Internal Deletions in the Gene for an *Escherichia coli* Outer Membrane Protein Define an Area Possibly Important for Recognition of the Outer Membrane by This Polypeptide*

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A series of overlapping deletions has been constructed in the *ompA* gene which encodes the 325-residue *Escherichia coli* outer membrane protein OmpA. Immunoelectron microscopy showed that the OmpA fragments were either located in the periplasmic space or were associated with the outer membrane. Apparently an area between residues 154 and 180 is required for this association; all proteins missing this area were found to be periplasmic. The nature of this association remained unknown; no membrane-protected tryptic fragments could be identified for any of these polypeptides. Hybrid genes were constructed encoding parts of the periplasmic maltose binding protein and an area of the *ompA* gene coding for residues 154–274. The corresponding proteins were not localized to the outer membrane but remained attached to the outer face of the plasma membrane, possibly because the normal mechanism of release from this membrane was impaired. In the OmpA protein the conspicuous sequence Ala<sup>153</sup>-Pro-Ala-Pro-Ala-Pro-Ala-Pro<sup>157</sup> exists. Frameshift mutants were constructed to eliminate this sequence. There was no effect on the incorporation of the mutant proteins into the outer membrane. Thus, this “hinge” region is not involved in sorting. A proposal suggesting the existence of a sorting signal common to several outer membrane proteins (Benson, S. A., Bremer, E., and Silhavy, T. J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 3830–3834) was subsequently rejected (Bosch, D., Leunissen, J., Verbakel, J., de Jong, M., van Erp, H., and Tommassen, J. (1986) *J. Mol. Biol.* 189, 449–455; Freudl, R., Schwarz, H., Klose, M., Movva, N. R., and Henning, U. (1985) *EMBO J.* 4, 3593–3598). Although it is not known whether or not the outer membrane association observed represents a step in the normal sorting mechanism, it is concluded that it remains an open question whether or not a sorting signal, as proposed originally, exists in outer membrane proteins.

Polypeptides to be translocated across the *Escherichia coli* plasma membrane are usually synthesized as precursors with an NH<sub>2</sub>-terminal signal sequence. The signal is removed, and the proteins are localized to the periplasmic space or to the outer membrane or, in some cases, to the external medium. It has been shown that directly after processing the periplasmic β-lactamase exists in a form which is still bound to the plasma membrane and which differs in conformation from the periplasmic species of the protein (1, 2). Localization, therefore, of this protein and probably others is most likely achieved by folding of the polypeptide chain to create a water-soluble molecule.

It is not at all clear, however, how sorting to the outer membrane occurs. Results of studies with the outer membrane protein LamB have suggested that a sorting signal, consisting of a relatively short stretch of amino acid residues near the NH<sub>2</sub> terminus, exists within the mature form of this maltoporin (3). On the other hand, results of an investigation employing the outer membrane protein PhoE have indicated that such a sorting signal does not exist in this protein (4, 5).

We have studied this question with the outer membrane protein OmpA. Again, very little is known concerning sorting of this protein. We have shown that it, like the β-lactamase, undergoes a conformational change during export, but it is unknown whether this change is causally related to the protein’s ability to “find” the outer membrane (6). We have also previously shown that the overall structure of this protein is not required for its localization to this membrane (7). In this communication we demonstrate that a small area of the polypeptide is able to associate internally deleted or COOH-terminally truncated OmpA proteins with the outer membrane.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**

Strain UH203 (lac<sup>supF</sup> recA ompA<sup>proA</sup> or B rpsL<sup>F</sup> lacI<sup>8</sup> lacZ<sup>Δ M15 proAB<sup>+</sup></sup> (8)) and its ompA<sup>+</sup> derivative were used. Strain BW212 (dut ung thi relA spot7<sup>F</sup> lysA (9)) was used for preparing uracil containing phage DNA. Cells were grown at 37 °C in L-broth (10) containing glucose (0.5%) or IPTG<sup>2</sup> (1 mM) and ampicillin (40 μg/ml). For induction of the *ompA*-medF hybrid genes were grown in L-broth containing 0.5% maltose.

**Construction of Deletions**

The construction of these plasmids is illustrated in Fig. 1. *ompAΔ66-153*—This gene was obtained from plasmid pTU500 (7) into which a 10-mer EcoRI linker was inserted between the codons for residues 153 and 154 of the OmpA protein (11). The plasmid was digested with HpaI and EcoRI, the latter site was made blunt-ended by treatment with the Klenow fragment of DNA polymerase I, and the DNA was religated.

*ompAΔ161-227*—Plasmid pR87<sup>2</sup> was restricted with BamHI,
and the ends were made blunt-ended as above. The DNA was then cut with SphI and treated with S1 nuclease and religated.

The construction of the frameshifts is shown in Fig. 2. For fs(+1), the appropriate mismatch oligonucleotide plus a universal 17-mer sequencing primer (13) were annealed to DNA of phage M13mp19 (14), carrying the ompA gene encoding the signal sequence and the mature protein up to residue 227 (a HindIII-BamHI fragment). According to Kunkel's method (15) to enrich for mutant sequences, the hybrid genes are shown schematically in Fig. 3. Plasmid pTU 500 carrying the oligonucleotide mentioned above, the EcoRI-HindIII fragment (Fig. 1) was isolated and inserted into pIN ompA1.

Construction of Frameshifts

The construction of these hybrids is illustrated in Fig. 3. Plasmid pPD1, carrying the malE gene (17), was treated as shown in the figure. The ompA fragment was isolated from a plasmid pTU500 into which an oligonucleotide was inserted between the codons for residues 153 and 154 of the OmpA protein (18); the Smal site is located in this insert. Part of the malE gene was deleted from the malE-ompA hybrid as outlined in the lower part of Fig. 3. In detail, all DNA manipulations were performed essentially as described by Maniatis et al. (19).

Immunelectron Microscopy

Cells pregrown in the presence of glucose were grown in the presence of IPTG for 2 h and processed as described previously (6, 7). In brief, for better differentiation of inner and outer membranes cells were plasmolyzed with 25% sucrose fixed with 2% formaldehyde and 0.05% glutaraldehyde, and embedded in Lowicryl K4M or HM20 at low temperature (20). Ultrathin sections were labeled with rabbit anti-OmpA serum and protein A-gold complexes. Colloidal gold par-


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RESULTS

Relevant Properties of the System—All plasmid-borne ompA genes are under the control of the lac regulatory elements (7). On pTU 500, the gene carries a TAG stop codon corresponding to position 7 of the protein, and in the presence of the suppressor supF wild type amounts of the protein are produced (25, 26). Deletions removing the stop codon lead to overproduction of the corresponding proteins. The ompA gene present on plasmid pHDS7 does not harbor this stop codon, and products from this gene or derivatives thereof are overproduced. The 325-residue OmpA protein (27) consists of a membrane part and a periplasmic part encompassing residues 1-177 and 178-325, respectively. Proteases acting on cell envelopes or on cells permeabilized with sucrose and EDTA but not on intact cells degrade the periplasmic moiety and leave an intact membrane-protected fragment (27 cf. Fig. 6). The wild type protein in the outer membrane exhibits the phenomenon of “heat modifiability” (23-30 cf. Fig. 6). When solubilized with SDS at 50 °C, it migrates on SDS-polyacrylamide gels as a 31-kDa species; when boiled in SDS, it migrates as a 36-kDa species. The completely unfolded 36-kDa protein can be renatured to yield the 31-kDa polypeptide by the addition of lipopolysaccharide (24).

Deletions in the ompA Gene and Their Products—The structures of the partially deleted genes, including those described earlier (7, 31), are shown in Fig. 4. The products of the genes not previously described are shown in Fig. 5. Overproduction of the OmpA protein or its fragments in all cases studied so far is toxic. The corresponding cells lyse either rather rapidly or after several hours following the addition of IPTG to liquid medium. Expression of the genes causing overproduction of the proteins shown in Fig. 4A was more toxic than most of those shown in Fig. 4B. In general, the latter strains grew from an inoculum of about 2 × 10^6/ml to 1-2 × 10^9/ml and then began to lyse, while the former started lysing at a density of 2-4 × 10^8/ml. It is not possible, however, to draw any conclusions from this difference because expression of the ompAΔ160-227 gene (Fig. 4B) also led to early lysis. As we noted with another toxic construction (31), a chromosomally ompA strain (UH203) carrying any of the “toxic plasmids” grew on solid medium containing IPTG. It was thus possible to test if the internally deleted OmpA proteins conferred sensitivity to OmpA-specific phages (32). None of them did. Also, none of them exhibited the heat modifiability typical for the wild type protein. Since this could have been an indication that the proteins had never come into contact with lipopolysaccharide, we tried to bring one protein, OmpAA4-45, in vitro into the heat-modifiable conformation. Cell envelopes of the strain possessing this protein were combined with an excess of pure lipopolysaccharide. The electrophoretic mobility of the polypeptide thus treated was, however, the same whether the preparations were solubilized at room temperature, 30, 40, 50, or 100 °C. This protein, therefore, appears to be unable to assume a conformation resembling that of the
wild type.

Proteases added to cells in the presence of sucrose and EDTA can penetrate the outer membrane but have no access to the cytosol (33). Cells plasmolyzed this way and harboring the OmpAΔ4-45, Δ43-84, Δ43-153, Δ2-153, Δ2-153/231-325, Δ46-226, Δ84-226, Δ161-226, and Δ161-325 proteins were treated with trypsin. Without exception, these polypeptides were degraded to such a degree that no fragment could be detected electrophoretically, i.e. no area of about 4 kDa or larger size was protected by the outer membrane.

It could also be shown for the OmpAΔ43-84 protein that a region of the polypeptide which is known to be exposed at the cell surface with the wild type protein is not exposed (at least in detectable levels) in the mutant polypeptide. For constructing some of the deletions we had inserted into the wild type gene a 75-bp polylinker between the codons for residues 153 and 154 (18). As expected from previous results (11), the protein possessing the additional residues could be cleaved by trypsin added to intact cells, yielding two fragments of the expected size (data not shown). The same polylinker was inserted into the ompAΔ43-84 gene. The corresponding protein could not be cleaved by trypsin acting on intact cells.

Frameshift Mutants—The OmpA protein possesses an unusual repeat of alanine and proline residues (Fig. 4) at a site where the polypeptide leaves the membrane and becomes periplasmic. The shortest COOH-terminally truncated protein which we have described and which is normally incorporated into the outer membrane is OmpAΔ194-325 (25). As this protein still carries the Ala-Pro repeat sequence, we have asked if the Ala-Pro repeat may play a role in sorting, e.g. as a nucleation site for folding of the membrane part of the protein. To this end, the OmpA mutants 180 fs(+1) and 180 fs(+1)/190 fs(−1) were constructed (Fig. 2). Both corresponding proteins are shown in Fig. 6. They both conferred sensitivity to OmpA-specific phages, exhibited heat modifiability (Fig. 6), and cells producing these proteins grew at wild type rates (note that these proteins are not overproduced). Also, treatment of cell envelopes with Pronase yielded, in both cases, a membrane-protected fragment of the same size as that derived from wild type OmpA (Fig. 6). Clearly, both mutants were normally assembled into the outer membrane, and the loss of the Ala-Pro repeat has, under laboratory conditions, no phenotype. The only noticeable difference from the wild type protein was the magnitude of the heat modification effect. Although the mutant polypeptide 180 fs(+1)/190 fs(−1) behaved in the same way as the wild type polypeptide, there was only a very small difference in electrophoretic mobility of the 180 fs(+1) polypeptide following solubilization at the two different temperatures (Fig. 6). This was clearly related to loss of the periplasmic moiety rather than to the mutation.

Location of Proteins—The results presented so far strongly suggested that none of the partially deleted OmpA proteins is assembled into the outer membrane. Immunoelectron microscopy, however, revealed that these mutant polypeptides fell into two classes. Those of one class, labeled A in Fig. 4, were found to be associated with the outer membrane and those of another, labeled B in Fig. 4, were located in the periplasm (Fig. 7). Even the small 76-residue fragment, encoded by the ompAΔ2-153/230-325 gene, was associated with the outer membrane. It should be noted that the population of cells harboring this fragment was somewhat heterogeneous; in wild type.

FIG. 6. Heat modifiability and Pronase sensitivity of frameshift mutants proteins. A, heat modifiability. Cell envelopes of strain UH203 were used carrying pTU500 (wild type OmpA protein, lanes 1 and 2); lanes 3 and 4, this plasmid with ompA 180 fs(+1)/190 fs(−1), or lanes 5 and 6, with ompA 180 fs(+1). Envelopes were heated in sample buffer either at 50 °C for 15 min (lanes 1, 3, and 5) or at 100 °C for 5 min (lanes 2, 4, and 6). Pulse-chase experiments (not shown) revealed that the OmpA 180 fs(+1) protein (open arrowhead) is degraded to a polypeptide indicated by the closed arrowhead. Numbers on the left margin, molecular weights in kDa; 36 and 31 indicate the different positions of the OmpA protein. B, Pronase sensitivity. The same strains as in A were used. Lanes 1 and 4, pTU500; lanes 2 and 5, ompA 180 fs(+1)/190 fs(−1); lanes 3 and 6, ompA 180 fs(+1). Dots, products of the ompA 180 fs(+1) gene; square, Pronase fragment. In all cases cells were grown for 3 h in the presence of IPTG. A stained 12.5% gel is shown. Lanes 1–3, controls; lanes 4–6, Pronase-treated.

FIG. 7. Localization of ompA gene products by immunoelectron microscopy. A, chromosomally ompA wild type; B and C, strain UH203 harboring the plasmid with ompAΔ. 161-277 grown in the presence of glucose or IPTG, respectively; D, the same strain induced for the expression of the plasmid-borne ompAΔ2-153/230-325 gene. A–C, Lowicryl HM20 sections; D, Lowicryl K4M section, all labeled with 15-nm gold beads.
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The data presented in this communication show that the area between residues 154 and 180 is able to associate various shortened OmpA proteins with the outer membrane. Even a small fragment (OmpAA2-153/231-325 possessing this area) consisting of only 76 residues plus 17 non-OmpA residues was located at the outer membrane. All proteins missing this area were found to be periplasmic. The interpretation of the latter results is, however, very problematic. While one would not expect the OmpAA2-229 protein to have any affinity for the outer membrane, all others could possess such an affinity. They may become insoluble too soon after translocation across the plasma membrane to become attached to the outer membrane. Also, one does not know in which state the proteins are which are visualized by the gold beads. It could be, for example, that these abnormal proteins fold in a way which masks a site(s) required for recognition of the membrane.

The solubility problem is best illustrated by the OmpA-maltose-binding protein hybrids. The OmpA fragment, encompassing residues 154-274, could not direct the hybrids to the outer membrane. They were always found in clumps attached to the outer face of the plasma membrane. Unexpectedly, expression of these hybrid genes was toxic; cells began to form filaments and finally lysed. This suggests that the normal mechanism of release from the plasma membrane was impaired (analogous to certain mutants of the periplasmic β-lactamase (2, 34, 35)), a situation possibly somehow interfering with cell division. It should also be mentioned that none of the shortened OmpA proteins shown in Fig. 1 are water-soluble, a property one would not expect from the OmpAA1-229 polypeptide. Despite these uncertainties concerning an interpretation of the cause for periplasmic location, it should be noted that the behavior regarding localization of all polypeptides is entirely consistent with the assumption that the area between residues 154 and 180 is required for an association with the outer membrane; all others could possess such an affinity.

From earlier studies we know that the OmpAA195-325 protein was and the OmpAA161-325 protein was not incorporated into the outer membrane (25). The area in between residues 160 and 194 contains the conspicuous Ala-Pro repeat (Fig. 3). We considered the possibility that this region could be important for the recognition of the membrane. The frameshift mutants demonstrate that this is not so. The Ala-Pro repeat is completely conserved in the OmpA proteins of five different Enterobacteria (36). The frameshift mutants have no phenotype, and it remains mysterious what the role of this area may be. Its function is likely to be related to that of the periplasmic moiety, for which no function is yet known.

The frameshift mutant proteins as well as the OmpAA195-325 protein are apparently correctly assembled into the outer membrane; cells possessing these proteins grew normally, are sensitive to OmpA-specific phages, and the polypeptides are heat-modifiable. The other internally deleted proteins are associated with this membrane in a way which is not yet understood. They are not heat-modifiable and are completely degraded by trypsin in permeabilized cells, as far as can be judged by SDS-polyacrylamide gel electrophoresis. Even with the OmpAA4-45 protein, no protected fragments could be detected, i.e. the protein somehow sticks to the membrane but cannot form stable loops within it, as the wild type does, although it misses only those residues which form the first NH₂-terminal loop (37, 38). The conformation of the protein

![Fig. 8. Structures of hybrid genes. Top, the complete malE gene with the inserted ompA fragment; bottom, hybrid with the partially deleted malE gene. Numbers indicate the amino acid positions in the respective proteins; positions in the OmpA protein are underlined. Black areas indicate areas coding for additional residues (cf. 21 bp and 15 bp in Fig. 3). P, the malB promoter.](image)

![Fig. 9. Localization of the maltose-binding protein-OmpA hybrid. Strain UH203 carrying the plasmid with the malE-ompA hybrid gene was grown for 2 h in the presence of 0.5% maltose. The Lowicryl HM20 section was labeled with 15-nm gold beads (anti-OmpA serum was used).](image)
before it is inserted into the membrane (the imp-OmpA protein) differs from that of the membrane form (6). Thus, it most likely “folds into” the membrane to form an amphipathic β-barrel (38). It is therefore not surprising that the OmpAΔ4-45 cannot properly incorporate into the outer membrane as in the absence of the first loop such a β-barrel could not be established.

Computer analyses have shown that areas homologous to each other exist in several outer membrane proteins (39). Among these is the OmpA sequence between residues 159 and 171. We had earlier dismissed this area as a common sorting signal (7), because it had been reported that this sequence in the LamB protein is not its sorting signal (3). Such a sequence is also present in the outer membrane protein PhoE (39, 40), and from an analysis of internally deleted PhoE proteins, which were all localized to the periplasmic space, it was concluded that no sorting signal exists in this protein (5). However, the situation with the LamB and PhoE proteins may be more complex than with the OmpA protein because the former two exist as trimers in the outer membrane (for review see Ref. 41) whereas no evidence for any defined oligomer of the latter exists. Trimerization may in fact be required for the assembly of these pore-forming proteins into the membrane (42), and none of the internally deleted PhoE proteins may be able to trimerize. Also, concerning the periplasmic location of abnormal proteins the same problems of interpretation exist as those discussed above for our proteins. We believe that it remains an open question whether or not common sorting signals exist in a number of outer membrane proteins.

The results reported cannot answer the crucial question of whether or not the association of the partially deleted proteins with the outer membrane represents a step in the physiological sorting mechanism. This association does not appear to be caused by some nonspecific “membrane stickiness”; except for the maltose-binding protein-OmpA hybrids none of the proteins were attached to the plasma membrane. It cannot be excluded, however, that the localization of some of the internal deletion mutants of OmpA to the outer membrane is the result of a fortuitous interaction of these polypeptides with another protein of the outer membrane. If this were so, our data demonstrate how careful one has to be in drawing any conclusions concerning the location of abnormal proteins even by using immunoelectron microscopy, and our previous interpretation of such data concerning sorting may be incorrect (7). However, evidence is presented in the accompanying paper (43) showing that the area in question is important for incorporation of the OmpA protein into the outer membrane.

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