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Amino Terminus of Outer Membrane PhoE Protein: Localization by Use of a *bla-phoE* Hybrid Gene

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Expression of a recently constructed *bla-phoE* hybrid gene results in synthesis and incorporation into the outer membrane of PhoE protein containing an amino-terminal extension of 158 amino acid residues of β -lactamase (Tommasen et al., EMBO J. 2:1275-1279, 1983). As the PhoE protein part of this hybrid protein is apparently normally incorporated into the outer membrane, the β -lactamase part of the protein can be considered as a label of the amino terminus of PhoE protein. By using trypsin accessibility experiments, this β -lactamase part was shown to be located at the periplasmic side of the membrane. Therefore, the amino terminus of PhoE protein most likely faces the periplasm.

PhoE protein (16) of *Escherichia coli* K-12 is an outer membrane pore protein (6) which can be induced by growing cells under phosphate limitation (8, 15). Like other outer membrane proteins, it has to be transported from the cytoplasm to the outer membrane. Except for the role of the signal sequence, little is known concerning this transport process. Several models for this process have been proposed, some of which result in a topology of the proteins with the carboxy terminus at the inside surface and the amino terminus at the outside surface of the membrane (see, e.g., reference 2), whereas others result in the reverse topology (see, e.g., reference 14). Thus, studies on the topology of these membrane proteins are important for understanding the transport process.

We have recently constructed a *bla-phoE* hybrid gene, encoding the signal sequence of β -lactamase, about two-thirds of the structural sequence of β -lactamase (i.e., 158 amino acid residues), as well as the complete structural sequence of PhoE protein (17). Expression of this gene, located on plasmid pJP43, results in the synthesis of a hybrid protein with an apparent molecular weight of 60,000 (Fig. 1, lane b), which reacts with both anti- β -lactamase serum and anti-PhoE protein serum. The PhoE protein part of this hybrid protein is normally incorporated in the outer membrane, since the protein is peptidoglycan associated and since it can serve as the receptor for the PhoE protein-specific phage TC45 (17). Therefore, the β -lactamase part of the protein can be used as a label for the localization of the amino terminus of PhoE protein.

To localize the β -lactamase part of the hybrid protein, trypsin accessibility experiments (3) were performed. Cells of strain CE1222 (F^- *thr leu del proA-phoE-gpt thi argE lacY galK xyl rpsL phoS21 recA ompR*) containing pJP43 (17) were harvested from 10-ml portions of an overnight culture, washed with either 10 mM Tris-hydrochloride-10 mM $MgCl_2$ (pH 8.0) or 10 mM Tris-hydrochloride-5 mM EDTA (pH 8.0), and resuspended in 10 ml of the same solutions containing 50 μ g of trypsin per ml. Like the other pore proteins, OmpC protein and OmpF protein (4), PhoE protein, present in cell envelopes, is resistant to trypsin treatment in the presence of Mg^{2+} as well as in the presence of EDTA (results not shown). As the presence of Mg^{2+} strongly inhibits the penetration of trypsin into the periplasmic space

(3), the hybrid protein would be degraded under these conditions if the β -lactamase portion were extending into the medium. Should the hybrid protein be degraded only in the presence of EDTA, which allows proteolytic attack also from the periplasmic side, the β -lactamase portion would face the periplasm. After incubation with trypsin for 30 min at 0°C, the cells were washed twice and resuspended in 10 ml of 10 mM Tris-hydrochloride (pH 8.0), and 200 μ l of a solution of 0.1 M diisopropyl fluorophosphate in isopropanol was added to abolish residual trypsin activity. Subsequently, cell envelopes were isolated and analyzed on sodium dodecyl sulfate-polyacrylamide gels, as described previously (5). The results (Fig. 1) show that in the presence of EDTA (lane d), the hybrid protein band has completely disappeared and a new band with an apparent molecular weight of 42,000 has appeared which could well represent the main degradation product, consisting of the complete PhoE protein with an apparent molecular weight of 40,000 and a small oligopeptide of β -lactamase (see below). Also, OmpA protein is degraded under these conditions, and a band with an apparent molecular weight of 24,000 appears (Fig. 1, lane d). This band represents the amino-terminal fragment of OmpA protein, which is known to be protected against trypsin (12). In the presence of Mg^{2+} (Fig. 1, lane c), OmpA protein is rather well protected. Thus, the fragment of OmpA protein which is sensitive to proteolytic attack extends in the periplasm, confirming earlier observations (11). Also, the material at the electrophoretic position of the hybrid protein is protected by the presence of Mg^{2+} , and no trace of a band with an apparent molecular weight of 42,000 could be detected (Fig. 1, lane c).

To discriminate between two possible explanations for sensitivity of the hybrid protein to trypsin, namely, (i) that EDTA exposes regions of the hybrid protein normally embedded in the membrane and (ii) that EDTA makes the outer membrane permeable for trypsin, cell envelopes of Mg^{2+} -trypsin-treated cells were prepared in the absence of diisopropyl fluorophosphate, which allows activity of the residual trypsin. Both OmpA protein and the 60,000-molecular-weight hybrid protein were found to be degraded. This result shows that EDTA actually acts by making the outer membrane leaky, allowing the conclusion that the hybrid protein can only be degraded by trypsin from the periplasmic side of the outer membrane.

For a more detailed analysis of the fate of the 60,000-

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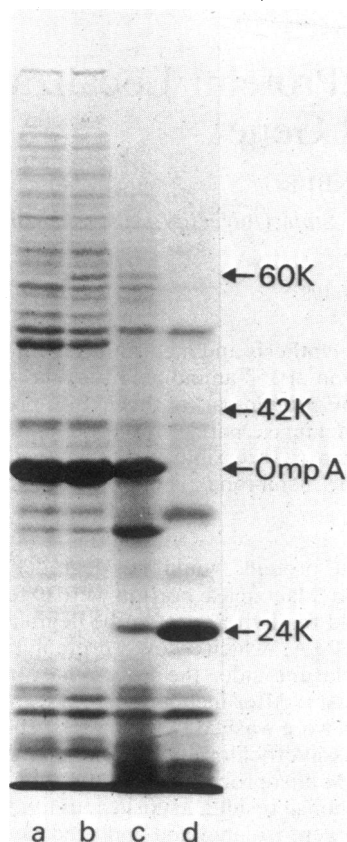


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of the cell envelope proteins of strain CE1222 (a), a pJP43-containing derivative of this strain (b), and pJP43-containing cells treated with trypsin in the presence of Mg^{2+} (c) or EDTA (d). The positions of the β -lactamase-PhoE hybrid protein and the trypsin-resistant fragment of OmpA protein are indicated by their apparent molecular weights (60,000 and 24,000, respectively).

molecular-weight hybrid protein and of the nature of its degradation products, the gel-immuno-radio-assay technique (10) was applied. Figure 2A shows the results of the reaction of a longitudinal gel slice with anti- β -lactamase serum. Comparison of the hybrid protein band before (lane b) and after (lane c) trypsin treatment in the presence of Mg^{2+} shows that trypsin hardly affects the hybrid protein. On the other hand, after trypsin treatment in the presence of EDTA (lane d), no bands are observed reacting with anti- β -lactamase serum. Also, the results with anti-PhoE protein serum (Fig. 2B) demonstrate that the hybrid protein is rather well protected against degradation by trypsin in the presence of Mg^{2+} (lane c), whereas after treatment of the cells with trypsin in the presence of EDTA, no material could be detected in the 60,000 position (lane d). However, in the latter case a reaction was observed in the position of 42,000, showing that this band is the main degradation product of the 60,000-molecular-weight hybrid protein and probably consists of the complete PhoE protein and a small oligopeptide of β -lactamase, the latter being too small to be detected by anti- β -lactamase serum.

As it was already concluded that the hybrid protein can only be degraded by trypsin from the periplasmic side and as degradation of most of the β -lactamase part of the hybrid protein apparently exists, the results are best interpreted by assuming that the complete β -lactamase part of the hybrid

protein is exposed to the periplasmic side of the outer membrane and thus that the amino terminus of the PhoE protein part of the hybrid molecule faces the periplasm. However, an alternative possibility should be considered, i.e., the first residue of the PhoE protein part could be on the outside surface of the cell, whereas the β -lactamase part folds back to span the bilayer such that the bulk of the β -lactamase part is present in the periplasm. In this case, the β -lactamase part on the outside would be very small, since the first trypsin-sensitive site is at amino acid residue 6 before the fusion site (corresponding to the arginine at residue 176 in pro- β -lactamase in reference 13). This possibility therefore implies that the region around this arginine residue traverses the membrane. However, as the β -lactamase part before the fusion site is extremely rich in charged amino acid residues (see reference 13), this alternative is highly unlikely.

Thus, whereas the first residue of the PhoE protein part of the hybrid molecule apparently faces the periplasm, the question remains whether this is also the case for the native PhoE protein. This extrapolation can only be made if PhoE protein is inserted in the outer membrane in the same orientation in both its native state and in the hybrid form. As the hybrid protein, like the native PhoE protein, is peptidoglycan associated and also serves as the receptor for phage TC45 (17), it seems likely that at least the major part of the protein is folded correctly. However, it does not guarantee that the extreme amino terminus is in its proper place. Therefore, an alternative should be considered, namely, that the bulk of the PhoE protein part is in its proper functional location, but that, due to the β -lactamase extension, this is not the case with the amino-terminal fragment. In that case, one membrane-spanning fragment of PhoE protein would not traverse the membrane but would extend in the periplasm in the case of the hybrid protein. However, as amino acid residue number 6 of PhoE protein is lysine (7), the main degradation product of the hybrid protein in the EDTA-trypsin-treated cells would be a fragment slightly smaller than the native PhoE protein, instead of the observed 42,000-

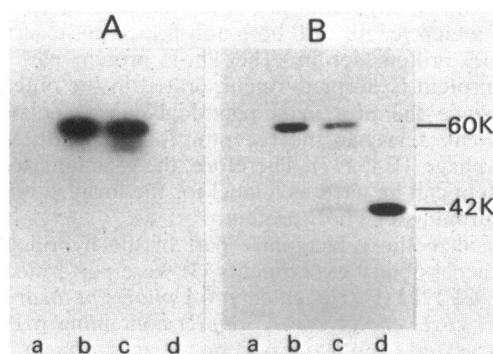


FIG. 2. Gel-immuno-radio-assays on longitudinal gel slices of gels containing the cell envelope proteins of strain CE1222 (a), a pJP43-containing derivative of CE1222 (b), and pJP43-containing cells treated with trypsin in the presence of Mg^{2+} (c) or EDTA (d). Gel slices were incubated with anti- β -lactamase serum (A) or anti-PhoE protein serum (B). Before use, the anti-PhoE protein serum was preabsorbed with peptidoglycan-lipoprotein complexes (1) to remove antilipoprotein activity from the antiserum (9). After incubation with the antisera, the gel slices were incubated with ^{125}I -labeled protein A and autoradiographed. The position of the β -lactamase-PhoE hybrid protein is indicated by its apparent molecular weight (60,000).

molecular-weight fragment, which is slightly larger. From these considerations, it seems likely that the extreme amino terminus of PhoE protein in the hybrid molecule is located at the correct side of the membrane, i.e., at the periplasmic side.

PhoE protein is the first outer membrane protein for which the location of the amino terminus has been studied. As the amino terminus apparently faces the periplasm, the topology of PhoE protein in the outer membrane seems not to be consistent with models for the assembly of outer membrane proteins, as described by Halegoua and Inouye (2), predicting a topology with the amino terminus at the outside surface.

The carboxy terminus has been localized for another outer membrane protein, i.e., OmpA protein. The amino terminal portion of OmpA protein is protected against trypsin treatment of cells or cell envelopes (12). As shown in Fig. 1, the carboxy-terminal fragment is protected against trypsin treatment of whole cells by Mg^{2+} ; thus, this fragment extends into the periplasm. This observation is consistent with earlier experiments of Reithmeier and Bragg (11). Therefore, if the peptide chains of PhoE protein and OmpA protein traverse the outer membrane only once, these proteins follow different pathways of assembly into this membrane. On the other hand, however, these proteins might traverse the membrane more than once, resulting in a topology with both the carboxy terminus and the amino terminus at the periplasmic side.

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