NEAREST NEIGHBORS OF MAJOR PROTEINS IN THE OUTER MEMBRANE OF *ESCHERICHIA COLI* K12

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1. Introduction

The outer cell envelope membrane of *E. coli* contains a set of only a few so-called major proteins, i.e., proteins present at high concentrations. In cells of K12 strains grown in a rich medium at 30°C usually 5 such major proteins are found: the closely-related polypeptides Ia and Ib ([1] for other nomenclatures of these 'porins' [2] see [3]), protein II* (other nomenclature [3]) protein III [4], and the lipoprotein [5]. Nearest neighbors of several of these proteins have been studied in some detail and the present knowledge is summarized in table 1. Several of the neighbor relationships listed in table 1 are derived from results of crosslinking experiments [7–9,14]. In a number of these experiments it has, however, not been excluded that at least part of the crosslinked complexes observed in addition to protein may contain lipopolysaccharide and/or phospholipid. Therefore, some of the apparent protein–protein interactions may not be real.

Here we show that the two non-protein components of the outer membrane do not, under the experimental conditions used, become crosslinked to protein to a degree invalidating the protein–protein interactions deduced. In addition we demonstrate that the absence of one or the other major protein has some, but in general no drastic influence on the behavior in crosslinking of the remaining major proteins.

2. Experimental

The *E. coli* K12 strains used were: P400 [15] and its derivatives missing proteins Ia and Ib (P530 [16])

Table 1
Interaction of outer membrane components

Major proteins	Interacting neighbors [ref.]			
	Lipopolysaccharide	Lipoprotein	II*	Ia,Ib
Ia,Ib	+ [11,12]	+ [10]	n.r.	+ [6–8]
II*	+ [12,13]	+ [9]	+ [9]	
Lipoprotein	n.r.	+ [9]		

n.r., not reported

Protein III and phospholipid are not included because a direct interaction of major fractions of these with the components listed has not yet been reported. Polypeptides Ia and Ib can be crosslinked to dimers, trimers, and higher oligomers; it has been shown that these products do not contain lipopolysaccharide or phospholipid [8]

or protein II* (P400 TuII^{*R}1.3 obtained by selection for resistance to phage TuII* [17]); JE5506 and its derivative JE5505 missing the lipoprotein [18]. Cells were grown at 30°C in antibiotic medium no.3 (Difco) or, for labeling of cells with [^{32}P]orthophosphate, in nutrient broth (Difco) with added glucose (0.5%) and NaCl (0.5%). Preparation of cell envelopes and dodecylsulfate–polyacrylamide gel electrophoreses were done as repeatedly described (e.g. [1]). Crosslinking with dithiobis (succinimidyl propionate) was according to [9]; in all cases 20 mg crosslinker was allowed to act for 1 min at room temperature on envelopes corresponding to ~10 mg protein (final vol. 3.5 ml). Further increasing the crosslinker concentration did not change results. Upon interruption of the reaction [9] envelopes were recovered by centrifugation (20 min, 37 000 × *g*) and resuspended in 6 ml 10 mM Tris–HCl (pH 7.5) containing 5 mM EDTA and 2% sodium dodecylsulfate. After boiling for 3 min samples were centrifuged for 90 min at 50 000 × *g* and the pellet was boiled once more in the same way. It was washed with water and lyophilized. Protein from the combined supernatants was precipitated with acetone (90%), washed 4 times with acetone, and lyophilized.

For following the fate of phospholipid after crosslinking cells grown in the presence of [^{32}P]orthophosphate (3.3 μCi/ml, medium spec. radioact. 1.5 Ci/M) were used. The amount of phospholipid in the various fractions was measured as radioactivity extractable with chloroform/methanol (2/1, v/v), and thin-layer chromatography showed that nothing labeled but phospholipid was extracted. Lipopolysaccharide was quantitated by determination of ketodeoxyoctonate [19] using the pure substance as standard.

3. Results

Cell envelopes were crosslinked with dithiobis (succinimidyl propionate), separated in fractions becoming soluble and remaining with the murein layer upon boiling in dodecylsulfate, and analyzed electrophoretically upon reduction with mercaptoethanol. The results are summarized in table 2 and examples shown in fig.1. Regardless of presence or absence of other major proteins polypeptide II* and the lipoprotein became almost quantitatively crosslinked to the murein layer. (Here and in the following lipoprotein refers to the major fraction of this protein not bound covalently to the murein [5].) The attachment of polypeptides Ia and Ib to this layer depended on the presence of the lipoprotein; in its absence the former were not detectable in the insoluble fraction. The concentration of protein I in this fraction was increased when protein II* was missing.

The distribution of lipopolysaccharide and phospholipid in the two fractions were determined in strain JE5506 and its derivative missing the lipoprotein. The results were similar for both strains and are summarized in table 3. Most of the phospholipid present in the envelopes was not recovered in either fraction and it was found to be lost during the precipitation of the proteins from the soluble fraction with acetone. Protein precipitates immediately while phospholipid remains in a fine suspension and

Table 2
Distribution of crosslinked proteins in envelope fractions

Strain	Protein missing	Major proteins in	
		Soluble fraction	Insoluble (murein) fraction
JE5506	–	I, III	I (~10%), II*, lipoprotein
JE5505	Lipoprotein	I, III	II*
P400 TuII^{*R}1.3	II*	I (~70%), III	I (~30%), lipoprotein
P530	I	III	II*, lipoprotein

Proteins Ia,Ib are listed as proteins I because upon crosslinking and reductive cleavage the two polypeptides were no longer separable electrophoretically (see fig.1)

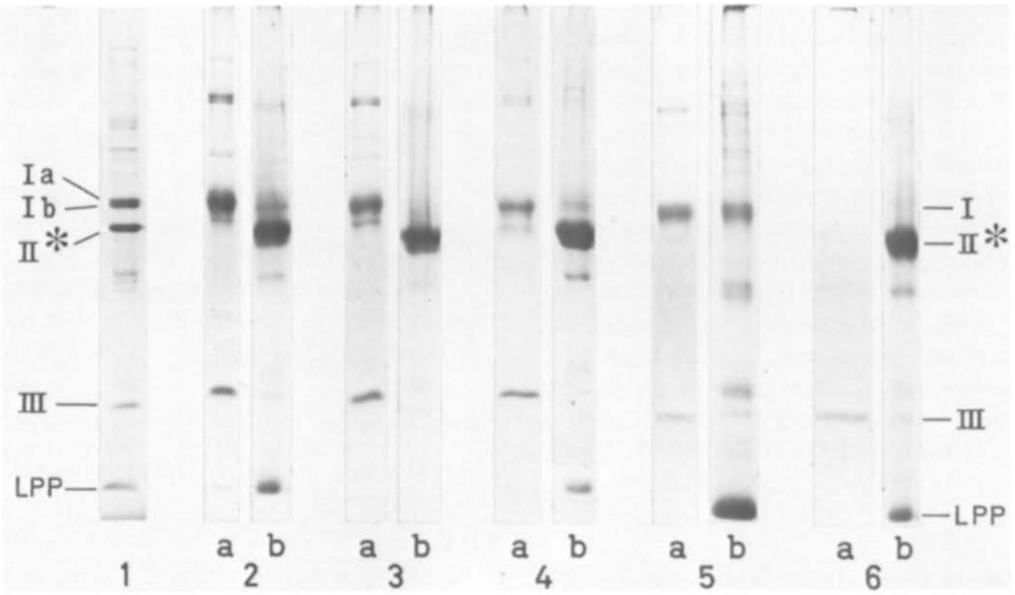

Fig.1. SDS–polyacrylamide gel electrophoreses. (1) Envelopes before crosslinking (strain P400). (a,b) Soluble and insoluble (murein-associated) fractions, respectively, after reductive cleavage. Based on wet wt cells the amounts of fractions a and b applied to the gels are about equivalent. (2) Strain JE5506; (3) strain JE5505 (lacking lipoprotein); (4) strain P400; (5) strain P400 Tull*^{R} 1·3 (lacking protein II*); (6) strain P530 (lacking proteins Ia,Ib). Polypeptides Ia,Ib upon crosslinking and cleavage are no longer separable. Electrophoresis of the soluble fractions before cleavage showed almost exclusively monomeric protein III and the monomeric, dimeric, and trimeric proteins I as in [8]. It has not been determined if the apparent increase in lipoprotein concentrations in 5b and 6b is due to an overproduction of the protein in these mutants. LPP, lipoprotein.

much of it attaches to the glass of the cylinder used. In other words, ~98% of the envelopes' phospholipid did not become crosslinked to protein. Also, ~50% of all lipopolysaccharide is not recovered and, although we have not followed the fate of the missing fraction, it must also be lost during precipitation and washing of the soluble proteins with acetone. Since in case of the mutant missing the lipoprotein practically only protein II* is crosslinked to the murein determination of protein concentration in this complex will give the approximate molar concentration of this protein. It was found that per mg dry wt

Table 3
Distribution of phospholipid and lipopolysaccharide in envelope fractions

Component	Strain	% component present in envelopes found in	
		Soluble fraction	Insoluble (murein) fraction
Ketodeoxyoctonate	JE5506	56	1.3
	JE5505	46	1.9
Phospholipid	JE5506	3.2	0.9
	JE5505	1.7	0.5

of this complex there were 0.38 mg = 12 nmol protein II*, 3 nmol ketodeoxyoctonate = 1 nmol lipopolysaccharide monomer, and 6.5 nmol phospholipid. This excess of protein over lipopolysaccharide or phospholipid must be considerably larger in the wild-type parent: on a molar basis about 5-times more lipoprotein than protein II* is present in the envelope [5,20]; practically all of it was found in the crosslinked murein–protein complex, and the concentrations of lipopolysaccharide and phospholipid did not differ much between wild type and the lipoprotein-less mutant. That is, in such a complex from wild type strains the molar excess of protein II* and lipoprotein over phospholipid plus lipopolysaccharide should at least be 10-fold.

4. Discussion

The results of these experiments for most proteins represent near all-or-none phenomena. Protein II* and the lipoprotein were found almost quantitatively in the murein–protein complex, regardless of presence or absence of lipoprotein or protein II*, respectively, or polypeptides Ia-Ib. We had reported before [14], that only 20–30% of protein II* can be crosslinked to the murein. The discrepancy between this result and that reported here was found to be due to the somewhat disturbing fact that this difference is caused by strain differences. The pair (with and without lipoprotein) used before is not isogenic with the pair used here, and the previous results have been reproduced with the respective strains. Obviously and unfortunately therefore, caution is required in accepting generalisations.

Some variability was found for proteins Ia,Ib. They were absent from murein–protein complexes in the mutant missing the lipoprotein and ~10% of protein I appeared to be bound to such complexes derived from wild-type cells, and this may well reflect the near neighbor relationship between lipoprotein and proteins I deduced already by other methods [10]. The amount of proteins I present in murein–protein complexes clearly increased in a mutant missing protein II*. We have not determined which interaction causes this increase; from the results discussed above, however, it would appear that in the absence of protein II* crosslinking between proteins I and the lipoprotein becomes more efficient.

The excess of protein over phospholipid and lipopolysaccharide in the fraction insoluble in hot dodecylsulfate after crosslinking demonstrates that much of the links made and involving proteins I, II* and the lipoprotein represent protein–protein or protein–murein bonds.

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