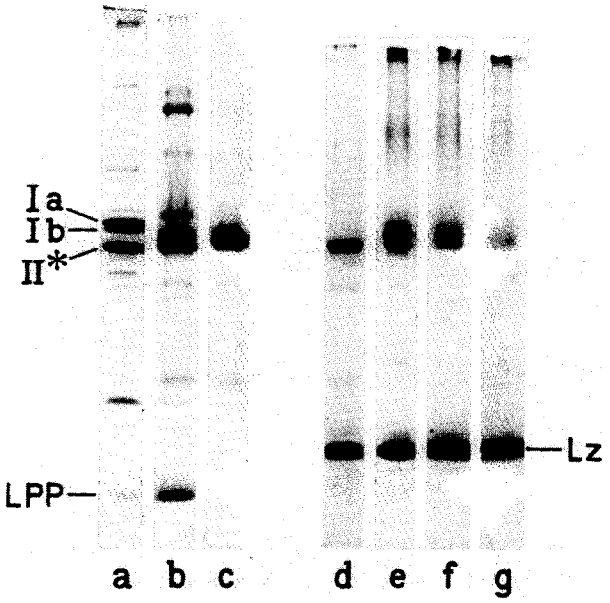

Fig.1. SDS–polyacrylamide gel electrophoreses. (a) cell envelopes from strain *aro* D *lpo*$^+$; (b) cell envelopes from same strain crosslinked with dithiobis (succinimidyl propionate) at 1 mg/mg protein and material not removable from murein with hot SDS subjected to electrophoresis after reduction; (c) same experiment as in (b) but with strain *aro*D$^+$ *lpo*. (d–g) cell envelopes from strain *aro*D$^+$ *lpo* crosslinked, material not removable from murein with hot SDS digested with lysozyme and electrophoresed after reduction (d) and without reduction (e–g). Ratios crosslinker to protein: (d) 0.38; (e) 0.08; (f) 0.38; (g) 3.0. LPP, lipoprotein; Lz, lysozyme.

can be crosslinked to the murein possibly through that fraction of the lipoprotein which is covalently bound to the murein. The same crosslinking experiment was then performed with the mutant completely missing the lipoprotein [16] and the result is shown in fig.1c: from the mutant murein almost only protein II* was recovered. Semiquantitative determination of the amount of protein II* associated with murein and the amount still removable with hot dodecylsulfate after crosslinking showed that 20–30% of all protein II* present had remained with the murein layer of the *lpo* mutant. This fraction may therefore have been linked directly to the murein, may have been linked intermolecularly to an extent no longer allowing release from murein by hot dodecylsulfate, or links to murein as well as a protein–intermolecular connection may have been formed.

3.2. *Direct linkage of protein II* to the murein*

In the first case mentioned, lysozyme should release the monomeric protein, in the second case the enzyme should not release protein migrating into the gel, and in the third case an unpredictable mixture of mono-, oligo- and higher polymers or possibly also no protein should appear in the gel upon the action of lysozyme. In addition, in this third case the degree of protein–intermolecular crosslinking should probably decrease with decreasing concentrations of the crosslinker. Figure 1e–g shows that apparently the last case is realized: Lysozyme action leads to the appearance of several bands in the position of protein II* and these bands decrease with increasing concentration of the crosslinker. They must represent protein II* since practically no other protein can reductively be released from the murein of the *lpo* mutant (fig.1d); protein liberated by lysozyme has to carry covalently bound fragments of murein and the multiplicity of the bands could be due to size differences of these fragments. Direct evidence for the latter assumption has not yet been obtained; as expected, however, it could easily be proven that muropeptides comigrate with protein released by lysozyme. A diaminopimelate–lysine auxotroph (see section 2) was used for the same crosslinking experiments upon growth on medium supplemented with radioactive diaminopimelate. Figure 2 shows the distribution of radioactivity in gels when protein was released by lysozyme and when the material was subjected to electrophoresis with and without prior reduction. (A diaminopimelate–lysine auxotroph missing the lipoprotein could not yet be constructed, therefore, radioactivity in the upper part of the gel with the non-reduced sample will not only reflect association of murein with protein II* but also associations involving the lipoprotein.)

All experiments described so far have been performed with envelopes and the question remains if the close proximity of protein II* and murein also exists in cells. We have therefore cross-linked cells (1.4 g wet wt cells in 10 ml 0.2 M triethanolamine buffer treated with 30 mg crosslinker) of both the *lpo* and *lpo*$^+$ strains. In both cases the most abundant protein remaining insoluble in hot dodecylsulfate (i.e., remaining attached to the murein) and liberated by reduction was protein II*. It is, of course, extremely unlikely that envelope preparation renders a protein transmem-

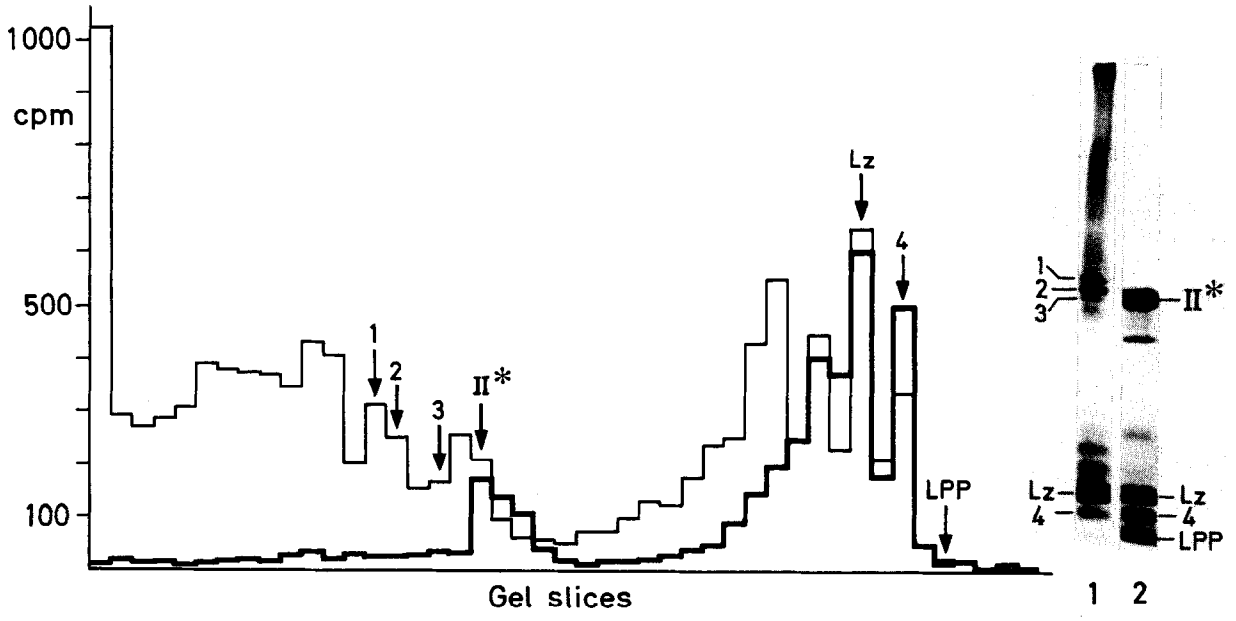

Fig.2. Distribution of radioactivity in SDS–polyacrylamide gels. Lysozyme-treated material as that used in fig.1e and from cells labeled with [^{3}H]diaminopimelate was subjected to electrophoresis before ((———) gel 1) and after ((———) gel 2) reduction. The gels were stained, cut in approx. 1.5 mm slices, and radioactivity was measured in a scintillation spectrometer. Band 4 may well represent the lipoprotein fraction covalently bound to murein [28]. If so, the gels would indicate that this bound fraction participates in crosslinking to a much lesser degree than the free lipoprotein. It is questionable whether radioactivity present in the protein II* band represents diaminopimelate residues still bound to the protein since the distribution of radioactivity in the non-reduced sample is very similar in this area of the gel. Lz, lysozyme; LPP, lipoprotein.

brane which is not transmembrane in vivo, and we conclude that the result with cells reflects the same crosslinking of protein II* as that described for envelopes.

4. Discussion

It has recently been shown that protein II* can serve as a phage receptor [11,19]. In addition, from the data [10], which demonstrates reactivity of *E. coli* B major outer membrane proteins in cells with CNBr-activated dextran, there is little doubt that protein II* belongs to this class of polypeptides. Therefore, part of the protein must be exposed at the cell surface. Direct linkage of the protein to the murein then proves its transmembrane arrangement. Recently evidence has been produced suggesting that protein II* may be involved in the diffusion of amino acids through the outer membrane [20], and as in the case of protein I (see section 1) such a function would, of course, be consistent with this arrangement.

It is of interest to note that mutants missing protein II* are deficient as recipients in conjugation [21] as well as tolerant to colicin L-JF 246 [22]. Similarly, mutants missing polypeptides Ia and Ib or Ia only are tolerant to several colicins including colicin E3 [23–25] for which there is little doubt that it is taken up into the cell [26,27]. It remains to be seen if there is a causal connection between the transmembrane topology of the proteins and the transmembrane character of these processes.

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