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Arrangement of Protein I in *Escherichia coli* Outer Membrane: Cross-Linking Study

E. T. PALVA* AND L. L. RANDALL

The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden

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The arrangement of protein I in the outer membrane of *Escherichia coli* was investigated by cross-linking whole cells, isolated cell wall, protein-peptidoglycan complexes, and protein I released from peptidoglycan with NaCl. Both cleavable azide cross-linkers and imidoester reagents were used. The data presented suggest that protein I exists in the outer membrane as a trimer.

The outer membrane of gram-negative bacteria is not a true permeability barrier. It excludes hydrophilic molecules of molecular weights >700 but allows free passage of small molecules (18). Thus, it seems that the outer membrane contains passive pores. Nakae (17) has shown that reconstitution of membrane vesicles that show permeability properties similar to those of outer membrane requires the presence of one of the major outer membrane proteins, protein I (7). He proposes that the same protein forms an aqueous pore in vivo, thus explaining the permeability of the outer membrane. In vivo studies with *Salmonella typhimurium* mutants that lack proteins analogous to protein I indicate that these proteins are involved in passage of β-lactam antibiotics through the outer membrane (19).

Protein I does appear to span the membrane. It is exposed to the outside as evidenced by its role as a receptor for phage (24, 25), and, at the same time, it is in tight association with the peptidoglycan (9, 23, 28). Rosenbusch, who has designated this protein the matrix protein, has isolated a protein I-peptidoglycan complex and analyzed it by electron microscopy (23, 27). Protein I is arranged in a periodic lattice structure with a basic repeating unit of three protein I molecules (27).

The isolation of cell-shaped ghosts that contain predominantly major outer membrane proteins (7, 10, 11) demonstrated that strong protein-protein interactions exist among the major proteins. If the ghosts are treated with high concentrations of imidoester cross-linking reagents, they retain their shape even when boiled in sodium dodecyl sulfate (SDS) (8).

In a previous study (21), the results of which have been recently confirmed and extended by Reithmeier and Bragg (22), we showed that protein I is specifically cross-linked to itself when whole cells are treated with cleavable azide reagents. We observed mainly dimers of protein I and low levels of higher multimers that appeared to be trimers and tetramers. We further investigated the arrangement of protein I by cross-linking whole cells, isolated cell wall, protein-peptidoglycan complexes, and protein I released from peptidoglycan with NaCl (9). Both cleavable azide cross-linkers (14) and the more reactive, but not truly cleavable, imidoester reagents (4) were used. We present evidence that the same cross-linked complexes of protein I can be found in all of these preparations and that the basic unit is probably a trimer.

MATERIALS AND METHODS

Chemicals. The cleavable cross-linkers tartryl-diazide (0.6-nm bridge length) and tartryl-di-(glycylazide) (TDGA; 1.3 nm) were a generous gift from Hans Fasold. Dimethylsuberimidate (DMS, 1.0 nm) and dimethyladipimidate (0.7 nm) were obtained from Sigma Chemical Co., St. Louis, Mo. Radioactive isotopes came from the Radiochemical Centre, Amersham, England.

Strains, media, and growth conditions. *Escherichia coli* K-12 strain Hfr G6 (13), which is partly constitutive for λ receptor (26), and *E. coli* B wild type were used. Cells were grown in tryptone broth (1% tryptone [Difco Laboratories, Detroit, Mich.], 0.5% NaCl), tryptone broth with 0.4% maltose, or M9 minimal medium (16) with maltose or glucose (0.4%) and required amino acids. Cells were grown to midlogarithmic phase (4×10^8 cells per ml) on a rotary shaker at 37°C before harvesting. To obtain radioactively labeled protein, cells were grown in M9 medium to 10^8/ml, [^{35}S]methionine was added (250 μCi/40 ml of medium), and growth continued to 4×10^8 cells per ml. ^{32}P labeling was done by adding 1 mCi of $^{32}P_i$ to 50 ml of low-phosphate medium M56 LP (2) containing 0.4% glucose, using the cell densities described above.

Isolation of cell wall and protein-peptidoglycan complex. Cells were washed with 10 mM tris-(hydroxymethyl)aminomethane-hydrochloride (pH 7.4)-1 mM ethylenediaminetetraacetic acid and broken by mild sonic treatment (two 10-s periods in a

salt-ice bath). Membranes were pelleted at 100,000 × *g* for 2 h, and the cell wall (outer membrane and peptidoglycan) was isolated on sucrose gradients as described by Osborn et al. (20). The protein-peptidoglycan complex was isolated by extraction with SDS at 60°C for 30 min as described by Rosenbusch (23), except that isolated cell wall was used as the starting material. In our hands, the isolated protein-peptidoglycan complex also contained the lipoprotein that has been characterized by Braun and Bosch (3).

When *E. coli* K-12 is grown on maltose, the isolated protein-peptidoglycan complex also contains the lambda receptor (C. A. Schnaitman, personal communication).

Purification of protein I. Proteins were released from the peptidoglycan complex by treatment with 0.5 M NaCl as described by Hasegawa et al. (9). The salt-treated protein-peptidoglycan complex was applied directly to a sucrose gradient [5 to 28% (wt/wt) sucrose in 0.2% SDS–10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4)], or the peptidoglycan was first removed by centrifugation (60,000 × *g* 30 min) and then the supernatant containing the released proteins was subjected to sucrose gradient centrifugation. Centrifugation was for 14 h at 130,000 × *g*, using an SW60 rotor in a Sorvall OTD-2 ultracentrifuge. Sedimentation coefficient calculation was by the method of McEwen (15). The partial specific volume of protein I was calculated from those of the individual amino acids by using the amino acid composition given by Rosenbusch (23).

Cross-linking. Cross-linking of whole cells at room temperature (21 to 23°C) was done as described previously (21). Isolated cell wall, protein-peptidoglycan complex, or purified protein I in amounts corresponding to 4 × 10^{10} cells was cross-linked in 1 ml of 1 M triethanolamine-hydrochloride (pH 8.5) for 1 h at room temperature, with reagents at the concentrations indicated. SDS (0.1%) was present during cross-linking of protein-peptidoglycan complexes or isolated protein I. The reaction was terminated by adding excess methylamine.

SDS-polyacrylamide gel electrophoresis. Discontinuous 7, 9, or 14% slab gels (1 mm thick) used were as described previously (21). For symmetrical two-dimensional gels, the first dimension was run as a slab and then individual tracks were cut out, cleaved for 15 min with sodium periodate, and applied to a second slab gel (21). Continuous gels (3 to 9%), according to Davies and Stark (4), were used as 1-mm slab gels. All samples were boiled for 5 min before application to the gel. Gels were stained with Coomassie brilliant blue by the method of Fairbanks et al. (5), but solution three was omitted. Periodic acid-Schiff staining was done by the method of Fairbanks et al. (5). Autoradiography was done by overlaying stained and dried gels with Kodak X-ray film RP 14. Films were developed by a standard technique.

RESULTS

Cross-linking with cleavable reagents. *E. coli* B whole cells, isolated cell wall, and protein-

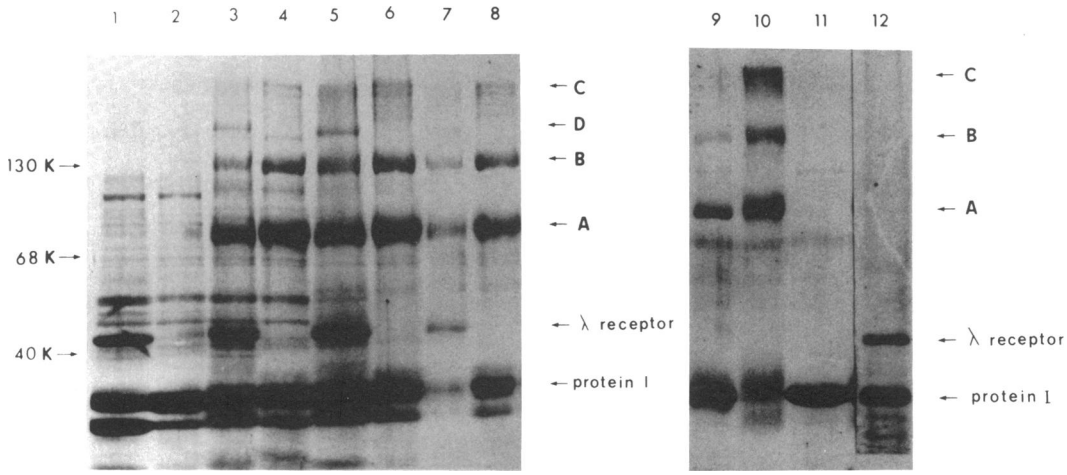

FIG. 1. *Discontinuous polyacrylamide gel (9%) showing protein patterns obtained after cross-linking. Cross-linking was done with E. coli K-12 or B whole cells, isolated cell wall, or protein-peptidoglycan complex, and then the samples were further processed as indicated before application to the gel. Whole cells were cross-linked, and the cell wall was isolated and applied to the gel (slot 3, E. coli K-12; slot 4, E. coli B). Isolated cell wall was cross-linked and applied directly (slot 5, E. coli K-12; slot 6, E. coli B), or the protein-peptidoglycan complex was isolated before application to the gel (slot 7, E. coli K-12; slot 8, E. coli B). The protein-peptidoglycan complex isolated from E. coli B was cross-linked and applied to the gel (slots 9 and 10). All samples were cross-linked with TDGA (35 mM) except the sample in slot 9, which was cross-linked with tartryl-diazide (60 mM). Untreated cell walls were applied to slot 1 (E. coli K-12) and slot 2 (E. coli B), and untreated protein-peptidoglycan complex was applied to slot 12 (E. coli K-12) and slot 11 (E. coli B). The arrows at the left indicate the positions of molecular weight standards: β-galactosidase (130,000 g/mol); bovine serum albumin (68,000 g/mol), and creatine kinase (40,000 g/mol). A, B, C, and D are cross-linked complexes. All samples were boiled for 5 min before application to the gel.*

peptidoglycan complexes were cross-linked with TDGA (35 mM). The cross-linked material was analyzed on discontinuous 9% gels (Fig. 1). In all TDGA cross-linked preparations, three new major bands (A, B, and C) could be seen. Bands A and B were always distinct, whereas C was usually diffuse.

Protein-peptidoglycan complexes were also cross-linked with a high concentration (60 mM) of reagent tartryl-diazide, which is shorter than TDGA (0.6 and 1.3 nm, respectively). Under these conditions, complex A was clearly present and a small amount of complex B was visible. Protein I was identified as the protein component of complexes A, B, and C by symmetrical two-dimensional gel electrophoresis (Fig. 2).

No other proteins appear to be involved in these complexes. Cross-linking of *E. coli* K-12,

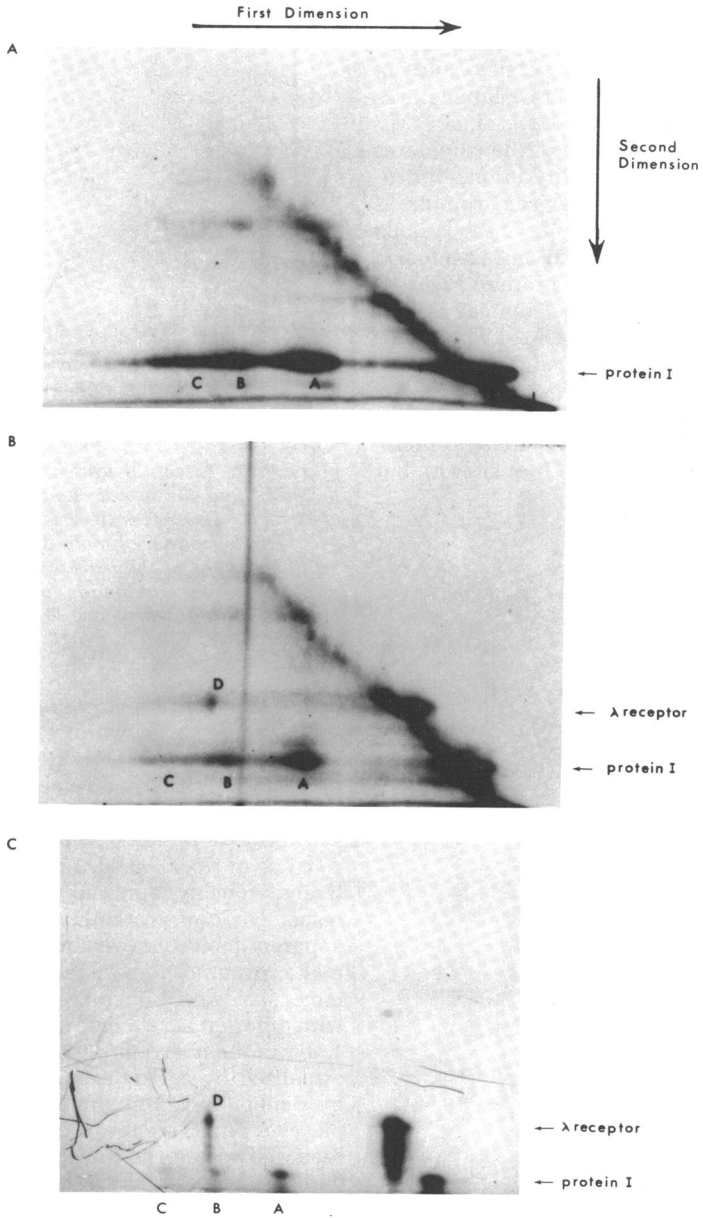

FIG. 2. *Symmetrical two-dimensional discontinuous SDS-polyacrylamide (7%) gels of cross-linked (35 mM TDGA) samples. (A) Cell wall of E. coli B; (B) cell wall of E. coli K-12; (C) protein-peptidoglycan complex of E. coli K-12. All cross-links were cleaved after the first-dimension run.*

which has the lambda receptor in its outer membrane as well as the other major proteins, gave complexes A, B, and C as described above, but, in addition, a new band appeared (band D in Fig. 1). Symmetrical two-dimensional gel electrophoresis was used to identify complexes A, B, and C as multimers of protein I and D as a multimer of lambda receptor (Fig. 2C).

Cross-linking with imidoesters. With high concentrations of TDGA (up to 100 mM), low levels of higher-molecular-weight complexes of protein I could be seen. This reagent is not reactive enough to allow more detailed study of these complexes; therefore, cross-linking was done with the more reactive DMS. Since DMS is not cleavable, identification of the complexes is based solely on their mobility in one-dimensional gels. Protein-peptidoglycan complexes isolated from *E. coli* B, which lacks the lambda receptor, were used to avoid any confusion caused by complexes of that protein. Figure 3 shows protein-peptidoglycan complex cross-linked with either TDGA or DMS. The multimers A, B, and C are observed with both reagents, but with DMS additional higher-molecular-weight material is seen. Cross-linking with a shorter reagent, dimethyladipimidate (0.7 nm), gave the same results as DMS (not shown), but

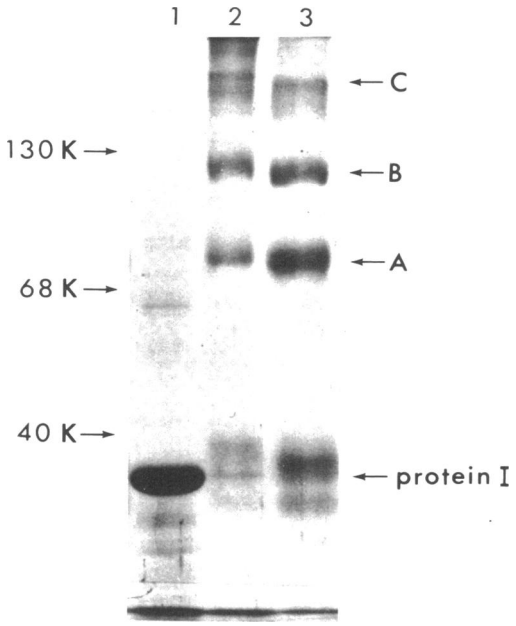

FIG. 3. *Discontinuous SDS-polyacrylamide (9%) gel showing cross-linked complexes from protein-peptidoglycan complex of E. coli B. Slot 1, Control; slot 2, cross-linked with DMS (25 mM); slot 3, cross-linked with TDGA (50 mM). Arrows at left show the positions of standard proteins as in Fig. 1.*

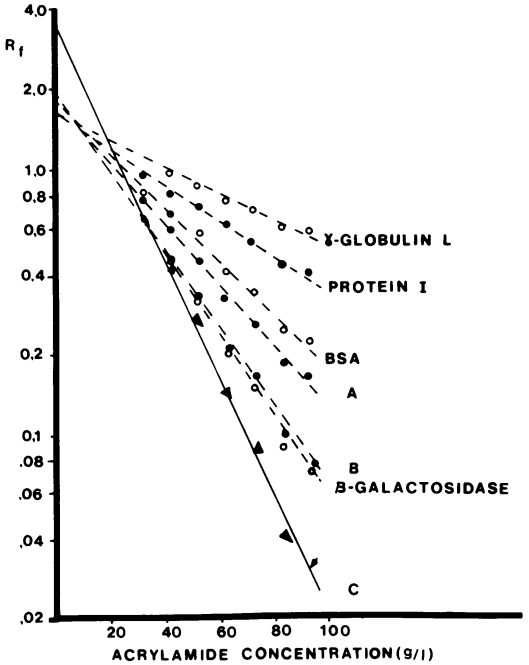

FIG. 4. *Ferguson plot of the cross-linked complexes (A, B, and C). Protein-peptidoglycan complexes from E. coli B were cross-linked with DMS (50 mM), and the boiled samples were subjected to electrophoresis in continuous SDS-polyacrylamide gels. The logarithm of mobility of the protein band relative to that of the tracking dye (R_f) was plotted against the total acrylamide concentration (12). Symbols: ○, standard proteins; ●, protein I and multimers A and B; ▲, multimer C.*

higher concentrations were required. Results similar to those shown in Fig. 3 were obtained if, before isolation of protein-peptidoglycan complexes, whole cells or isolated cell walls were treated with DMS.

Multimers A and B, with apparent molecular weights of 75,000 and 120,000 g/mol, respectively, probably represent the dimer and trimer, respectively, of protein I. Complex C, with an apparent molecular weight of around 170,000 g/mol, proved not to be a tetramer. The apparent molecular weight of complex C on gels changes with different acrylamide concentrations. Figure 4 shows the aberrant behavior of complex C as visualized by a Ferguson plot (6) as described by Banker and Cotman (1). Protein I and complexes A and B seem to have normal free mobilities well within the range of those of the standard proteins. In contrast, the mobility of C is clearly aberrant, being the same as that of B in 3% gels but lower in higher acrylamide concentrations (Fig. 4). Thus, complex C appears as a separate band in 6% gels (Fig. 5).

It is possible that the aberrant mobility of C is due to the presence of nonprotein moieties in the cross-linked complex. The complexes were examined for carbohydrate by staining gels by the periodic acid-Schiff staining procedure (5). No carbohydrate was detected. To assay the presence of phospholipid or lipopolysaccharide, cells were labeled with $^{32}P_i$. Less than 1% of the label in the cell wall remained with the isolated protein-peptidoglycan complex. When this material was cross-linked with DMS, less than 1% of the radioactivity in the protein-peptidoglycan complex remained with the protein I multimers. It is not likely that the phosphate-containing material, which is cross-linked to complex C, is responsible for its aberrant behavior, since the amount in C was no different from that in A or B.

The higher multimeric complexes of protein I obtained by DMS cross-linking could best be resolved on 3% gels, where the aberrant mobility of C did not interfere. Figure 5 shows a protein-peptidoglycan complex cross-linked with 50 mM DMS. There appears to be a continuous series of multimers, with every third one more abundant than the two immediate lower ones. A plot of logarithm of calculated molecular weight versus observed mobility (Fig. 6) clearly shows that the molecular weights of the multimers are multiples of the monomeric molecular weight (37,000 g/mol). The highest complex clearly seen is a nonamer, but there is no evidence that the series does not continue indefinitely.

Cross-linking of purified protein I. It has been shown by Mizushima and co-workers that protein I can be released from peptidoglycan with salt treatment (9). By isolating the salt-released protein on a sucrose gradient, we were able to study the arrangement of pure protein I. Under the conditions used, the protein sedimented as a distinct peak (Fig. 7A) with a sedimentation coefficient of approximately 10S, indicating that it is in multimeric form. The lipoprotein, which was also released by salt treatment, remained at the top of the gradient. Boiling the protein in SDS before gradient centrifugation resulted in a reduction in sedimentation rate (Fig. 7C). This slowly sedimenting form is

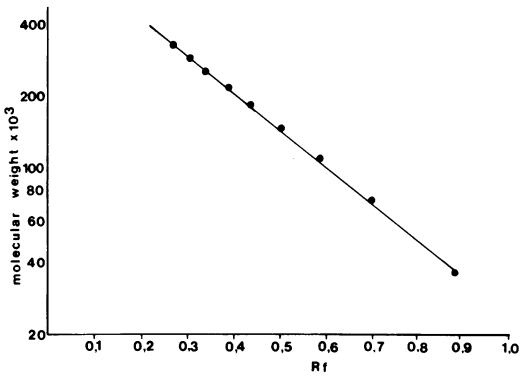

FIG. 6. *Plot of the logarithm of molecular weight versus migration (R_f) for the multimers formed (as in Fig. 5) during cross-linking of protein-peptidoglycan complexes from E. coli B with DMS (50 mM).*

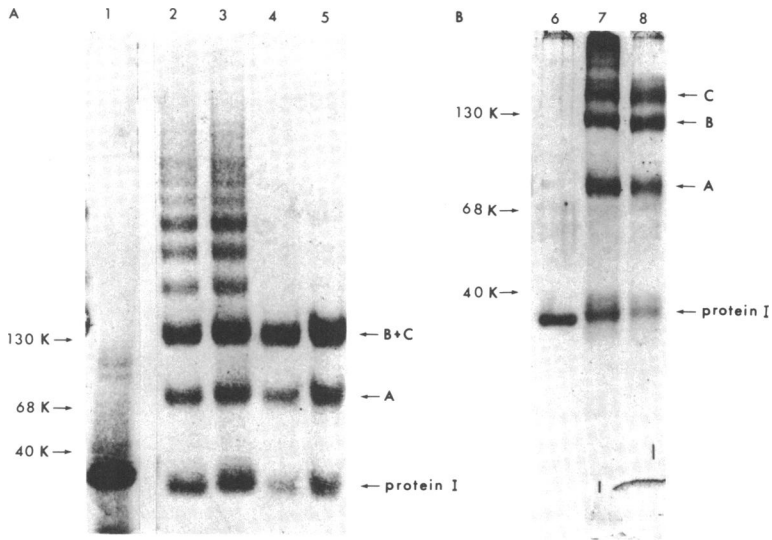

FIG. 5. *DMS (50 mM) cross-linking of protein-peptidoglycan complexes and purified protein I from E. coli B, analyzed in 3% (A) and 6% (B) continuous SDS gels. Slots 1 and 6, Control protein-peptidoglycan complex; slots 2, 3, and 7, cross-linked protein-peptidoglycan complexes; slots 4, 5, and 8, cross-linked purified protein I. Arrows at left show the migration of standards as in Fig. 1.*

probably a monomer-SDS complex. When the boiled material was treated with 50 mM DMS, no multimeric forms were observed (Fig. 7C, last slot).

Cross-linking the released, purified protein (10S complex) with DMS (Fig. 5 and 7A) yielded only complexes A, B, and C. No higher multimers were found (Fig. 5), even when high concentrations of DMS (up to 100 mM) were used. Similar results were obtained when material that was salt released but not purified by gradient centrifugation was cross-linked.

When protein I was cross-linked before sucrose gradient centrifugation, a distinct peak with approximately the same sedimentation value as that in Fig. 7A was obtained (Fig. 7B). The cross-linked multimeric species A, B, and C could be found in this peak. When this sample was boiled before centrifugation, the profile shown in Fig. 7D was observed. When the gradient fractions were analyzed on 9% gels, it was seen that the monomer, complex A, and complex B sediment as separate species in the gradient. The slightly slower sedimentation of the boiled

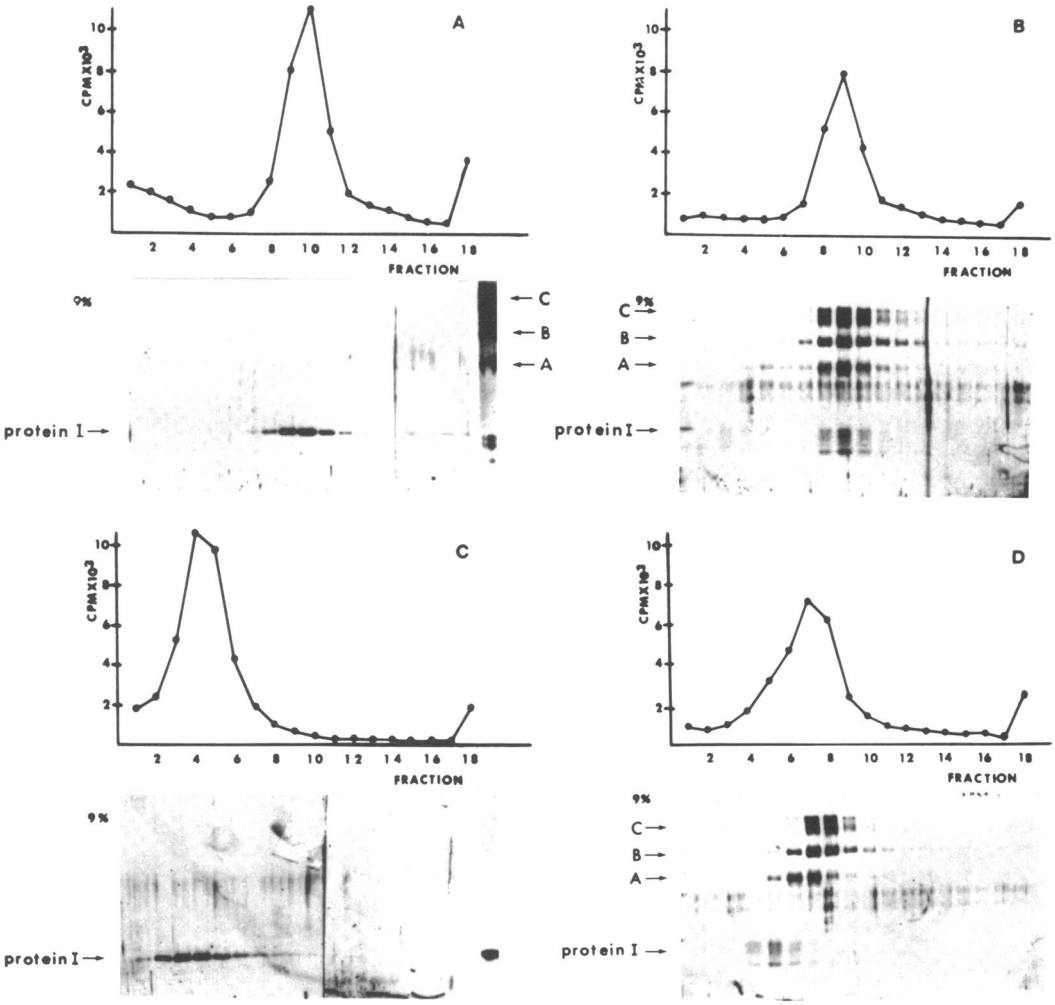

FIG. 7. *Sucrose gradient centrifugation of protein I complexes. Cells (E. coli B) were labeled with [^{35}S]methionine, the protein-peptidoglycan complex was isolated, and protein I was released as described in the text. Released protein I was applied to the sucrose gradients after treatment as follows: (A) applied directly, no further treatment; (B), cross-linked with DMS (50 mM); (C) boiled for 5 min; (D) cross-linked with DMS (50 mM) and then boiled for 5 min. A sample of each gradient fraction was analyzed by SDS-discontinuous gel electrophoresis. Autoradiograms of the gels are shown below each corresponding gradient profile. The last slots in A and C show the material from the peak which was cross-linked (50 mM DMS) after isolation on the sucrose gradient and then applied to the gel.*

material relative to that of the protein before boiling (cf. Fig. 7B and D) is probably because the boiled protein is in a more extended denatured form.

DISCUSSION

Our results clearly show that in both *E. coli* K-12 and B similar multimeric complexes of protein I can be found in cross-linked whole cells, isolated cell wall, protein-peptidoglycan complex, or purified protein I. The native arrangement of this protein seems to be conserved in all of these structures, even in the presence of SDS used for protein-peptidoglycan complex preparation. Multimers A and B most probably represent the dimer and trimer, respectively, of protein I. Our results are consistent with those obtained by Reithmeier and Bragg (22), who found dimers and trimers of protein I when cross-linking cell wall, isolated outer membrane, or protein-peptidoglycan complexes with a different type of cleavable reagent. Haller (Ph.D. thesis, University of Tübingen, Tübingen, Germany, 1975) also observed dimers and trimers of protein I when trypsin-treated ghosts were cross-linked with DMS. The yield of multimers B and C in these experiments is greater than we previously observed with TDGA (21), because of a more reactive batch of reagent.

The exact nature of multimer C, which is probably the same as the "tetramer" observed by Reithmeier and Bragg (22), is difficult to determine. The most likely explanations for its aberrant mobility in gels is either the presence of nonprotein moieties or an aberrant conformation that is preserved even when boiled in SDS. The fact that complex C appears during cross-linking of purified protein I as well as during cross-linking of whole cells speaks against the presence of nonprotein moieties. In addition, we found neither carbohydrate by the periodic-acid Schiff staining procedure, nor a different amount of ^{32}P cross-linked to C relative to A or B. These observations are consistent with absence of detectable substituent groups on purified, denatured protein I (23).

Perhaps the explanation is conformational. If the basic structure of protein I is a trimer, complex B might be a trimer cross-linked into a linear structure with normal migrational properties in SDS gels, and complex C might be a trimer cross-linked into a circular structure that cannot completely unfold.

During cross-linking of whole cells, cell wall, or protein I in association with the peptidoglycan, multimers of protein I are observed that are higher than trimer. These probably represent cross-linking between neighboring trimers that are very closely packed in the outer membrane (27). The high levels of trimers, hexamers, and nonamers relative to other multimers suggests that the basic unit is a trimer. The interaction between trimers would be abolished when the protein is released from peptidoglycan and is free in solution. Thus, a trimer is the highest multimer seen when the released protein is cross-linked.

The data presented here suggest that protein I exists in the outer membrane as a trimer. This is in agreement with electron microscopic data (27) that show a threefold symmetry in the basic unit of the protein I-peptidoglycan complex.

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