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NOTES

Nearest-Neighbor Analysis of *Escherichia coli* Outer Membrane Proteins, Using Cleavable Cross-Links

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

The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden

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A specific dimer of the 37,000-dalton, major outer membrane protein was demonstrated by chemical cross-linking with cleavable reagents.

Proteins in the outer membrane of *Escherichia coli* seem to form an interacting network (2, 4, 5). Henning and his co-workers have demonstrated the presence of all the major outer membrane proteins in one cross-linked complex after extensive treatment with high concentrations of dimethyl imidoester cross-linkers (3). This work indicates a high degree of protein-protein interaction but does not provide information concerning specific pairwise interactions.

We have studied the nearest-neighbor relationship of outer membrane proteins in *E. coli* K-12 strain Hfr G6 (6) by controlled cross-linking with two classes of cleavable reagents. We have used a series of reagents with different bridge lengths: first, the tartaric acid derivatives, in which the reactive groups are carboxylic acid azides (8), tartryl-diazide (TDA; 0.6 nm), tartryl-di-(glycylazide) (TDGA; 1.3 nm), and tartryl-di-(ε-amino-caproylazide) (TDCA; 2.3 nm); and second, the cleavable diimidoesters, bis-methyl-3,8-diaza-4,7-dioxo-5,6-dihydroxydecane-bisimidate (DEBE; 1.4 nm) and bis-methyl-4,9-diaza-5,8-dioxo-6,7-dihydroxydodecane-bisimidate (DOBE; 1.7 nm).

Cross-linking was done with whole cells grown to early logarithmic phase, harvested by low-speed centrifugation, and suspended in 1 M triethanolamine, pH 8.5, to a final concentration of 3×10^{10} cells/ml. Cross-linking was allowed to proceed for either 1 h at 4°C or 30 min at room temperature (21 to 23°C), with reagents at the concentrations indicated. Results were the same at either temperature. The reaction was terminated with excess methylamine, and the cells were centrifuged, suspended, and sonically disrupted. Outer membrane was isolated on a discontinuous sucrose gradient (10), and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

In one-dimensional gels, a new band of molecular weight 72,000 g/mol could be seen in samples cross-linked with a low level of TDGA (10 mM). The band grows more intense with increasing concentrations of cross-linkers (10 to 140 mM). The same complex is visible after treatment with TDCA (10 to 50 mM) and DEBE or DOBE (5 to 50 mM). In contrast, even with high concentrations (200 mM) of the short cross-linker TDA, no difference could be detected between control and cross-linked samples.

To identify the constituents of the complex, a symmetrical two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (8) was used. Proteins are first subjected to electrophoresis in a cylindrical gel, which is incubated in a sodium meta-periodate solution to cleave the cross-link before running the second dimension. Unmodified proteins migrate the same distance in both dimensions and thus lie on a diagonal. Proteins from a complex cleaved between the two electrophoretic steps migrate further in the second dimension relative to the first. Thus, they appear below the diagonal on the same vertical line. The constituents are identified by horizontal alignment with spots on the diagonal.

Figure 1 shows that no off-diagonal spots were detected in cleaved, control samples (Fig. 1A and B), as was also the case with uncleaved, cross-linked samples (not shown). In contrast, cleaved, cross-linked samples (Fig. 1C and D) contain a prominent off-diagonal spot arising from the 72,000-dalton complex. This was apparently the only cleavage product from this complex, since no other spots were detected on the same vertical line. Comparison with the diagonal allows identification of this spot as the 37,000-dalton major protein (Fig. 1, arrow) (protein 1 [11] or protein I [2]). We conclude that the complex represents a dimer of this protein. Two weaker spots of the 37,000-dalton protein were also seen originating from complexes of higher

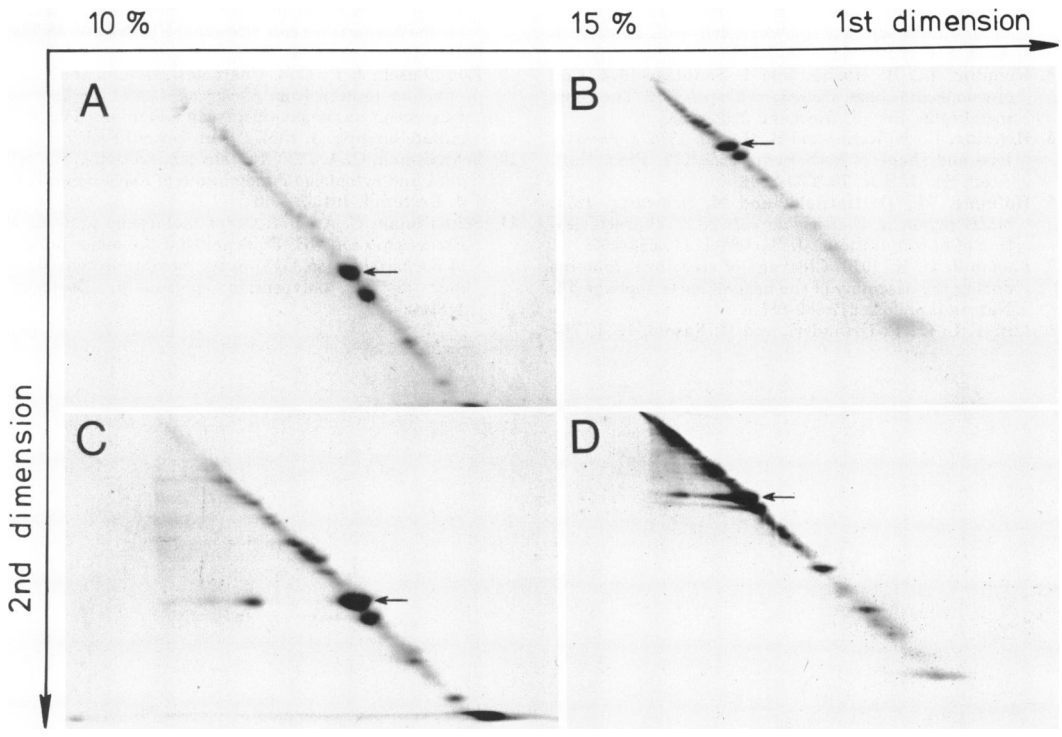

FIG. 1. *Symmetrical two-dimensional sodium dodecyl sulfate (SDS)-gel electrophoresis in 10 and 15% acrylamide gels of a control (A and B) and a cross-linked (140 mM TDGA) sample (C and D). All the samples were cleaved after running the first dimension in a cylindrical gel, using 15 mM $NaIO_4$ in 20 mM sodium phosphate buffer (pH 6.0) and 0.1% SDS. The incubation was for 1 h at room temperature. The discontinuous SDS-gel system used was essentially that described by Laemmli (7), with the following modifications. The separation gel had a pH of 8.9. The stacking gel (pH 7.8) was 5% acrylamide, 62.5 mM tris(hydroxymethyl)aminomethane (Tris), 56 mM sodium phosphate, and 0.1% SDS. The electrode buffer (pH 8.6) contained 29 mM Tris, 220 mM glycine, and 0.1% SDS. The samples (pH 7.8) contained 1% SDS, 1% β-mercaptoethanol, 20 mM Tris, 8 mM sodium phosphate, and 9% glycerol. Phenol red was used as the tracking dye. Proteins were solubilized by incubating for 5 min at 100°C. The staining of the gels was done according to Fairbanks et al. (1).*

molecular weight. No other components were detected, and, therefore, based on molecular weight calculations, we conclude that these spots represent trimer and tetramer of this same protein.

With the highest concentration (140 mM) of TDGA used, a very weak off-diagonal spot corresponding to the 33,000-dalton major protein (protein 3 [11], or protein II* [2]) was also detected. The calculated molecular weight (45,000) of this complex makes it unlikely that it is a dimer. No other spot was visible in the low-molecular-weight region, but it may be so diffuse that it could not be detected under these conditions.

Rosenbusch (9) showed that the 36,500-dalton major protein in *E. coli* B (our 37,000-dalton protein) forms a hexagonal array over the peptidoglycan. Calculation of the size of morphological subunits seen with the electron microscope suggest that they are composed of two polypeptides. Our results provide chemical evidence that, in fact, there is a specific dimer of this protein in *E. coli* outer membrane.

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