JOURNAL OF BACTERIOLOGY, Aug. 1992, p. 5485–5487
0021-9193/92/165485-03$02.00/0

Vol. 174, No. 16

Membrane Topology of the *Escherichia coli* ExbD Protein

KARLHEINZ KAMPFENKEL AND VOLKMAR BRAUN*

Mikrobiologie II, Universität Tübingen, 7400 Tübingen, Germany

Received 13 April 1992/Accepted 12 June 1992

The ExbD protein is involved in the energy-coupled transport of ferric siderophores, vitamin B_{12}, and B-group colicins across the outer membrane of *Escherichia coli*. In order to study ExbD membrane topology, ExbD–β-lactamase fusion proteins were constructed. Cells expressing β-lactamase fusions to residues 53, 57, 70, 76, 78, 80, 92, 121, and 134 of ExbD displayed high levels of ampicillin resistance, whereas fusions to residues 9 and 19 conferred no ampicillin resistance. It is concluded that the only hydrophobic segment of ExbD, encompassing residues 23 to 43, forms a transmembrane domain and that residues 1 to 22 are located in the cytoplasm and residues 44 to 141 are located in the periplasm.

Hydrophilic substrates with molecular masses of less than 700 Da diffuse through pores in the outer membrane formed by the porin proteins (18). In contrast, the uptake of larger molecules, such as ferric siderophores, vitamin B_{12}, and B-group colicins, and infection by phages T1 and φ80 require energy, receptor proteins, and the TonB, ExbB, and ExbD proteins (2). Evidence for an interaction between these components has been obtained by the suppression of point mutations in the TonB box of receptors (12, 14, 17, 24) and that of colicin B (16) by point mutations in TonB and by the stabilization of TonB by FhuA (3, 9) and ExbB (7, 25) and that of ExbD by ExbB (7). On the basis of these data, a protein complex composed of TonB, ExbB, and ExbD was proposed. In the energized conformation, this complex induces the release of receptor-bound substrates into the periplasm (2, 3, 7). This model is an extension of the previously suggested mode of action of TonB (10, 21). Variants of this model have been discussed by several authors (11, 14, 19).

A crucial point for the refinement of the model is the transmembrane arrangement of the components. It has been shown that the TonB protein is anchored in the cytoplasmic membrane by the N-terminal domain and that the remainder of the protein is located in the periplasm (1, 11, 22). The ExbB and ExbD proteins were found predominantly in the cytoplasmic membrane fraction (6), but no detailed analysis of the arrangement of these proteins has been performed.

With the ultimate aim of arriving at a molecular model of the presumed TonB-ExbB-ExbD energy transduction complex, we studied the membrane topology of the ExbD protein. For this purpose, we constructed fusions between the TEM β-lactamase and the ExbD protein. Since only periplasmic β-lactamase confers resistance to ampicillin, it is inferred that fusions which render cells ampicillin resistant contain β-lactamase in the segments of the ExbD protein which are exposed to the periplasm. The method of using a β-lactamase as a probe to study membrane protein assembly and protein export was developed by Broome-Smith and Spratt (4) and has been successfully applied in studies of the transmembrane topology of various cytoplasmic membrane proteins (5).

Plasmid pKE61 (6) contains the *exbB* and *exbD* structural genes downstream of the gene 10 promoter of phage T7, allowing their overexpression by the T7 RNA polymerase. Plasmid pJBS633 contains the β-lactamase gene *blaM* and markers for tetracycline and kanamycin resistance (4). The *Pvu*II-*Bam*HI fragment of pKE61 was cloned into the *tet* gene of plasmid pJBS633 cleaved with *Eco*RV and *Bam*HI. The resulting plasmid, pKK2, contained *exbB* and *exbD* downstream of the tandem *tet* and gene 10 promoters. It was cleaved by *Stu*I downstream of *exbD* or by *Bst*EII within codon 82 of *exbD* and digested for various periods of time with either *Bal* 31 exonuclease or exonuclease III. Blunt ends were generated by a fill-in reaction with deoxynucleotides and Klenow polymerase. The derivatives were cleaved with *Pvu*II upstream of *blaM*, ligated, and transformed into *Escherichia coli* 5K *hadR lacZ rpsL thi thr fhuA* (13). Kanamycin- and subsequently ampicillin-resistant transformants were selected on TY agar plates (8 g of Bacto Tryptone, 5 g of yeast extract, 5 g of NaCl, and 50 mg of kanamycin or 200 mg of ampicillin, each per liter). About 20% of the kanamycin-resistant transformants were ampicillin resistant. Ampicillin selects not only in-frame *exbD-blaM* fusions forming periplasmatic β-lactamase fusions; ampicillin-lysed cells release cytoplasmic β-lactamase into the agar, where it protects cells from being killed by ampicillin. At high densities, cells producing cytoplasmic in-frame ExbD–β-lactamase fusions can be recognized by the growth zones surrounding them.

In order to differentiate between periplasmic and cytoplasmic β-lactamase fusions, individual colonies were tested on TY agar plates containing increasing concentrations of ampicillin (5, 200, 400, 800, and 1,600 μg per ml). Resistance to 200 μg per ml indicates that the β-lactamase is located in the periplasm, and sensitivity to 5 μg per ml indicates that it is located in the cytoplasm (4, 5). DNAs of the insertion sites of the plasmids carrying in-frame fusions were sequenced by using the dideoxy chain termination method (23) with a primer (5′ dCTCGTGCACCCAACTGA 3′) complementary to codons 15 to 21 of mature β-lactamase. Eleven derivatives were characterized as containing β-lactamase fusions to residues 9, 19, 53, 57, 70, 76, 78, 80, 92, 121, and 134 of ExbD. The first two fusions conferred no ampicillin resistance to single cells, whereas transformants carrying the other fusions were highly resistant (1,600 μg of ampicillin per ml), except for the transformants carrying the fusion to residue 121 (800 μg per ml).

The fusion proteins in transformants of *E. coli* BL21 F⁻ *hsdS gal* carrying the T7 RNA polymerase gene on the chromosome under isopropyl-β-D-thiogalactoside (IPTG) control were determined (26). Cells were treated with IPTG

* Corresponding author.

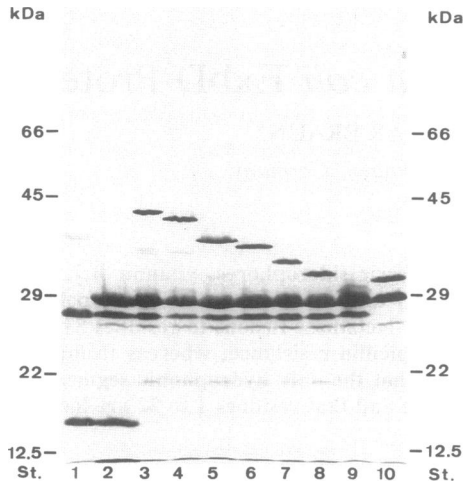

FIG. 1. Autoradiograph of labeled β-lactamase fused to ExbD at residues 134, 121, 92, 76, 53, 19, and 9 (lanes 3 to 9, respectively) compared with the unfused ExbB and ExbD proteins (lanes 1 and 2) and with the precursor and mature forms of β-lactamase (lane 10). Transformants of *E. coli* BL21 carrying the plasmids pKE61 *exbB exbD* (lane 1) and pKK2 *exbB exbD* (lane 2), the fusion plasmids (lanes 3 to 9), and pT7-3 *bla* (27) were grown (2 ml of culture) in the presence of IPTG to an A_{578} of 0.4 and then labeled with 170 kBq of [^{35}S]methionine for 15 min. Cells were washed, dissolved in sample buffer, and subjected to SDS-PAGE on a 15% polyacrylamide gel. St., the standard proteins bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (22 kDa), and cytochrome *c* (12.5 kDa).

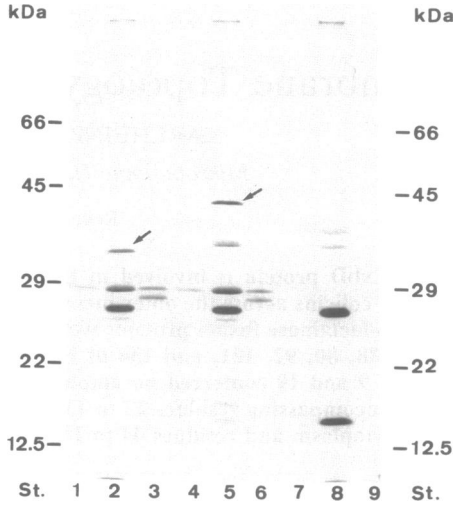

FIG. 2. Autoradiograph of the subcellular localization of the fusion proteins consisting of β-lactamase linked to residues 53 (lanes 1 to 3) and 134 (lanes 4 to 6) of ExbD. As a control, *E. coli* BL21 transformed with pKE61 *exbB exbD* is shown in lanes 7 to 9. Periplasmic fractions are shown in lanes 1, 4, and 7; membrane fractions are shown in lanes 2, 5, and 8; and cytoplasmic fractions are shown in lanes 3, 6, and 9. Cells were labeled as described in the legend to Fig. 1, and the same standard proteins (lane St.) were employed.

and labeled with [^{35}S]methionine. The proteins were separated by using discontinuous polyacrylamide gel electrophoresis (PAGE) (15) in the presence of sodium dodecyl sulfate (SDS). Figure 1 shows the autoradiograph of seven ExbD–β-lactamase fusion proteins (Fig. 1, lanes 3 to 9). The ExbD protein (15.5 kDa) expressed by the original plasmid pKE61 (Fig. 1, lane 1) and plasmid pKK2 (Fig. 1, lane 2) in *E. coli* BL21 has disappeared, and new bands with electrophoretic mobilities corresponding to the molecular weights of the fusion proteins appeared, as deduced from the insertion sites determined by using nucleotide sequencing. All of the transformants contained the ExbB protein (26.1 kDa) (Fig. 1, lanes 1 to 9) which appeared below the kanamycin resistance protein (29 kDa; phosphotransferase of Tn*903* [8]) and the mature β-lactamase (29 kDa) (Fig. 1, lane 10). The precursor of β-lactamase (32 kDa) (Fig. 1, lane 10) appeared between the ExbD–β-lactamase fusion protein to residue 19 (33.5 kDa) and the fusion protein to residue 9 (30.9 kDa). These results indicate that residues 1 to 53 are involved in translocating the remainder of ExbD across the cytoplasmic membrane.

For subcellular localization of the fusion proteins, [^{35}S]methionine-labeled cells of *E. coli* BL21 carrying plasmids expressing the β-lactamase fusions to residues 53 and 134 were fractionated into membranes, cytoplasm, and periplasm as described previously (28). Fractionation was examined by staining the gel with Coomassie blue, which revealed the exclusive presence of the highly expressed outer membrane proteins OmpF, OmpC, and OmpA in the membrane fraction, a few more strongly expressed protein bands in the periplasmic fraction which were absent from the cytoplasmic fraction, and many weak bands in the cytoplasmic fraction. The ExbB and ExbD proteins expressed by the BL21 transformant carrying plasmid pKE61 were contained exclusively in the membrane fraction (strongest bands in Fig. 2, lane 8). Also, the two fusion proteins and the unaltered ExbB protein were found only in the membrane fraction (indicated by arrows in Fig. 2, lanes 2 and 5). The periplasmic fractions contained only trace amounts of the kanamycin resistance protein (Fig. 2, lanes 1 and 4), of which most was found in the cytoplasmic fraction (Fig. 2, lanes 3 and 6). The additional band below the kanamycin resistance protein of the cytoplasmic fraction is probably a degradation product, since it occurred only when the kanamycin resistance gene was expressed (compare lanes 3 and 6 with lane 9 in Fig. 2). These results clearly demonstrate the membrane association of the fusion proteins and support the previous localization of the ExbB and ExbD proteins to the cytoplasmic membrane (6).

The only hydrophobic region of ExbD spans residues 18 to 43 (6). Here, we have shown that β-lactamase fusions to residues 9 and 19 of ExbD conferred no ampicillin resistance, implying that the β-lactamase portions of these fusion proteins were not transported across the cytoplasmic membrane. In contrast, cells expressing proteins with β-lactamase fusions to residues between positions 53 and 134 of ExbD were highly ampicillin resistant, showing that the β-lactamase portion is contained in the periplasm. On the basis of these results, we propose that residues 1 to 22 of ExbD are located in the cytoplasm, residues 23 to 43 form a transmembrane region crossing the cytoplasmic membrane once, and residues 44 to 141 are located in the periplasm (Fig. 3).

The procedure of Rao and Argos (20) predicts a transmembrane helix in ExbD between residues 23 and 43 (data not shown), which agrees with the conclusions drawn from the topology studies described in this paper and the hydrophobicity profile of ExbD analyzed by using the Kyte-Doolittle method (6). According to our model, the transmembrane

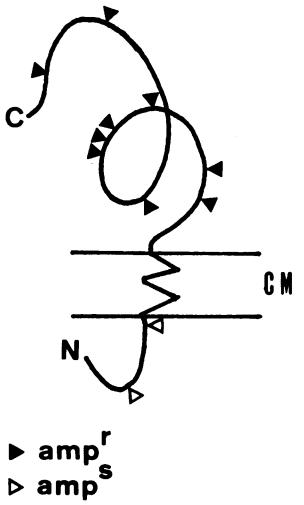

FIG. 3. Arrangement of the *E. coli* ExbD protein across the cytoplasmic membrane (CM) with the cytoplasmic N-terminal domain (N) and the periplasmic C-terminal portion (C). The ExbD–β-lactamase fusion sites resulting in ampicillin resistance (amp^r) and ampicillin sensitivity (amp^s) are indicated.

arrangement of ExbD is similar to that of TonB. Since the ExbD amino acid sequence is 25% identical and 70% similar to the TolR sequence (6, 29) and since both proteins show similar hydrophobicity profiles and agree in the predicted transmembrane helix, we propose that TolR will show a transmembrane arrangement similar to that of ExbD. This aspect is interesting in light of the evolutionary relationship between the ExbD and TolR proteins (and the ExbB and TolQ proteins) in that TolQ and TolR can replace the missing ExbB and ExbD functions in *exbB exbD* mutants (2, 3).

We thank J. K. Broome-Smith for providing plasmid pJBS633 and a detailed protocol on how to use her method and R. Tsolis for helpful comments on the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 323 and Graduiertenkolleg Mikrobiologie).

REFERENCES

1. **Anton, M., and K. J. Heller.** 1991. Functional analysis of a C-terminally altered TonB protein of *Escherichia coli*. Gene **105**:23–29.
2. **Braun, V.** 1989. The structurally related *exbB* and *tolQ* genes are interchangeable in conferring *tonB*-dependent colicin, bacteriophage, and albomycin sensitivity. J. Bacteriol. **171**:6387–6390.
3. **Braun, V., K. Günter, and K. Hantke.** 1991. Transport of iron across the outer membrane. Biol. Metals **4**:14–22.
4. **Broome-Smith, J. K., and B. G. Spratt.** 1986. A vector for the construction of translational fusions to TEM β-lactamase and the analysis of protein export signals and membrane protein topology. Gene **49**:341–349.
5. **Broome-Smith, J. K., M. Tadayyon, and Y. Zhang.** 1990. β-Lactamase as a probe of membrane protein assembly and protein export. Mol. Microbiol. **4**:1637–1644.
6. **Eick-Helmerich, K., and V. Braun.** 1989. Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* genes, respectively. J. Bacteriol. **171**:5117–5126.
7. **Fischer, E., K. Günter, and V. Braun.** 1989. Involvement of ExbB and TonB in transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exb* mutants by overproduced *tonB* and physical stabilization of TonB by ExbB. J. Bacteriol. **171**:5127–5134.
8. **Grindley, N. D. F., and C. M. Joyce.** 1980. Genetic and DNA sequence analysis of the kanamycin resistance transposon Tn 903. Proc. Natl. Acad. Sci. USA **77**:7176–7180.
9. **Günter, K., and V. Braun.** 1990. In vivo evidence for FhuA outer membrane receptor interaction with the TonB inner membrane protein of *Escherichia coli*. FEBS Lett. **274**:85–88.
10. **Hancock, R. E. W., and V. Braun.** 1976. Nature of the energy requirement for the irreversible adsorption of bacteriophages T1 and φ80 to *Escherichia coli*. J. Bacteriol. **125**:409–415.
11. **Hannavy, K., G. C. Barr, C. J. Dorman, J. Adamson, L. R. Mazengera, M. P. Gallagher, J. S. Evans, B. A. Levine, I. P. Trayer, and C. F. Higgins.** 1990. TonB protein of *Salmonella typhimurium*. A model for signal transduction between membranes. J. Mol. Biol. **216**:897–910.
12. **Heller, K. J., R. J. Kadner, and K. Günter.** 1988. Suppression of the *btuB*451 mutation by mutations in the *tonB* gene suggest a direct interaction between TonB and TonB-dependent receptor proteins of the outer membrane of *Escherichia coli*. Gene **64**:147–153.
13. **Hubacek, J., and S. W. Glover.** 1970. Complementation analysis of temperature-sensitive host specificity mutations in *Escherichia coli*. J. Mol. Biol. **50**:111–127.
14. **Kadner, R. J.** 1990. Vitamin B_{12} transport in *Escherichia coli*: energy coupling between membranes. Mol. Microbiol. **4**:2027–2033.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Mende, J., and V. Braun.** 1990. Import-defective colicin B derivatives mutated in the TonB box. Mol. Microbiol. **4**:1523–1533.
17. **Nau, C. D., and J. Konisky.** 1989. Evolutionary relationship between the TonB-dependent outer membrane transport proteins: nucleotide and amino acid sequences of the *Escherichia coli* colicin I receptor gene. J. Bacteriol. **171**:1041–1047.
18. **Nikaido, H.** 1992. Porins and specific channels of bacterial outer membranes. Mol. Microbiol. **6**:435–442.
19. **Postle, K.** 1990. TonB and the Gram-negative dilemma. Mol. Microbiol. **4**:2019–2025.
20. **Rao, J. K. M., and P. Argos.** 1986. A conformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta **869**:197–214.
21. **Reynolds, P. R., P. M. George, and C. Bradbeer.** 1980. Transport of vitamin B_{12} in *Escherichia coli*. J. Biol. Chem. **255**:4313–4319.
22. **Roof, S. K., J. D. Allard, K. P. Bertrand, and K. Postle.** 1991. Analysis of *Escherichia coli* TonB membrane topology by use of PhoA fusions. J. Bacteriol. **173**:5554–5557.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
24. **Schöffler, H., and V. Braun.** 1989. Transport across the outer membrane of *Escherichia coli* via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane. Mol. Gen. Genet. **217**:378–383.
25. **Skare, J. T., and K. Postle.** 1991. Evidence for a TonB-dependent energy transduction complex in *Escherichia coli*. Mol. Microbiol. **5**:2883–2890.
26. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
27. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
28. **Van Hove, B., H. Staudenmaier, and V. Braun.** 1990. Novel two-component transmembrane transcription control: regulation of iron dicitrate transport in *Escherichia coli* K-12. J. Bacteriol. **172**:6749–6758.
29. **Webster, R. E.** 1991. The *tol* gene products and the import of macromolecules into *Escherichia coli*. Mol. Microbiol. **5**:1005–1011.