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Cell Envelope and Shape of *Escherichia coli* K12. Crosslinking with Dimethyl Imidoesters of the Whole Cell Wall

(envelope proteins/long-range protein–protein interaction)

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ABSTRACT ***E. coli* cells treated with the bifunctional crosslinking reagents dimethyl malonimidate, succinimidate, adipimidate, suberimidate, and sebacinimidate served for the isolation of rod-shaped "ghosts." These ghosts proved to be crosslinked over their entire surface; i.e., a macromolecule (resistant to boiling 1% Na dodecyl sulfate) the size of the cell had been created. Also, ghosts could similarly be crosslinked. In both cases, the final "sacs" contained about 60–70% protein, and very little or no lipopolysaccharide. When ghosts from which phospholipid had been removed were crosslinked, the covalently closed ghosts were almost pure protein; 80–90% of their dry mass was accounted for by protein. Ammonolysis of the crosslinked material (whether stemming from crosslinked cells or ghosts) showed that the same four proteins (Na dodecyl sulfate gel bands) had been crosslinked that are found in normally prepared ghosts. These observations practically exclude the hypothesis that a fluid mosaic model of membrane structure can be applied to the outer membrane of the *E. coli* cell envelope; rather, extensive protein–protein interactions must exist over the whole surface of this membrane. These findings are consistent with the possibility that the ghost polypeptide chains are involved in the determination of cellular shape.**

We have shown that rod-shaped "ghosts," which are surrounded by the outer membrane of the cell envelope, devoid of murein, and free from all cytoplasmic material except for remaining fragments of the cytoplasmic membrane, can be isolated from *Escherichia coli* cells (1, 2). These ghosts consist of about 25% phospholipid, 25–30% lipopolysaccharide, and 45–50% protein. We have shown that the protein of ghosts is separable into four main bands (I, II, III, and IV) in Na dodecyl sulfate–polyacrylamide gel electrophoresis (see Fig. 2). We have speculated that one or more of these polypeptide chains, i.e., presumably by their self assembly, could be the final products of the genetic information specifying cellular shape. One prediction following from this hypothesis is that protein–protein interactions should be existent over the whole cell envelope between one or more of the proteins mentioned. We show in this communication that such appears, indeed, to be the case.

MATERIALS AND METHODS

Cells, Media, Growth Conditions, and Preparation of Ghosts. The *E. coli* K12 strain W945-T3282 [a diaminopimelate plus lysine auxotroph (3)] was used in the same way as described (1, 2). Ghosts were isolated following the recently described (1) procedure II. In brief, it involves treatment of cells with Triton X-100 in 40% sucrose, urea, trypsin, and finally lysozyme.

Crosslinking. All diimidoesters were prepared essentially according to Davies' and Stark's (4) version of the method of McElvain and Schroeder (5), and all dinitriles were purchased from Schuchardt (München, Germany). Whole cells for crosslinking were, after harvesting, washed once with 150 mM NaCl and once with 1 M triethanolamine, pH 8.5. They were suspended (about 100 mg of wet weight per ml) in the same buffer, and an equal volume of 200 mM dimethyl imidoester dissolved in the same buffer was added (4); if required, the pH was adjusted with NaOH. The reaction was allowed to proceed with stirring at room temperature (23°) for 3 hr (longer incubation had no effect) and for 8 hr at 4° or −10°. Cells were recovered by centrifugation and subjected to the ghost purification procedure. Ghosts (at about 50 mg of wet weight per ml) were processed identically. They were used either before trypsin digestion or after the trypsin and lysozyme steps (see *Results*). After crosslinking, the former were then subjected to lysozyme. Upon centrifugation, the material was suspended with 1% Na dodecyl sulfate. It was kept at 100° for 5 min, centrifuged, and again boiled for 5 min in 1% Na dodecyl sulfate. After centrifugation it was lyophilized from aqueous suspension, washed with acetone, and suspended in water for further investigation.

Ammonolysis (6). About 100 mg of lyophilized material was suspended in 20 ml of concentrated NH_4OH–glacial acetic acid (15:1, v/v). The suspension was stirred for 15 min at room temperature. Considerably longer incubation does not visibly increase the yield of liberated polypeptide chains, but instead can lead to increasing fragmentation of band II protein, as was revealed by control experiments in which non-crosslinked ghosts were subjected to the ammonolysis procedure. Upon centrifugation, the supernatant was lyophilized and the sediment was suspended with 10 ml of 1% Na dodecyl sulfate, kept for 5 min at 100°, and centrifuged again. This supernatant was lyophilized and dissolved with water; acetone was added to a final concentration of 90%. The precipitated protein was freed from most of the detergent by one more washing with acetone (7) and then lyophilized. The two lyophilized supernatants were combined or used separately for electrophoretic analysis.

Other Methods. For electrophoresis, Method II of the previously described procedures (2) was followed. Electron microscopy was performed with a Philips EM 201 microscope, by described methods (8). Amino-acid analyses were conducted with acid hydrolysates (usually 24 hr, 110°, 6 N

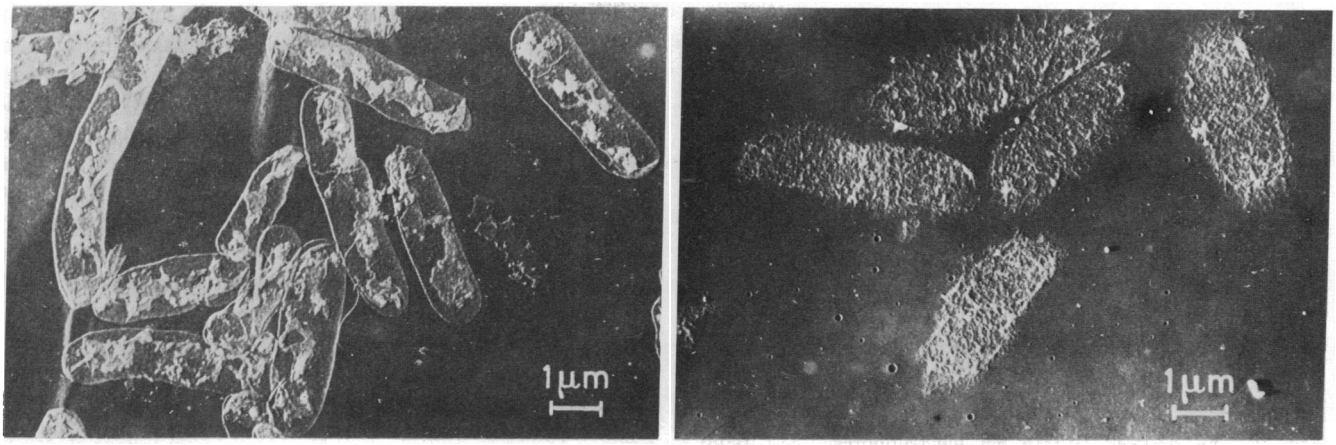

FIG. 1. Shadowed ghosts. (*Left*) normal ghosts, which are completely soluble in Na dodecyl sulfate and which yield protein patterns like those shown in Fig. 2-*2*. (*Right*) ghosts prepared from cells treated with dimethyl suberimidate after boiling in 1% Na dodecyl sulfate. Before detergent treatment, these ghosts look identical to those on the left.

HCl) on an Unichrom Analyzer (Beckman Instruments). Lipopolysaccharide was determined essentially according to Osborn (9).

RESULTS

Crosslinking of Whole Cells and Ghosts. Whole cells treated with dimethyl suberimidate were subjected to the ghost purification procedure. The ghosts did not differ morphologically from those we have described before (1, 2), and lysozyme removed murein as completely as we have shown for normal ghosts. Normal ghosts are soluble, at room temperature, in ionic detergents, e.g., 1% Na dodecyl sulfate. Ghosts from cells treated with dimethyl suberimidate, however, can be kept in boiling 1% Na dodecyl sulfate (Fig. 1). The same results were obtained when cells were treated with dimethyl malonimidate, succinimidate, adipimidate, or sebacinimidate.

Normal ghosts, when treated with any of the imidoesters, did not become resistant to Na dodecyl sulfate. Ghost purification involves a trypsin step, and we have shown (ref. 2, compare Fig. 2) that one of the ghost proteins (protein II) is a trypsin fragment of protein II*; trypsin reduces the apparent molecular weight of II* (about 40,000) by about 30%. Therefore, ghosts that had not been subjected to trypsin digestion were treated with the above-mentioned imidoesters, and all effected resistance to boiling Na dodecyl sulfate. The yield of ghosts resistant this way has been determined quantitatively (particle counting) with preparations obtained from cells and those from nontrypsinized ghosts crosslinked with dimethyl suberimidate. It was found to be 70–80% in both cases, and visual inspection showed that the yields for all other imidoesters cannot be greatly different.

Composition of Crosslinked Ghosts. All experiments described below were performed with material cross-linked with dimethyl suberimidate, and the results were the same whether whole cells were crosslinked or whether nontrypsinized ghosts were used for crosslinking. Crosslinked ghosts extracted with boiling Na dodecyl sulfate were found to contain at most 5% (as dry weight) lipopolysaccharide (normal ghosts contain about 30% lipopolysaccharide); in several preparations, lipopolysaccharide was no longer detectable at all. It was not clear whether or not some phospholipid remains in ghosts extracted with Na dodecyl sulfate. A quantitative determination would be made somewhat difficult by the fact that most of the phospholipid components occur also in the lipoprotein (our protein IV) described by Braun *et al.* (10, 11). Since ghosts do not lose shape upon complete removal of phospholipid (2), we asked whether ghosts without phospholipid can be crosslinked the same way as normal ghosts. This was found to be the case (only dimethyl suberimidate was tested), and the following results show that some phospholipid is not removable from crosslinked ghosts that had not been extracted with chloroform–methanol before treatment with the diimidoester. Amino-acid analyses of crosslinked ghosts that had been extracted with Na dodecyl sulfate and hydrolyzed by acid revealed that 60–70% of their dry weight could be accounted for by protein. When ghosts without phospholipid were used for crosslinking, the same analyses showed that after treatment with Na dodecyl sulfate 80–90% of their dry weight could be accounted for by protein. The moisture content of such preparations was somewhat difficult to quantitate because the material, upon drying under reduced pressure at 60°, is rather hygroscopic. Therefore, the 5–10% water found represent minimum values. Considering, in addition, the presence of known (11) and possibly unknown non-amino-acid substituents of the ghost proteins, as well as the fact that in normal ghosts there is no other major constituent besides protein, phospholipid, and lipopolysaccharide (2), it is rather safe to assume that ghosts such as those shown in Fig. 1 (*right*) are practically pure protein sacs.

This conclusion is consistent with the following observation. Normal ghosts are resistant morphologically to all proteases we have used (1, 2). Crosslinked ghosts do not differ in this respect from normal ghosts. After extraction with hot Na dodecyl sulfate, however, they are completely dissolved within a few minutes when trypsin is added.

Crosslinked ghosts were subjected to ammonolysis after Na dodecyl sulfate extraction. Na dodecyl sulfate–polyacrylamide gel electrophoresis of the material solubilized upon this treatment showed that *all four* proteins known to be present in the ghost membrane had been tied into the crosslinked material (Fig. 2). The relative amounts of the four bands measured by microdensitometer did not differ significantly

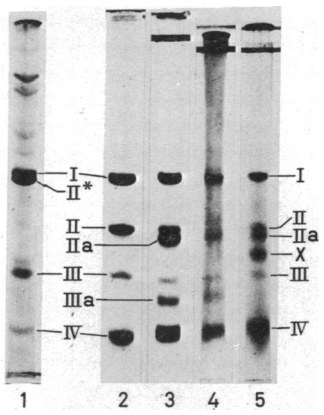

FIG. 2. Na dodecyl sulfate gel electrophoresis. (*1*) Band pattern from ghosts not treated with trypsin. (*2* and *3*) Typical band patterns of normal ghosts. Band II is a trypsin fragment of band II*, which is hardly separable from I (2). Band IIa often is completely lacking, and it probably is also a trypsin fragment of II* (2). Band IIIa is lysozyme (2). (*4*) Ghosts prepared from crosslinked (dimethyl suberimidate) cells and subjected to ammonolysis; band pattern of proteins soluble in the ammonia–acetic acid mixture (see *text*). (*5*) Same preparation, band pattern of material obtained by Na dodecyl sulfate extraction of ghosts subjected to ammonolysis. Band X has also been observed to occur in noncrosslinked ghosts that had been subjected to ammonolysis, and it very likely arises at the expense of protein II. Essentially the same patterns as *4* and *5* were obtained when nontrypsinized ghosts were crosslinked and subjected to ammonolysis.

from those found in the parallel preparations that had not been crosslinked. As a *caveat* it should be mentioned that the yields of protein solubilized by ammonolysis were only about 10% of the total protein input, and minor protein bands may not have been detectable.

Crosslinking at Low Temperatures. The dimethyl malonimidate molecule is about 0.3 nm long. We felt it a rather astonishing fact that even with such a reagent a covalently linked container of the size and shape of the cell can be obtained that consists mainly of protein and that contains all known ghost proteins, indicating an extraordinarily close packing of these proteins. We considered the possibility that we may have created an artifact if lateral diffusion of the proteins (not known whether such is the case in this membrane) would be considerably faster than the half-life of the diimidoesters. A monosubstituted lysine residue of one protein may, with its methyl imidoester end, be able to "catch" another protein as soon as it diffuses near enough. We have, therefore, treated cells as well as ghosts (nontrypsinized) with all initially mentioned imidoesters at room temperature, at 4°, and at −10° (in the presence of 30% glycerol). At all temperatures and with all reagents, cells and ghosts could be crosslinked, i.e., ghosts were obtained that, after digestion with lysozyme, proved to be resistant to boiling Na dodecyl sulfate.

DISCUSSION

We believe it is clear that in the outer membrane of the *E. coli* cell envelope a protein network must exist with extensive protein–protein interactions between *the same* four proteins (plus perhaps others not detectable after ammonolysis) that are present in the ghost membrane. This fact not only tends to favor the view that they may belong to the final products of the genetic information specifying cellular shape, but it also strongly indicates that the occurrence of the four proteins in the ghost membrane is not just accidental. Namely, upon purification of a membrane with techniques like those we have used, one may simply end up with a number of proteins that are very water insoluble and/or protected in their host membrane and that have no functional relationship at all.

It appears fairly remarkable that the whole outer membrane can be crosslinked not only with an agent spanning only about 0.3 nm, but also with all reagents covering spanning distances from 0.3 to 1.8 nm. The experiments at low temperature strongly argue against the possibility that, as discussed above, this is due to extensive lateral movement of the proteins in the membrane. The very small distances coverable by the crosslinking agents then suggest a sponge-like structure of the proteins in the outer membrane. That is, the distances from protein to protein cannot be uniformly 0.3 nm or less in one plane of the membrane because there would not be enough space left for even the penetration of simple metabolites. Another possibility would be that the protein lattice is not uniform over the whole cell envelope, but that areas with very dense protein packing are interrupted by areas with a less dense arrangement of polypeptide chains.

Trypsinized ghosts can no longer be crosslinked with the imidoesters used (it is still possible to completely crosslink them with glutaraldehyde), and it is very likely that this is caused by the trypsin removal of about 30% of the amino-acid residues from protein II* although there is no absolute proof because trypsin also destroys a number of other, minor protein bands (see Fig. 2). None of these minor proteins is present in amounts that would make any one of them a candidate for a repeating subunit; therefore, it would not appear that their presence is a requirement for successful crosslinking. The trypsin fragment of protein II*, protein II, has been isolated on a preparative scale and it has been found that it contains about 10 lysine residues (Garten, W. & Henning, U., in preparation). Thus, it seems that trypsin fragmentation of protein II* leads to a spatial situation preventing complete crosslinking. We have shown before (2) that the molar ratio of polypeptides I, II*, and IV in ghosts is roughly 1:1:8–10 and that there are smaller amounts of protein III, which, in contrast to I, II*, and IV, show considerable variations from preparation to preparation. It may be, therefore, that the extended, crosslinked network requires repeating I–II* sequences.

We have shown before (2) that a phospholipid bilayer is not the basic structure of the ghost membrane, and we have shown in this communication that apparently extensive protein–protein interactions exist in the outer membrane of the *E. coli* cell envelope (a situation that we found not to be unique for *E. coli;* it holds true for probably most gram-negative bacteria). It is thus reasonable to assume that such membranes do not fit the fluid mosaic model (12) of membrane structure but rather constitute an extreme case of the type of models presented by Capaldi and Green (13).

It should finally be pointed out that throughout this work we have somewhat carelessly used the designation "protein" for protein material, the homogeneity of which (concerning number of polypeptide chains) has not yet been proven. We have shown before (2) that protein IV is a homogeneous polypeptide chain, namely the lipoprotein described by Braun *et*

al. (10, 11). We have also isolated proteins I and II on a preparative scale (Garten, W. & Henning, U., in preparation), and all protein chemical data available so far at least show that none of them can be a mixture of three or more polypeptide chains (14–16). There is thus no doubt that repeating subunits have been crosslinked.

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