Folding of a bacterial outer membrane protein during passage through the periplasm

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The transport of bacterial outer membrane proteins to their destination might be either a one-step process via the contact zones between the inner and outer membrane or a two-step process, implicating a periplasmic intermediate that inserts into the membrane. Furthermore, folding might precede insertion or vice versa. To address these questions, we have made use of the known 3D-structure of the trimeric porin PhoE of *Escherichia coli* to engineer intramolecular disulfide bridges into this protein at positions that are not exposed to the periplasm once the protein is correctly assembled. The mutations did not interfere with the biogenesis of the protein, and disulfide bond formation appeared to be dependent on the periplasmic enzyme DsbA, which catalyzes disulfide bond formation in the periplasm. This proves that the protein passes through the periplasm on its way to the outer membrane. Furthermore, since the disulfide bonds create elements of tertiary structure within the mutant proteins, it appears that these proteins are at least partially folded before they insert into the outer membrane.

Keywords: DsbA/*Escherichia coli*/outer membrane/PhoE protein/protein folding

Introduction

In Gram-negative bacteria, such as *Escherichia coli*, outer membrane proteins (OMPs) are synthesized in the cytoplasm as precursor proteins with an N-terminal extension, the signal sequence. These precursors, like those of periplasmic proteins, are translocated across the inner membrane via the Sec machinery, and the signal sequence is cleaved off. Intensive research over the past decade has led to the unraveling of the mechanism of this transport process into much molecular detail (Schatz and Beckwith, 1990; Pugsley, 1993a; Driessen, 1994). In contrast, the knowledge concerning the subsequent steps in the biogenesis of OMPs is very scarce. Two pathways for the sorting of OMPs to their destination have been proposed. On the one hand, these proteins might directly be inserted into the outer membrane via the contact sites between the two membranes (Bayer *et al.*, 1982). This model is supported by electron-microscopic studies, which reveal the presence of newly exported porins above the fusion sites (Smit and Nikaido, 1978). Furthermore, pulse-chase

experiments suggested that nascent OMPs pass through a membrane fraction of intermediate density (de Leij *et al.*, 1978), which is supposed to contain the fusion sites, and that they are incorporated into the outer membrane even before their synthesis is completed (de Leij *et al.*, 1979). Alternatively, OMPs might pass through the periplasm on their way to the outer membrane. In this type of model, periplasmic folding might precede membrane insertion. Whereas the latter type of model is presently being favored (Nikaido, 1992; Tommassen and de Cock, 1995), direct evidence is lacking. The fact that mutant OMPs that fail to assemble correctly accumulate in the periplasm favors the periplasmic pathway (Freudl *et al.*, 1985; Bosch *et al.*, 1986), but could also be explained by a default pathway of defective OMPs. The observation that OMPs, synthesized *in vitro* or secreted by spheroplasts, can be assembled into isolated outer membranes (Sen and Nikaido, 1990; de Cock *et al.*, 1996) is also consistent with a free soluble assembly intermediate, but could also reflect an artificial pathway, especially since low amounts of detergents were required to stimulate insertion.

The periplasm contains molecular chaperones and folding catalysts, such as disulfide bond isomerases and peptidyl-prolyl *cis/trans* isomerases (Liu and Walsh, 1990; Bardwell *et al.*, 1991; Rudd *et al.*, 1995) which assist in protein folding. If any of these proteins were directly involved in the folding of OMPs, this would demonstrate that these OMPs pass through the periplasm on their way to the outer membrane and that folding precedes insertion. However, unequivocal evidence for such an involvement is lacking. Whereas it has been reported that mutations in genes encoding putative periplasmic chaperones or folding catalysts can influence the amount of OMPs assembled into the outer membrane, such effects could be indirect. For example, a mutation in the *dsbA* gene, which encodes an enzyme that catalyzes disulfide bond formation, drastically affected the expression of OmpF, even though this porin does not contain any cysteines (Pugsley, 1993b). Similarly, the observation that an *skp* mutation affects the expression of several OMPs (Chen and Henning, 1996) might be explained by a role of the *skp* gene product in the biogenesis of lipopolysaccharides (LPSs), since the *skp* gene is located in a cluster of LPS biosynthesis genes and LPS is involved in the biogenesis of OMPs (de Cock and Tommassen, 1996).

In the present study, we have investigated whether a periplasmic enzyme, DsbA, can modulate the folding of an OMP on its way to the outer membrane. As a model OMP, the trimeric porin PhoE is used in our laboratory. This protein does not contain any cysteines, but its structure has been resolved (Cowan *et al.*, 1992), which enabled us to introduce cysteines at positions that are in sufficiently close proximity to allow disulfide bond formation when the protein folds into its correct conform-

Table I. Plasmids used

Plasmids	Relevant characteristics ^a	Reference
pJP29	Cam ^r , wild-type <i>phoE</i> gene	Bosch <i>et al.</i> (1986)
pVG1	Amp ^r , wild-type <i>phoE</i> gene lacking signal sequence	Van Gelder <i>et al.</i> (1994)
pEE1	Cam ^r , mutant <i>phoE</i> with K18C substitution	this study
pEE2	Cam ^r , mutant <i>phoE</i> with E110C substitution	this study
pEE3	Cam ^r , mutant <i>phoE</i> with F111C substitution	this study
pEE4	Cam ^r , mutant <i>phoE</i> with D302C substitution	this study
pEE5	Cam ^r , mutant <i>phoE</i> with K18C and E110C substitution	this study
pEE6	Cam ^r , mutant <i>phoE</i> with E110C and D302C substitution	this study
pEE7	Cam ^r , mutant <i>phoE</i> with F111C and D302C substitution	this study

^aCam^r and Amp^r indicate resistance to chloramphenicol and ampicillin, respectively.

ation. Each monomer of PhoE consists of a 16-stranded β -barrel with short turns at the periplasmic side and long loops at the cell surface. One of these loops, L3, forms a constriction within the barrel at half the height of the membrane. The sequence PEFGG (residues 109–113) at the tip of L3 is highly conserved in a superfamily of bacterial porins (Jeanteur *et al.*, 1991). Residues Glu110 and Phe111 are at hydrogen-bonding distance from Lys18 and to Asp302, respectively, in the barrel wall (Karshikoff *et al.*, 1994). By constructing a series of double mutants, we were able to engineer disulfide bridges within PhoE. We demonstrate that disulfide bond formation in the mutant PhoE proteins is strongly stimulated by the periplasmic DsbA protein. Hence, it appears that PhoE passes through the periplasm on its way to the outer membrane. Furthermore, since the DsbA protein creates an element of tertiary structure within the mutant PhoE proteins, these proteins can at least be folded partially prior to their insertion into the outer membrane.

Results

Expression of cysteine-containing mutant PhoE proteins

Site-directed mutagenesis was applied to substitute cysteines for Lys18, Glu110, Phe111 and Asp302 in PhoE protein and double mutants with cysteines at positions 18 and 110, 110 and 302 and 111 and 302 were constructed by combining appropriate restriction fragments. The mutant plasmids (Table I) were introduced in *phoR* strain CE1265 by transformation, and cell envelopes were isolated and analyzed by SDS–PAGE. All mutant proteins were efficiently expressed (Figure 1A). To study whether the mutant proteins were properly inserted into the outer membrane, their sensitivity to trypsin was assessed. Wild-type PhoE protein is resistant to proteases when it is correctly assembled into the outer membrane (Tommassen and Lugtenberg, 1984). Similarly, all the mutant proteins were protected when cell envelopes were treated with trypsin (Figure 1A), indicating that they were correctly assembled. Furthermore, wild-type PhoE functions in the outer membrane as the receptor for phage TC45 (Chai and Foulds, 1978). CE1265 cells expressing either one of the mutant PhoE proteins were sensitive to this phage (data not shown), which confirms that the mutant proteins were correctly assembled. This conclusion was further substantiated by the observation that all mutant proteins could be isolated from the membranes as native trimers (see Figure 1B for examples). However, the stability of some of the

mutant trimers was slightly affected. The wild-type trimer denatured into monomers only after incubation for 10 min at 70°C in 2% SDS (Figure 1B). The PhoE mutant trimers with substitutions at position 18 or at position 302 were equally as stable as the wild-type trimer. The mutations at the tip of the third loop, i.e. at the positions of residues 110 and 111, affected the stability of the trimers since the mutant proteins denatured at 60–65°C (Figure 1B). All three double cysteine PhoE mutants denatured at 60°C. Together, these results show that all mutant proteins are correctly assembled into the outer membrane but that the stability of some of the proteins was slightly affected by the mutations.

Disulfide bond formation

Frequently, proteins with an intramolecular disulfide bond have a higher electrophoretic mobility than the reduced form of these proteins, probably because of their more compact shape (see, for example, Derman and Beckwith, 1995). To examine whether disulfide bonds are formed between the two cysteines in the mutant PhoE proteins, trimers isolated from the outer membrane were denatured at 95°C in the presence or absence of dithiothreitol (DTT) and analyzed by SDS–PAGE. For all double mutants, faster migrating forms were detected when they were analyzed in the absence of the reducing agent, although with varying efficiency (Figure 2A). In the case of the mutant with cysteines at positions 18 and 110, the faster migrating form was formed with an efficiency of 80–100%. The difference in the mobility of the reduced and the oxidized form was small and could only be visualized when the proteins were separated on gels containing 8 M urea. Also for the mutants with cysteines at positions 110 and 302, or 111 and 302, faster migrating forms were detected on the gel (Figure 2A). In these cases, the efficiency varied from experiment to experiment between 30 and 70%. The mobility differences were more pronounced (Figure 2A). In all cases, both bands reacted on a Western blot with a PhoE-specific monoclonal antibody (Figure 2B), which confirms that they both represent different forms of the mutant PhoE proteins.

Since the bands with higher electrophoretic mobilities disappeared upon treatment of the samples with DTT, whereas those with the lower mobilities increased in intensity (Figure 2A), we assumed that they represent the oxidized and the reduced forms, respectively, of the mutant PhoE proteins. The presence of free SH groups in the protein samples was assessed with the thiol-specific reagent *N*-[6-(biotinamido)]hexyl-3'-(2'pyridyl)dithio)-pro-

Fig. 1. Expression and assembly of cysteine-containing mutant derivatives of PhoE protein. (A) Cell envelopes were isolated from plasmid-containing derivatives of strain CE1265 expressing mutant PhoE proteins. Samples were treated with trypsin when indicated, after which the samples were analyzed by SDS-PAGE. The sample buffer was supplemented with 20 mM DTT. (B) Trimers of (mutant) PhoE proteins were isolated and incubated for 10 min at the indicated temperatures in the presence of DTT. T and M indicate the positions of the trimeric and the denatured monomeric forms of PhoE, respectively.

pionamide (HPDP) to which a biotin was attached. Trimers isolated from the outer membrane were denatured at 95°C either in the absence or presence of DTT and subsequently incubated with biotin-HPDP. The proteins were separated by SDS-PAGE, blotted onto nitrocellulose paper, and proteins that had bound biotin were detected by using a streptavidin-horseradish peroxidase conjugate. The mutant protein with cysteines at positions 18 and 110 could only be labeled with biotin-HPDP after the protein had been denatured in the presence of DTT (Figure 2C). This result confirms that the cysteines in this mutant protein were efficiently oxidized into a disulfide bond. In the case of the PhoE mutants with cysteines at positions 110 and 302, or 111 and 302, only a single band was detected, which increased in intensity when the samples were denatured in the presence of DTT (Figure 2C). The monomers with

Fig. 2. Formation of disulfide bonds in cysteine-containing derivatives of PhoE protein. (A) Trimers were isolated and dissolved in sample buffer either supplemented or not with DTT, and the samples were boiled for 10 min and analyzed by SDS-PAGE. (B) Western blot analysis of the same samples, using a monoclonal antibody directed against PhoE. M and M* indicate the positions of the denatured form of PhoE and the faster migrating form with an intramolecular disulfide bridge, respectively. In both (A) and (B), the wild-type PhoE and the mutant with cysteines at positions 18 and 110 were analyzed on gels containing 8 M urea and the other proteins on gels without urea. (C) Isolated trimers of (mutant) PhoE were heated at 95°C in the presence or absence of DTT, after which a buffer was added which contained the thiol-specific reagent biotin-HPDP. After incubation for 1 h at room temperature, samples were loaded onto gels, blotted to nitrocellulose membranes and a streptavidin-peroxidase conjugate was used to detect biotin-labeled PhoE forms. The band underneath PhoE in the DTT-treated samples represents trace amounts of OmpA that were present in the samples, as was evidenced by Western blotting with an OmpA-specific monoclonal antibody (data not shown).

the higher mobility on gels (Figure 2A) were never visualized with the biotin-labeled, thiol-specific reagent. Together, the results show that the anticipated disulfide bonds were indeed formed in the mutant proteins.

Time course of disulfide bond formation

The time course of the disulfide bond formation in the double cysteine PhoE mutants was determined in pulse-chase experiments. Plasmid-containing derivatives of strain CE1224 expressing the varying (mutant) PhoE proteins were pulse-labeled, immunoprecipitated and the formation of the disulfide bond was followed during the

Fig. 3. Time course of the formation of the disulfide bonds and the assembly of the mutant PhoE proteins into a trypsin-resistant form *in vivo*. (A) The time course of disulfide bond formation in CE1224 cells expressing the PhoE mutants was studied in pulse-chase experiments. Cells were starved for phosphate and pulse-labeled for 30 s with [35 S]methionine, followed by chase periods as indicated. After immunoprecipitation with a polyclonal antiserum directed against PhoE, samples were boiled in sample buffer without DTT and analyzed by SDS-PAGE, followed by autoradiography. (B) The assembly of the PhoE mutants into a trypsin-resistant form. After various chase periods, samples of the cells were lysed and incubated in buffer containing trypsin. After adding trypsin inhibitor, the samples were boiled in sample buffer without DTT and analyzed by SDS-PAGE, followed by autoradiography. Indicated are the positions of the reduced monomeric (M), oxidized monomeric (M*) and precursor (prePhoE) forms of PhoE.

chase. In all three PhoE mutants, the disulfide bond was formed very rapidly since the oxidized forms were detected on the autoradiogram of SDS-polyacrylamide gels in the samples taken directly after the pulse. During the subsequent chase, no or hardly any increase in the amount of the oxidized forms was observed (Figure 3A). To study the kinetics of the assembly of the mutant PhoE proteins into their native conformation, samples from the same pulse-chase experiments were treated with trypsin. Small amounts of trypsin-resistant PhoE forms were already detected directly after the pulse, but these amounts drastically increased during the chase (Figure 3B). Apparently, disulfide bond formation in the mutant PhoE proteins is very rapid and precedes the maturation into the trypsin-resistant form.

Involvement of DsbA in disulfide bond formation

To determine whether disulfide bond formation in the mutant PhoE proteins is stimulated by the periplasmic enzyme DsbA, the proteins were expressed overnight in P_i -limited medium (Levinthal *et al.*, 1962) in strain CE1224 and in its *dsbA* mutant derivative CE1442. Iodoacetamide (IAA) was added after isolation of the cells to prevent oxidation of the cysteines during the following procedures. The cell envelope protein patterns of the cells were analyzed by SDS-PAGE (Figure 4). The oxidized form of the mutant proteins with cysteines at positions 18 and 110 or at 110 and 302 could not be detected after expression in the *dsbA* mutant. A disulfide bond was still formed in the case of the mutant protein with cysteines at positions 111 and 302 although with a reduced efficiency. The residual disulfide bond formation in this case might be explained by spontaneous oxidation or by the activity of other enzymes that catalyze disulfide bond formation, such as DsbC (Missiakas *et al.*, 1994). These results demonstrate that the periplasmic enzyme DsbA stimulates the formation of the disulfide bonds in the mutant PhoE proteins.

Discussion

OMPs are sorted to their final destination after cleavage of the signal sequence. Repeatedly, it has been postulated

Fig. 4. Involvement of DsbA in the formation of the disulfide bonds in the mutant PhoE proteins. After overnight growth, cell envelopes were isolated from derivatives of strain CE1224 and of *dsbA* mutant CE1442 expressing (mutant) PhoE proteins, and dissolved in sample buffer either with or without DTT. After heating for 10 min at 95°C, samples were loaded onto gels, which in the case of wild-type PhoE and the mutant with cysteines at position 18 and 110 contained 8 M urea, and analyzed by Western blotting using a monoclonal antibody directed against PhoE. The reduced and oxidized forms of the mutant PhoE proteins are indicated by M and M*, respectively.

that these proteins pass through the periplasm on their way to the outer membrane, and that folding precedes outer membrane insertion (e.g. Tommassen, 1986). However, definite proof for this model has so far not been described. Here, by making use of the known 3D-structure of the outer membrane protein PhoE (Cowan *et al.*, 1992), we were able to engineer artificial disulfide bonds into this protein. Our results show that the formation of the disulfide bonds depends on the periplasmic enzyme DsbA. Previously, it has been demonstrated that DsbA catalyzes the formation of a disulfide bond in another OMP, i.e. OmpA (Bardwell *et al.*, 1991). However, the two cysteines in OmpA are located in the C-terminal part of OmpA, which is supposed to extend in the periplasm (Morona *et al.*, 1984; Vogel and Jähnig, 1986). Hence, from those experiments it cannot be concluded that DsbA modifies the OmpA protein on its way to the outer membrane. In contrast, we created the disulfide bonds in PhoE at a position that is not

expected to be accessible from the periplasm, once the protein is correctly assembled into the membrane. Since DsbA nevertheless stimulated disulfide bond formation in the mutant PhoE proteins, our results prove that the protein passes through the periplasm on its way to the outer membrane. Furthermore, since the DsbA protein creates an element of tertiary structure within the mutant PhoE proteins, these proteins are at least partially folded before they insert into the outer membrane.

It was important to establish whether the mutant proteins were properly assembled and localized into the outer membrane. This condition was met by demonstrating that all the mutant proteins formed trimers, that they were trypsin-resistant and functional as phage receptors. However, the temperature stability of the trimers of the mutant proteins with substitutions of Glu110 or Phe111 was somewhat decreased. These residues belong to a highly conserved sequence motif, PEEGG, at the tip of loop L3 (Jeanteur *et al.*, 1991). They are probably contributing to a hydrogen-bonding network with residues in the barrel wall (Karshikoff *et al.*, 1994). Disruption of these interactions by the mutations might affect the stability of the trimers.

A study of the time course of disulfide bond formation in pulse-chase experiments revealed that this process was very rapid. A large number of the mutant proteins were oxidized during the pulse, and the proportion of proteins with a disulfide bond did not or only marginally increased during the chase. Previously, pulse-chase experiments have revealed a similarly rapid rate of disulfide bond formation in alkaline phosphatase, β -lactamase and OmpA in a *dsbA*⁺ strain, whereas this rate was severely retarded in a *dsbA* mutant (Bardwell *et al.*, 1991). In contrast, the maturation of the (mutant) PhoE proteins into their trypsin-resistant form was a slower process. Apparently, disulfide bond formation occurred early in the folding process, which is consistent with the idea that DsbA has no access to the cysteines once the protein is correctly folded.

Our observations prove the existence of periplasmic intermediates of OMPs during their biogenesis. However, these periplasmic intermediates are not necessarily soluble in the periplasm, but may remain associated with the periplasmic side of the inner membrane and fold by interaction with nascent LPS (de Cock and Tommassen, 1996; Bolla *et al.*, 1988). Since the DsbA protein fixates an element of tertiary structure in the mutant PhoE proteins, our results demonstrate that these proteins are indeed at least partially folded when they insert into the outer membrane. This is in agreement with the hydrophilic amino acid composition of PhoE (Overbeeke *et al.*, 1983) as well as of other OMPs, which expose hydrophobic domains only after folding into their tertiary structure. It will be interesting to determine, along similar lines to those described here, whether quaternary structure formation (trimerization) also precedes insertion into the outer membrane.

Materials and methods

Strains, phages and growth conditions

The *E. coli* K-12 strains CE1224 and CE1265 (Tommassen *et al.*, 1983) are deleted for the *phoE* gene and do not produce the related OmpF and OmpC proteins as a result of *ompR* mutations. Strain CE1265 carries in

addition a *phoR* mutation, resulting in constitutive expression of the *pho* regulon. Strain CE1442 is a *dsbA::kan* derivative of CE1224 and was constructed by P1 transduction (Miller, 1972) using strain JCB572 (Bardwell *et al.*, 1991) as a donor. Sensitivity of strains to the PhoE-specific phage TC45 (Chai and Foulds, 1978) was determined by cross-streaking. Bacteria were grown at 37°C under aeration in L-broth (Tommassen *et al.*, 1983), in a synthetic medium in which the phosphate concentration could be varied (Tommassen and Lugtenberg, 1980) or in the phosphate-limited medium described by Levinthal *et al.* (1962). When necessary for plasmid maintenance, the antibiotics chloramphenicol (25 µg/ml), ampicillin (100 µg/ml) or kanamycin (25 µg/ml) were added to the media.

Plasmids and DNA manipulations

Plasmid pJP29 is derived from the cloning vector pACYC184 and carries the *phoE* gene (Bosch *et al.*, 1986). Plasmid pVG1 is derived from the vector pBluescriptII SK(+)/T7 and also carries the *phoE* gene but lacks the signal sequence-encoding part (Van Gelder *et al.*, 1994). Plasmid DNA was isolated as described (Birnbom and Doly, 1979), followed by anion-exchange chromatography on Qiagen columns (Diagen, Düsseldorf, Germany). PCR fragments were isolated from low melting point agarose by using Qiaex (Diagen). Standard DNA manipulations were performed according to Maniatis *et al.* (1982). Restriction endonucleases, T4 DNA ligase and *Pfu* DNA polymerase were used according to the manufacturers' protocols (Fermentas and Stratagene). To obtain PhoE mutants, site-directed mutagenesis was used according to the three-primer PCR method described by Landt *et al.* (1990). Oligonucleotides were purchased from Isogen (Amsterdam). Either pJP29 or pVG1 were used as templates for the PCR-based mutagenesis, and restriction fragments of the PCR products containing the mutations were substituted for the wild-type fragments in pJP29. The resulting plasmids, pEE1, pEE2, pEE3 and pEE4 (Table I), encode mutant PhoE proteins with a single cysteine. Double-mutant plasmids were obtained by exchanging appropriate restriction fragments. Plasmid pEE5 was constructed by substituting the *MluI*-*BglII* fragment of pEE2 for the corresponding fragment in pEE1. The plasmids pEE6 and pEE7 were constructed by substituting the *BglII*-*XbaI* fragment of pEE4 for the corresponding segments of pEE2 and pEE3, respectively. Successful mutagenesis was confirmed by DNA sequence analysis using the T7 DNA Polymerase Sequencing Kit (Pharmacia).

Isolation and characterization of cell fractions

After overnight growth, cells were collected by centrifugation and resuspended in 50 mM Tris-HCl, 5 mM EDTA pH 8.0, supplemented when indicated with 100 mM IAA (Sigma). Cell envelopes were isolated by centrifugation after ultrasonic disintegration of the cells (Lugtenberg *et al.*, 1975). To test the accessibility of proteins in the cell envelope fractions for trypsin, the cell envelopes were resuspended in 1 ml 10 mM Tris-HCl, 10 mM MgCl₂ pH 8.0 containing 50 µg trypsin (Struyvé *et al.*, 1991). The samples were incubated on ice for 20 min, after which the cell envelopes were reisolated by centrifugation. Protein patterns of cell fractions were analyzed by SDS-PAGE (Lugtenberg *et al.*, 1975) using the sample buffer described by Lugtenberg *et al.* (1975), except that the β -mercaptoethanol was omitted or replaced when indicated by 20 mM DTT.

Isolation of the PhoE trimers

Trimers were isolated as described previously (Agterberg *et al.*, 1990). Briefly, cell envelope fractions were incubated for 30 min at 40°C in a buffer containing 2% SDS as described, except that the β -mercaptoethanol was omitted. Peptidoglycan-protein complexes were pelleted by ultracentrifugation and PhoE was dissociated from the peptidoglycan by incubation for 30 min at 40°C in the SDS buffer supplemented with 0.6 M NaCl. After ultracentrifugation, the trimers were precipitated from the supernatant with 66% ethanol and dissolved in a buffer containing 20 mM Tris-HCl pH 8.0 and 0.1% SDS. When appropriate, the trimers were denatured into monomers by heating for 10 min at 95°C (or different temperatures when indicated) in the absence or presence of 20 mM DTT. Proteins were analyzed by SDS-PAGE and Western immunoblotting (Agterberg *et al.*, 1990). The monoclonal antibody used to detect PhoE protein was mE2-1, which recognizes the denatured protein.

Pulse-chase experiments

To study the time course of disulfide bond formation in the mutant PhoE proteins, cells were grown under phosphate limitation at 37°C as described (Bosch *et al.*, 1986) to induce the expression of mutant PhoE

proteins. Cells were labeled with [³⁵S]methionine (10 µCi/ml) for 30 s at 37°C and subsequently chased with an excess of non-radioactive methionine. At various time points during the chase, samples were taken after which they were mixed with 100 mM IAA and incubated for 20 min at 0°C. The proteins in the samples were precipitated with 5% TCA and PhoE forms were immunoprecipitated as described by Bosch *et al.* (1989) with slight modifications. Briefly, the pellet obtained after TCA precipitation was dissolved in 15 µl 2% SDS, 50 mM Tris-HCl, 1 mM EDTA pH 8.0 and boiled for 10 min. Subsequently, 485 µl Triton buffer containing 2% Triton X-100, 50 mM Tris-HCl, 0.15 M NaCl, 0.1 mM EDTA pH 8.0 was added. After centrifugation for 15 min in the Eppendorf centrifuge, a polyclonal antiserum directed against denatured PhoE was added to the supernatant. After overnight incubation under gentle shaking at 4°C, 2.5 mg protein A-sepharose CL-4B (Pharmacia), dissolved in Triton buffer, was added and incubated for 1 h at room temperature under gentle shaking. The pellet obtained after centrifugation for 1 min in an Eppendorf centrifuge was washed twice in Triton buffer and once in 10 mM Tris-HCl pH 8.0, and finally resuspended in sample buffer without DTT, boiled and analyzed by SDS-PAGE and autoradiography. To analyze the time course of the assembly of the mutant PhoE proteins into a trypsin-resistant form, samples from the pulse-chase experiments were mixed with IAA and frozen at -20°C. Subsequently, the samples were thawed in the presence of 5% Triton X-100, 10 mM EDTA, 125 mM Tris-HCl pH 8.0 to which 15 mg/ml trypsin (Serva) was added. These mixtures were incubated at 0°C for 30 min. After adding a 3-fold excess of trypsin inhibitor (Serva) in 50 mM Tris-HCl, 20 mM MgCl₂ pH 8.0, membranes were pelleted by 30 min centrifugation at 15 000 r.p.m. in an Eppendorf centrifuge. The pellet was resuspended in sample buffer and boiled for 10 min before SDS-PAGE and autoradiography.

Detection free thiols

To detect free thiols in the mutant PhoE proteins, isolated trimers were incubated for 10 min at 95°C in the presence or absence of DTT as described. Subsequently, 20 mM phosphate-buffered saline (PBS), 10 mM EDTA, supplemented with 0.8 mM biotin-HPDP (Pierce) was added, and the samples were incubated for 1 h at room temperature. Subsequently, the proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters (Schleicher and Schuell, 0.45 µm) using a semi-dry electroblotting apparatus (2117 Multiphor II, LKB). Streptavidin-horseradish peroxidase was used to detect biotin-labeled proteins. The peroxidase activity was developed with a solution of 4-chloro-1-naphthol (0.5 mg/ml) in 15% MeOH, 85% PBS and 0.01% H₂O₂.

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