

The Early Interaction of the Outer Membrane Protein PhoE with the Periplasmic Chaperone Skp Occurs at the Cytoplasmic Membrane*

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Spheroplasts were used to study the early interactions of newly synthesized outer membrane protein PhoE with periplasmic proteins employing a protein cross-linking approach. Newly translocated PhoE protein could be cross-linked to the periplasmic chaperone Skp at the periplasmic side of the inner membrane. To study the timing of this interaction, a PhoE-dihydrofolate reductase hybrid protein was constructed that formed translocation intermediates, which had the PhoE moiety present in the periplasm and the dihydrofolate reductase moiety tightly folded in the cytoplasm. The hybrid protein was found to cross-link to Skp, indicating that PhoE closely interacts with the chaperone when the protein is still in a transmembrane orientation in the translocase. Removal of N-terminal parts of PhoE protein affected Skp binding in a cumulative manner, consistent with the presence of two Skp-binding sites in that region. In contrast, deletion of C-terminal parts resulted in variable interactions with Skp, suggesting that interaction of Skp with the N-terminal region is influenced by parts of the C terminus of PhoE protein. Both the soluble as well as the membrane-associated Skp protein were found to interact with PhoE. The latter form is proposed to be involved in the initial interaction with the N-terminal regions of the outer membrane protein.

The majority of the proteins destined for the extracytoplasmic environment in *Escherichia coli* are synthesized as precursors in the cytoplasm that are translocated across the inner IM¹ in a non-native state. To reach their final conformation and destiny they need to be prevented from premature folding and degradation in the cytoplasm. It is well established that molecular chaperones and folding catalysts are required to channel proteins into productive export, folding, and assembly pathways. In the cytoplasm one of the first events that takes place when a nascent PhoE emerges from the ribosome is the inter-

action with trigger factor, a cytoplasmic chaperone. Using a cross-linking approach, the latter protein was found to interact with nascent PhoE chains as short as 57 amino acids (1–3). Subsequently, at a late co-translational or early post-translational stage the precursor protein interacts with SecB (4–6). This SecB-precursor complex is then targeted to SecA, the peripheral subunit of the membrane-embedded SecYEG complex (7). PhoE precursors are then translocated across the IM in a non-native state. When appearing at the periplasmic side of the IM, these polypeptides undergo complex folding processes and have to be prevented from misfolding, aggregating, and degrading. Proteins destined for the outer membrane or the extracellular environment require correct targeting to these locations. Periplasmic chaperones and folding catalysts have been implicated to be involved in the folding and assembly pathway of OMPs. It was demonstrated previously that OMPs are exposed to the periplasmic chaperones during the assembly process (8). A periplasmic system consisting of molecular chaperones and folding catalysts has been described (9, 10). Among them, the peptidyl-prolyl isomerases (FkpA, SurA, RotA, and PpiD) carry out the isomerization around the Xaa-Pro peptide bond. In addition to the isomerase activity the FkpA and SurA proteins were found to possess chaperone activity (11, 12). The second type of folding catalysts are the protein disulfide isomerases (Dsb proteins) carrying out disulfide bond formation and thiol-disulfide exchanges (13).

In addition, Skp functions as a molecular chaperone in the periplasm of Gram-negative bacteria, possibly specifically required for the biogenesis of OMPs. Skp is a basic soluble protein in the periplasm, but it was also found in IM fractions (14, 15). The protein exists in two different states as was demonstrated by their different sensitivity toward proteases (16). The conversion between these states can be modulated *in vitro* by phospholipids, lipopolysaccharides, and bivalent cations (16). The function of these two different forms of Skp *in vivo* is not yet known. Previously, it was demonstrated that Skp binds selectively to OmpA and proteins of the bacterial porin family (16, 17). In addition, non-OMPs seem to act as a substrate for binding (18). Skp interacts with OmpA in close vicinity of the IM, and it was found to be involved in the release of the OMP from this membrane (15). In addition, cells defective in Skp and the periplasmic protease DegP accumulate protein aggregates in the periplasm (15). These results suggest that Skp is a molecular chaperone involved in generating and maintaining solubility of early folding intermediates of OMPs in the periplasm.

The molecular mechanism of the PhoE assembly pathway

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¹ The abbreviations used are: IM, inner or cytoplasmic membrane; OMP, outer membrane protein; IPTG, isopropyl-1-thio- β -D-galactopyranoside; BS³, bis(sulfosuccinimidyl) suberate; RNC, ribosome nascent chain complex; DSS, disuccinimidyl suberate; DHFR, dihydrofolate reductase.

has been investigated extensively *in vivo* and *in vitro*. However, little is known about which periplasmic chaperones and folding catalysts are involved in the late stages of translocation over the IM, in the release from the IM, and in the early stages of folding in the periplasm. Furthermore, the order of interactions with accessory proteins and the timing of the various reactions are largely unknown. In the studies described in this report, we used an unbiased approach to study these processes. We present evidence that the initial interaction of the non-native porin protein PhoE at the periplasmic side of the IM is with Skp. This interaction occurs already early during translocation of PhoE, *i.e.* when PhoE is still in a transmembrane orientation in the translocase. At the N terminus of PhoE, two Skp-binding sites could be identified. In contrast to OmpA, release of PhoE from the IM appeared not to be dependent on Skp.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The *E. coli* K12 strain MC4100 ($F^- \Delta lacU169 araD136 rpsL thi relA$) was used for the spheroplast labeling experiments and for the isolation of lysate and inner membrane vesicles (19). MC4100 and RC354c, containing a deletion in the *skp* gene (17), were used for the isolation of periplasmic extracts (20). BG87 (21) was used for the isolation of trigger factor-depleted lysate as described before (2, 19). Top10F⁺ (Invitrogen, Carlsbad, CA) was used for routine cloning procedures. Strains were routinely grown in LB medium and the appropriate antibiotics. For pulse labeling experiments a GB1 minimal medium was used (22) supplemented with 0.4% maltose as the carbon source and an 18-amino acid mixture (0.1 mM) (without *Met* and *Cys*).

Construction of Plasmids—Plasmid pNN100 containing the *phoE* gene under control of the *tac* promoter was constructed previously (23). Plasmids pC4Meth300PhoE, pC4Meth100PhoE Δ ss, and pC4Meth137PhoE Δ ss were obtained by polymerase chain reaction using pVG01 (24) as a template essentially as described in a previous study (2). The latter two plasmids contain instead of a signal sequence a *Met* and a *Ser* residue just as in pJP370 (25). Construction of plasmid pAra300PhoE.DHFR was as follows: pC4Meth300PhoE was digested with *Bam*HI and *Hind*III and ligated to the *Bam*HI-*Hind*III fragment of pGEM4.DHFR containing the mouse *dhfr* gene (a gift from T. Langer, München, Germany). Subsequently, the *phoE-dhfr* fusion gene was cloned downstream of the arabinose promoter on plasmid pMPMK4 (26). Plasmid pGAH317, carrying the wild-type *skp* gene, has been described (27).

Spheroplast Labeling, Cross-linking, and Release—For pulse labeling of spheroplasts cells were grown in GB1 medium with casamino acids during 7 h and then diluted for overnight growth in such a way that the culture turbidity reached a value of ~ 0.5 (at 660 nm) the next morning. 1.5 ml of cells was collected at $10,000 \times g$ for 2 min, washed once with phosphate-buffered saline (0.15 M sodium chloride, 0.1 M sodium phosphate, pH 7.0) and resuspended in 250 μ l of 100 mM Tris-HCl (pH 8.0). After addition of 250 μ l of 100 mM Tris-HCl (pH 8.0), 1 M sucrose, the mixture was incubated for 10 min at 0 °C. After addition of EDTA (4 mM) and lysozyme (50 μ g/ml), 500 μ l of cold H₂O was added immediately. Cells were incubated on ice for 15 min, and the formation of spheroplasts was followed by phase contrast microscopy. MgCl₂ was added to a final concentration of 20 mM to stabilize the spheroplasts, which were subsequently collected at $10,000 \times g$ for 2 min and resuspended in 0.5 ml of spheroplast labeling medium (GB1 medium containing 250 mM sucrose, 10 mM MgCl₂). After 10-min incubation at 30 °C the spheroplasts were induced for the synthesis of PhoE by adding 1 mM IPTG or of PhoE-DHFR by adding 0.1% arabinose. After 5-min incubation with IPTG or 10 min with arabinose, the spheroplasts were pulsed for 1 min with 16.5 μ Ci of [³⁵S]methionine (Amersham Pharmacia Biotech), followed by a 5-min chase with 2 mM cold methionine. During the chase 50 μ l of periplasmic proteins (~ 1 mg/ml) were added when indicated, and in the case of a cross-link experiment also 1 mM bis(sulfosuccinimidyl) suberate (BS³) dissolved in labeling medium (Pierce). After 10 min the cross-linking reaction was quenched on ice with quench buffer (0.1 M glycine, 10 mM NaHCO₃, 250 mM sucrose, pH 8.5). The spheroplasts were subsequently collected by centrifugation for 2 min at $10,000 \times g$. Pellet and supernatant were either trichloroacetic acid-precipitated and immunoprecipitated or used for flotation gradient centrifugation. In release experiments the samples were not treated with a cross-linker. The pellet and supernatant fractions were trichloro-

acetic acid-precipitated and either directly loaded (1/15 of the total material) on SDS-polyacrylamide gel electrophoresis (PhoE) or immunoprecipitated (OmpA). The release values for PhoE presented in the text are an average of at least three experiments.

Pulse-chase Labeling—Cells were grown exactly as described above and pulsed for 30 s with [³⁵S]methionine (30 μ Ci per ml of cells) followed by a chase with cold methionine (final concentration, 2 mM). Samples were taken at several time intervals and precipitated with trichloroacetic acid.

Flotation Gradient Centrifugation—To purify membranes and membrane-associated protein complexes; crude membranes were subjected to flotation gradient analysis as described previously (28).

Pellet fractions from the spheroplast labeling experiment were resuspended in spheroplast-labeling medium and subsequently osmotically lysed by dilution with 9 volumes of cold water. The lysed cells were sonicated three times for 10 s. Non-lysed cells were removed by low speed centrifugation ($6000 \times g$ for 2 min). Subsequently, membranes were pelleted by centrifugation in a TLA120.2 rotor for 30 min at $300,000 \times g$ and loaded onto the bottom of a tube that was overlaid with sucrose. After centrifugation, the two top fractions, containing the membranes and membrane-associated proteins, were trichloroacetic acid-precipitated.

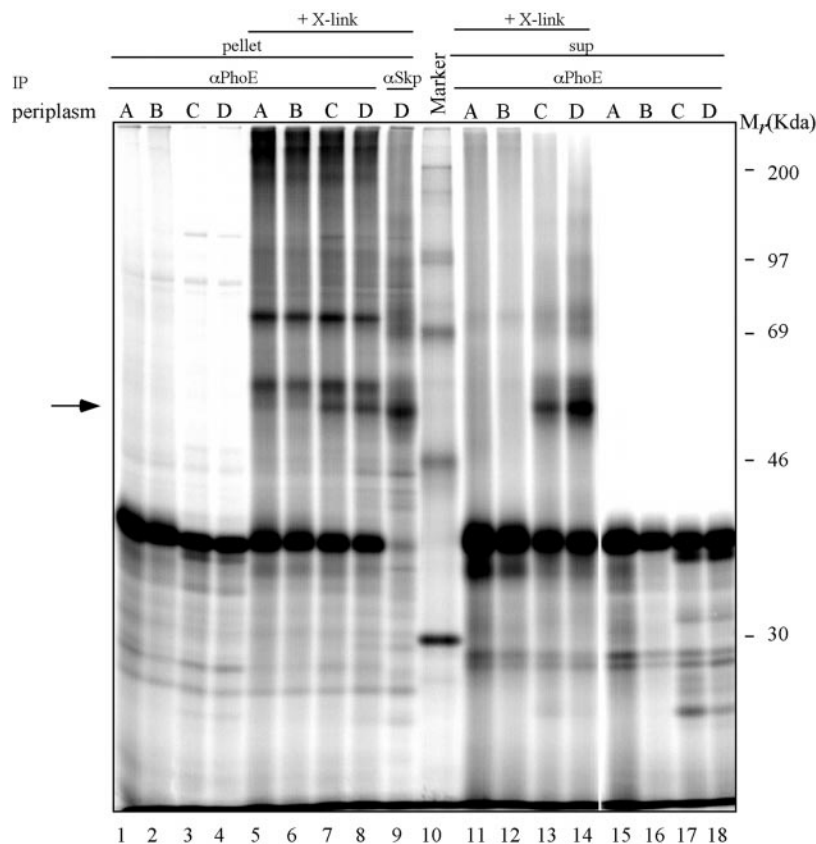
In Vitro Transcription and Translation—Ribosome nascent chain complexes (RNCs) were prepared as follows. pC4MethPhoE-derivative plasmids were linearized with *Hind*III and transcribed using a T7 polymerase MEGAscript kit of Ambion Inc. (Austin, TX). Translations were carried out as described previously (1) with the following modifications. A lysate was used from a trigger factor-depleted strain, and 100 μ M aurin tricarboxylic acid was added 2.5 min after the start of the translation to inhibit initiation. RNCs were purified by centrifugation over a sucrose cushion (29). Per standard translation reaction of 12.5 μ l the RNCs were resuspended in 2 μ l of RN buffer (100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM Hepes-KOH, pH 7.9), mixed with 10.5 μ l of periplasmic extract (~ 1 mg/ml), and treated with 1 mM BS³. *In vitro* translation of prePhoE was carried out as described previously (16). Either plasmid pJP29 (30) (Fig. 7) or pVG01 (24) (Fig. 3) was used. For targeting to the IM, vesicles were added at the start of the translation reaction, and cross-linking was carried out with 1 mM DSS as described (1). Translation reactions were transferred to ice and incubated for 5 min with chloramphenicol (30 μ g/ml) to stop further translations. Subsequently, mixtures were incubated with proteinase K (0.2 mg/ml) in the absence or presence of Triton X-100 (1% w/v). The protease reaction was stopped after 30-min incubation on ice by the addition of 1 mM phenylmethylsulfonyl fluoride.

Immunoprecipitations and Protein Analysis—Trichloroacetic acid pellets were either directly solubilized with SDS-sample buffer for SDS-polyacrylamide gel electrophoresis-precipitated proteins or solubilized in SDS-buffer and used in immunoprecipitation reactions under denaturing conditions. Immunoprecipitations were carried out as described previously (31). All samples were analyzed on 10 or 15% SDS-polyacrylamide gels. Co-immunoprecipitations of *in vitro* synthesized PhoE proteins with antibodies directed against Skp were performed as described previously (16). Radiolabeled proteins were visualized by phosphorimaging using a Molecular Dynamics PhosphorImager 473 and analyzed using the ImageQuaNT software from Molecular Dynamics.

RESULTS

Skp Interacts with PhoE at the Periplasmic Side of the Cytoplasmic Membrane—PhoE is translocated over the IM in a non-native state. Upon entering the periplasm, the protein has to be released from this membrane to assemble into the outer membrane in its native trimeric structure. We wanted to determine which chaperones and folding catalysts assist in these processes and to study the timing of the interaction with these accessory proteins. To that end, we used an unbiased approach to analyze the molecular environment of the newly translocated PhoE protein or PhoE translocation intermediates at the periplasmic side of the IM. MC4100 cells containing an inducible *phoE* gene were converted into spheroplasts, induced for the expression of PhoE, pulse-labeled with [³⁵S]methionine, and chased with cold methionine. Subsequently, cells were treated with the membrane-impermeable cross-linker BS³ either in the presence or absence of added periplasmic extract. After immunoprecipitating the PhoE proteins in the pellet or

FIG. 1. PhoE interacts with Skp in close proximity of the cytoplasmic membrane. *E. coli* cells of wild type MC4100 containing plasmid pNN100 were converted to spheroplasts, induced with IPTG, and pulse-labeled. During the chase either buffer (A) or a periplasmic extract was added from an *skp* mutant (B), wild type (C), or an Skp-overproducing strain (D). In addition, during the chase the cross-linker BS³ was added (lanes 5–9, 11–14). The spheroplasts were collected by centrifugation, and the pellet and membrane fractions were used in immunoprecipitation experiments with either α PhoE or α Skp. The cross-link product of PhoE with Skp is indicated with an arrow.



supernatant fractions with anti-sera against PhoE, proteins that interact with this OMP were identified. As shown in Fig. 1, lanes 13 and 14, a cross-linking product with a molecular mass of ~52 kDa was found in the supernatant fraction. The 52-kDa product corresponds in size to the combination of one molecule of PhoE (36 kDa) and one Skp (16 kDa). The band was absent when periplasmic proteins were not added (lane 11) or when a periplasmic extract of a *skp* mutant was used (lane 12). These data indicated that Skp closely interacts with PhoE during or after the process of secretion into the spheroplast medium, as was shown previously (16).

In the spheroplast pellet fraction cross-linking products of ~71, 57, and 52 kDa were detected (lanes 5–8), suggesting that these products are still associated to the IM. The intensity of the 71- and 57-kDa bands was independent of the addition of periplasmic proteins. This indicates that the cross-linking adducts were not periplasmic, but membrane-associated proteins. The cross-linking adducts were not further identified, but the size of the 71-kDa product suggests that this represents a PhoE dimer. The band of 52 kDa could be immunoprecipitated with a specific antiserum raised against Skp (lane 9). A faint reaction was also obtained without the addition of a periplasmic extract containing Skp (lanes 5 and 6), indicating that the residual amount of Skp associated with the spheroplasts interacted with PhoE. These results showed that Skp interacts with PhoE at the level of the IM. It is, however, also conceivable that the Skp-PhoE cross-linking product in the pellet fraction represented an interaction of Skp with PhoE aggregates. To distinguish between these possibilities, spheroplasts were lysed and IMs were purified by flotation gradient centrifugation (Fig. 2). The vast majority of PhoE protein was associated with the membranes present in fraction 2 (see lanes 3, 4, and 6). In this fraction the IM protein YidC (32) was detected as a control protein (results not shown). Furthermore, the typical three cross-linking products of 72, 57, and 52 kDa were also detected

in this membrane fraction. The 52-kDa band could be immunoprecipitated with either α PhoE (lane 6) or α Skp (lane 8). The three cross-linking bands could also be obtained when the cross-linking was carried out after the flotation (lane 4). These results showed that the Skp-PhoE interaction occurs in close proximity of the membrane, resulting in a membrane-associated PhoE-Skp complex.

The interaction of Skp with PhoE at the membrane was also investigated *in vitro*. The precursor of PhoE was synthesized in an *E. coli*-derived translation system in the presence of inverted IM vesicles. The precursors could be imported into these vesicles with an efficiency of ~25% as was monitored by their resistance against externally added proteinase K (Fig. 3, lanes 1, 4, and 7). This efficiency of targeting and insertion was also found in other studies with *in vitro* synthesized prePhoE (33). Cross-linking with the membrane-permeable cross-linker DSS gave rise to a cross-linking product of 52 kDa that could be immunoprecipitated with anti-serum directed against PhoE (lanes 2, 5) and Skp (lanes 3 and 6). This cross-linking product must have been formed, at least for a large part, inside the vesicles (periplasmic side), because it was resistant to externally added proteinase K (lanes 5 and 6). Importantly, this proteinase K-resistant cross-linking product could not be obtained when vesicles were used isolated from a *skp* mutant (lanes 13 and 14). The 52-kDa cross-linking product that could be immunoprecipitated with anti-Skp (lane 11) was degraded by the protease (lane 14), indicating that the cross-linking adduct origins from the lysate, which was isolated from the wild type.

Skp Interacts with a Translocation Intermediate of PhoE—We subsequently wanted to determine whether Skp interacts with PhoE during translocation across the IM. Previously, it was demonstrated that a PhoE-LacZ hybrid protein spans the IM, with the PhoE moiety in the periplasm and the β -galactosidase moiety in the cytoplasm. In addition, such LacZ

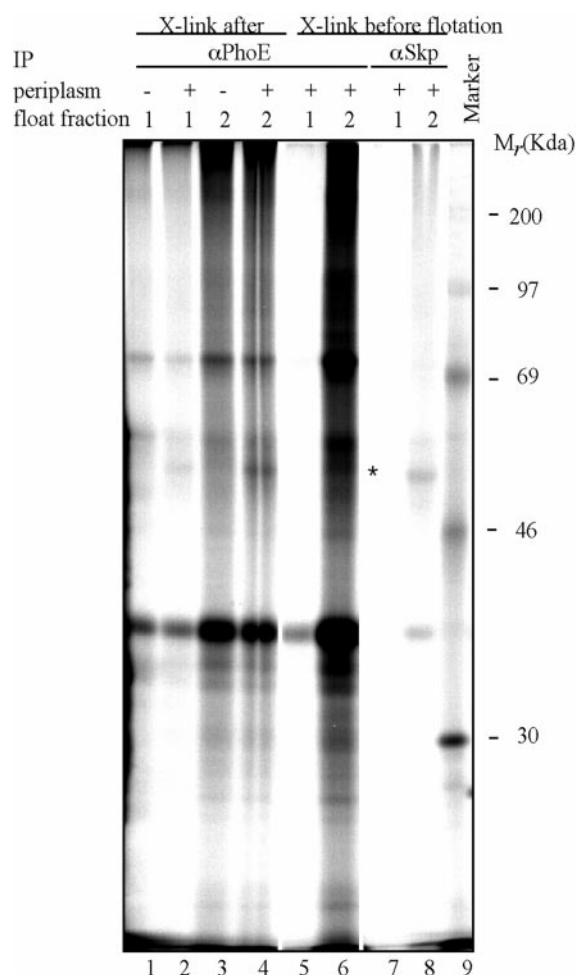


FIG. 2. The PhoE-Skp complex is membrane-associated. *E. coli* cells of wild type MC4100 containing plasmid pNN100 were converted to spheroplasts, induced with IPTG, and pulse-labeled. During the chase periplasmic extract was added from an Skp-overproducing strain together with the cross-linker BS³ (lanes 5–8). Membranes were isolated and loaded on a flotation gradient. The gradient was fractionated into four fractions, and the upper two fractions are shown. The fractions were used in immunoprecipitation experiments with either α PhoE (lanes 5, 6) or α Skp (lanes 7, 8). Lanes 1–4 contain the proteins that are present in membrane fractions that were cross-linked after the flotation. An asterisk indicate the cross-link product of PhoE with Skp.

fusion proteins were shown to block translocation of other proteins across the IM, due to jamming of the Sec pathway (34). In other studies dihydrofolate reductase (DHFR) was used to create OmpA-DHFR translocation intermediates that have similar effects as LacZ fusions (35) (36). We made use of this strategy to study the environment of PhoE translocation intermediates. We constructed a hybrid protein containing the N-terminal 300 amino acid residues of PhoE fused to mouse DHFR under control of the inducible arabinose promoter. Upon induction with arabinose, cells harboring the *phoE300-dhfr* gene fusion synthesized the hybrid protein and stopped growing (results not shown). In a pulse-chase experiment it could be demonstrated that under non-inducing conditions relatively low amounts of the hybrid protein were synthesized (Fig. 4a). In these cells, the OmpA protein was rapidly processed to the mature form (Fig. 4c). Upon induction with arabinose the PhoE-DHFR hybrid protein was synthesized to increased amounts and both the hybrid precursor as well as the mature form were present even after 30 min (Fig. 4b). In addition, the synthesis of OmpA was reduced and proOmpA accumulated, indicating that it could not be translocated across the IM (Fig. 4d). These results indicated that the synthesis of the PhoE-

DHFR hybrid protein causes jamming of the Sec translocase. Proteinase K treatment of spheroplasts that were induced for synthesis of the hybrid protein and subsequently pulse labeled, showed that the PhoE moiety of the mature hybrid protein was degraded by the protease, whereas the DHFR moiety was not (results not shown). These data demonstrate that the PhoE-DHFR hybrid protein is in a transmembrane orientation, because it is accessible for protease added from the outside and it blocks the translocation apparatus. The vast majority of the translocons must be occupied with this fusion protein, because an efficient block in protein export is observed.

To analyze the environment of these translocation intermediates, spheroplasts were induced with arabinose, pulse-labeled in the presence of additional periplasmic proteins, and subsequently treated with the soluble cross-linker BS³. In the pellet fractions several cross-linked bands could be identified after immunoprecipitation with antisera raised against DHFR (Fig. 5, lane 3). The strong cross-linking product of ~97 kDa possibly represented a dimer of the hybrid protein. The very weak band of ~65 kDa could also be immunoprecipitated with antiserum raised against Skp (lane 2). In addition, by using sequential immunoprecipitation we found that this cross-linking product could be immunoprecipitated first by anti-Skp and subsequently by anti-PhoE (lane 1). The 65-kDa cross-linking product was membrane-associated, because it was found in the same two fractions of a flotation gradient as the inner membrane protein YidC (32) (results not shown). These results demonstrated that the PhoE-DHFR hybrid protein interacted with Skp and that the interaction of Skp with PhoE occurred during translocation, *i.e.* when PhoE is in a transmembrane orientation. The amount of PhoE-DHFR-Skp complex found in this experimental setup is much less compared with the amount of PhoE-Skp found in the experiments described above and shown in Fig. 1. It is possible that cross-linking targets are not properly exposed in the hybrid protein, but it is also conceivable that a C-terminal PhoE-truncate binds less efficiently to Skp compared with the full-length protein.

The Skp Binding Site(s) in PhoE—The results obtained so far indicate an early interaction between Skp and the first 300 amino acids of PhoE protein. To determine which regions of the PhoE protein are important for Skp binding, we investigated whether Skp is able to bind to shorter, nascent chains of PhoE. PhoE ribosome nascent chain complexes (RNCs) were synthesized *in vitro*, isolated by centrifugation over a sucrose cushion, incubated with a periplasmic extract, and subsequently treated with BS³. In the past we found that nascent chains as long as 50 amino acids could interact with trigger factor (2). Because trigger factor will block binding sites on the nascent chain in these experiments, it is required to use a trigger factor-depleted lysate. In addition we used constructs that lack a signal sequence to mimic the situation at the periplasmic side of the membrane as good as possible. As shown in Fig. 6a, an RNC containing 137 amino acids of PhoE gave rise to cross-linking products of ~32 and ~48 kDa (lane 6). Both cross-linking products could be immunoprecipitated with α Skp (lane 7) and represent most probably complexes between the 137-mer of PhoE and monomeric and oligomeric forms of Skp. The cross-linking products were not detected when the isolated RNC complexes were incubated with control buffer (lane 2–4). Similar results were obtained with a shorter version of the PhoE protein. RNCs of a 100-mer of PhoE resulted in cross-linking products of 29 and 45 kDa that could be immunoprecipitated with α Skp (Fig. 6b, lanes 5, 6). These products were found when a wild type periplasm was used but were absent when an extract was used of a *skp* mutant strain (lanes 2, 3). The 100-mer of PhoE exposed ~65 amino acid residues out of the

FIG. 3. *In vitro* synthesized PhoE binds to Skp at the periplasmic side of the inner membrane. PrePhoE was synthesized *in vitro* using an S135 extract in the presence of inner membrane vesicles isolated from wild type (lanes 1–8) or from an *skp* mutant (lanes 9–16). After translocation, the samples were treated with the membrane permeable cross-linker DSS and either directly immunoprecipitated or after treatment with proteinase K. An asterisk indicates the cross-link product of PhoE with Skp. Molecular masses are indicated in kDa.

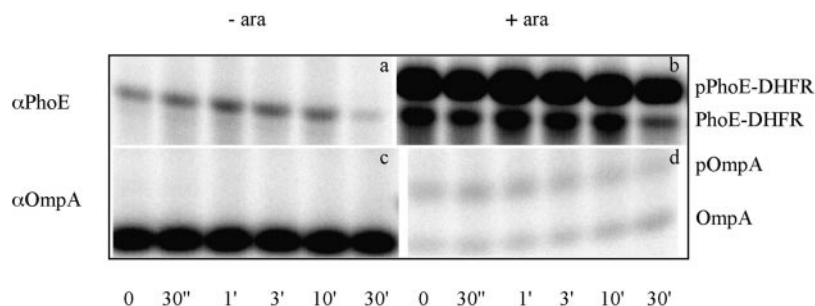
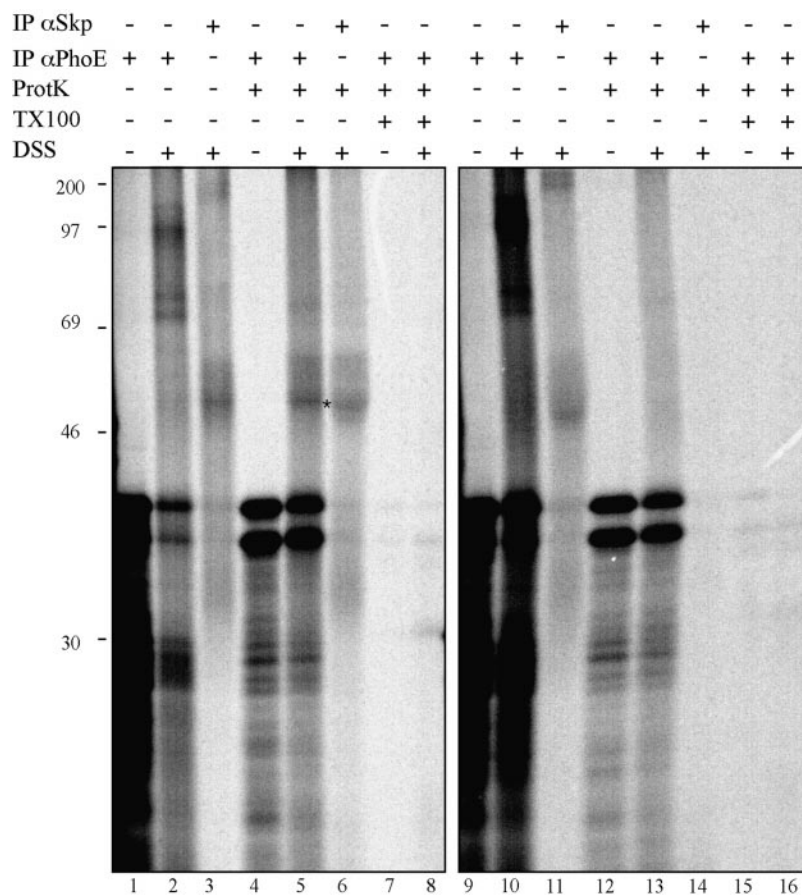


FIG. 4. 300PhoE-DHFR forms a transmembrane translocation intermediate. The translocation of 300PhoE-DHFR and OmpA was studied in a pulse-chase experiment. MC4100 containing para300PhoE-DHFR was incubated with (b, d) or without (a, c) arabinose. The cells were immunoprecipitated with either α PhoE (a, b) or α OmpA (c, d).

ribosome, indicating that a binding site for Skp is present at the extreme N terminus of PhoE.

Interaction of Skp with Mutant PhoE Proteins Synthesized *In Vitro*—De Cock *et al.* (16) demonstrated that Skp interacts with PhoE proteins that were synthesized *in vitro*, because these PhoE proteins were specifically precipitated with antibodies directed against Skp. The absence or presence of the signal sequence did not interfere with complex formation. We used this approach to study the Skp-binding site of PhoE in more detail by using a set of PhoE mutants with internal deletions in the mature part of the protein. Wild type and mutant PhoE proteins were synthesized in an *E. coli* derived *in vitro* system (Fig. 7, TL). After synthesis, purified Skp was added followed by the addition of puromycin to release all nascent chains. PhoE proteins associated to Skp were immunoprecipitated with antibodies directed against Skp. In all cases PhoE:Skp complexes could be precipitated (Fig. 7, α Skp). However, the relative amount of PhoE that could be co-precipitated varied between the different mutants (Table I). Deletion of amino acids 3–11 in the mature portion of PhoE protein reduced Skp binding efficiency only moderately by \sim 35% as compared with full-length PhoE protein. However, larger deletions in the N-

terminal part of PhoE protein reduced the binding efficiencies much further, *i.e.* $>90\%$. A deletion of amino acids 48–146 reduced the efficiency of binding another 20-fold to $>99\%$. This can be explained by assuming that two independent Skp-binding sites exist in the N-terminal part of PhoE protein, *i.e.* between 3–91 and 110–174 PhoE. Deletion of amino acids 48–146 would delete a major part of both sites resulting in an almost complete reduction of binding to Skp.

A mutant PhoE protein containing the N-terminal 204 amino acids, but lacking the C-terminal part (Fig. 7, lane 9) leads to a reduction of the binding efficiency with \sim 65%, suggesting that indeed the N-terminal part of PhoE protein contains the most important Skp-binding sites. Interestingly, however, a smaller deletion in the C-terminal part of PhoE, amino acids 249–298 (Fig. 7, lane 7), reduced the efficiency much further ($>90\%$). In conclusion, the N-terminal region seems to be important for Skp binding whereas different parts of the C terminus of PhoE seem to influence the binding to these N-terminal sites. However, it cannot be excluded that differences in folding characteristics of the various deletion mutants affect Skp binding due to alterations in exposure of the Skp-binding sites.

Skp Is Not Responsible for the Release of PhoE from the Inner

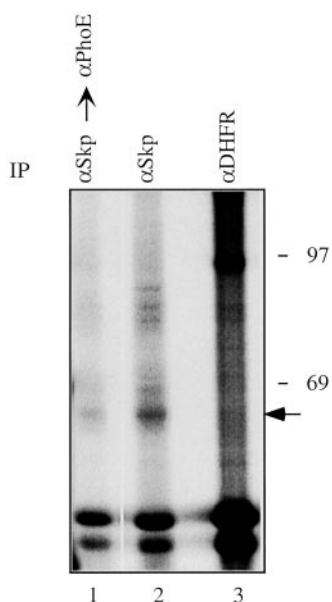


FIG. 5. The 300PhoE-DHFR translocation intermediate interacts with Skp. MC4100 containing pAra300PhoE-DHFR was converted to spheroplasts, induced with arabinose, and pulse-labeled. During the chase, periplasm from an Skp-overproducing strain and the cross-linker BS³ was added (*lanes 1–3*). Pellet fractions were used in immunoprecipitation experiments with α DHFR (*lane 3*), α Skp (*lane 2*), or α Skp followed by α PhoE (*lane 1*). The cross-link product of 300PhoE-DHFR with Skp is indicated with an arrow.

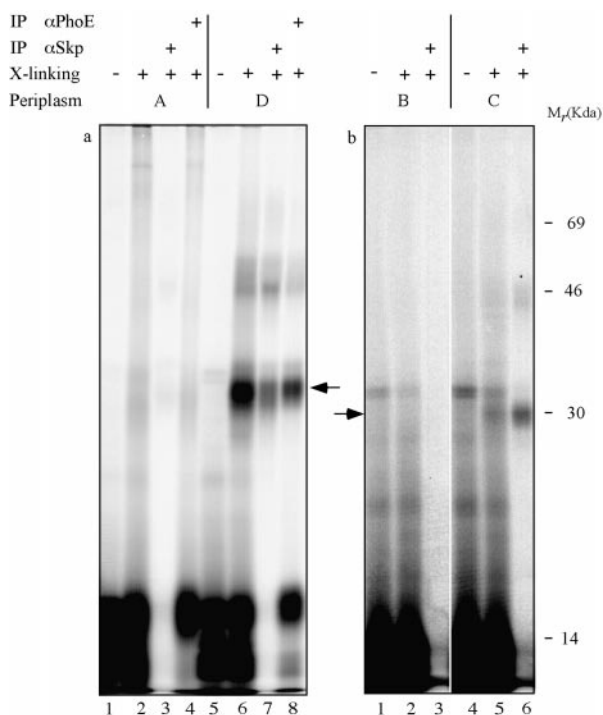


FIG. 6. Skp interacts with ribosome nascent chain complexes of PhoE. *a*, ribosome nascent chain complexes of 137 amino acids of PhoE were synthesized, purified over a sucrose cushion, incubated with buffer (A) (*lanes 1–4*) or a periplasmic extract of an Skp-overproducing strain (D) (*lanes 5–8*), and subsequently cross-linked with BS³. *b*, a 100-amino acid nascent chain was used and either incubated with a periplasmic extract of an *skp* mutant (B) (*lanes 1–3*) or of a wild type strain (C) (*lanes 4–6*). Cross-linked products were identified by immunoprecipitation with either α PhoE or α Skp. The cross-link product of PhoE with Skp is indicated with an arrow.

Membrane—Recently, it was proposed that Skp is required for the release of OmpA protein from spheroplasts. Furthermore, Skp was proposed to be involved in the acquirement of a soluble

periplasmic form of newly translocated OMPs (15). From the data presented in Fig. 1 (*lanes 1 and 15*) we observed that PhoE remained associated to the membrane to a large extent. Addition of a periplasmic extract isolated from a Skp-overproducing strain (Fig. 1, *lanes 4 and 18*) did not significantly influence the amount of released PhoE. To investigate the release of PhoE from the spheroplasts we carried out release experiments and found that 55% of PhoE remained associated to the membrane in a wild type strain. It has been reported that the amount of Skp in spheroplasts is lower in the presence of bivalent cations (14). When we leave out magnesium ions from our spheroplast preparations, the amount of PhoE that remained associated to the membranes was slightly increased (63%). In these spheroplast preparations the release of OmpA into the supernatant was 90%. On the contrary, in the strain lacking Skp protein, PhoE and OmpA were both for a large part detected in the pellet fraction (65 and 61%, respectively). The data indicate that Skp is required for the release and formation of a soluble form of OmpA protein, whereas the release of PhoE seems to be independent of Skp.

DISCUSSION

Proteins destined for the extracytoplasmic environment pass the IM in a non-native conformation. Upon entering the periplasm, these proteins have to be protected immediately against degradation, aggregation, and misfolding. To that end, periplasmic chaperones are supposed to be involved in directing these proteins into a productive folding and/or assembly pathway. Little is known which periplasmic chaperones and folding catalysts are involved in the late stages of IM translocation and the early stages of folding. We used an unbiased approach to study the early interactions in the periplasm of accessory proteins with the OMP PhoE, and we found an early interaction with the periplasmic chaperone Skp. It has been suggested that Skp plays an important role in these early events as a specific chaperone for OMPs (15, 16), but the timing and the location of this interaction was not clear. The data presented in this paper showed that the initial interaction of PhoE with Skp occurs at the periplasmic side of the IM. A hybrid protein consisting of 300 N-terminal amino acids of PhoE protein fused to mouse DHFR formed a transmembrane translocation intermediate. This hybrid protein interacted with Skp as monitored by protein cross-linking, indicating that PhoE interacts with the chaperone already during translocation. In addition, the data implicate the presence of one or more Skp-binding sites in the N-terminal region of PhoE. Indeed, we found that Skp was able to closely interact with an N-terminal PhoE fragment of 65 amino acid residues in *in vitro* cross-linking experiments. Furthermore, deletions in the N-terminal as well as the C-terminal part of the PhoE protein resulted in reduction of precipitation of PhoE with α Skp. The data can be explained by assuming the presence of two Skp-binding regions in the N terminus of PhoE. Absence of certain parts of the C terminus in PhoE mutant proteins might influence the conformation of this protein, and, as a consequence, the exposure of binding sites is different. The immunoprecipitation experiments were performed in the absence of membranes, which might influence the manner and extend to which Skp interacts with PhoE protein. However, it should be emphasized that detergents are present during immunoprecipitations, which can be regarded as a membrane-mimicking environment. Taken together, the results demonstrated that Skp interacts with PhoE protein once the extreme N-terminal part, containing these binding sites, appears in the periplasm. We therefore suggested that early translocation intermediates of OMPs are already recognized by this molecular chaperone. It is unlikely that the specific interaction of Skp with OMPs is due to recog-

FIG. 7. Immunoprecipitations of *in vitro* formed complexes between Skp and wild type or mutant PhoE proteins with Skp antiserum. Wild type and mutant PhoE proteins were synthesized as described under "Experimental Procedures." Part of translations mixtures (TL) were analyzed directly (5 μ l) demonstrating the position of all translation products (indicated by a small arrow) and chloramphenicol acetyltransferase (Cat). The other part (45 μ l) was analyzed after immunoprecipitations with Skp antiserum (α -Skp). Lanes: 1, mPhoE; 2, Δ 3-11; 3, Δ 3-18; 4, Δ 3-42; 5, Δ 8-91; 6, Δ 110-174; 7, Δ 249-298; 8, Δ 63-287; 9, Δ 204-330; 10, Δ 56-93; 11, Δ 48-146.

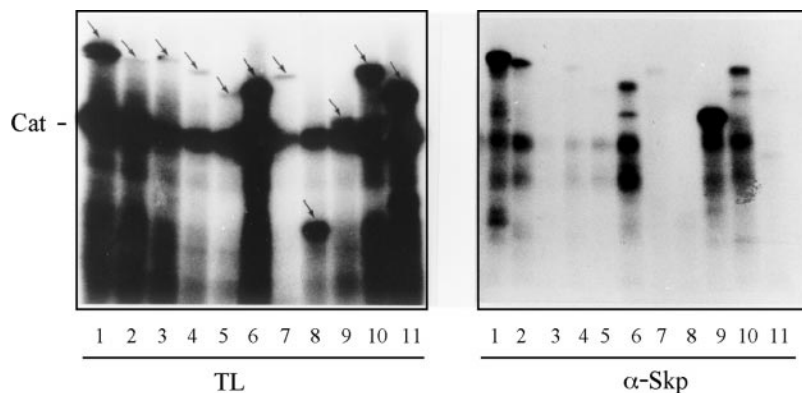


TABLE I

Relative efficiency of immunoprecipitations of PhoE proteins with Skp

Deleted areas in the mutant proteins are indicated by numbers, *i.e.* the deletion extends from amino acid in position *X* to the *Y*th amino acid in the mature part. The efficiency of immunoprecipitation of wild type (WT) is set at 100% (amount precipitated with Skp anti-serum as a fraction of full-length PhoE protein synthesized: on average 17% \pm 3%). The efficiency of immunoprecipitation of the various mutant proteins is expressed as a fraction of that amount.

Protein	Relative efficiency
WT (mPhoE)	100
Δ 3-11	65
Δ 3-18	7
Δ 3-42	8
Δ 8-91	8
Δ 110-174	4
Δ 249-298	5
Δ 63-287	3
Δ 204-330	33
Δ 56-93	5
Δ 48-146	0.2

dition of a linear amino acid sequence, because the extreme N terminus of PhoE protein does not reveal a conserved sequence. It is therefore more likely that the basis for the selective interaction of Skp with OMPs is at the level of recognizing certain structural elements, possibly β -strands or β -sheets.

Previously, it was demonstrated that Skp exists in two different states characterized by their different sensitivities to proteases *in vivo* (16). In addition, Skp was localized as a soluble periplasmic as well as a membrane-associated protein. Both forms have apparently the ability to interact with OMPs. In co-immunoprecipitation and cross-linking experiments to ribosome-nascent chains, the soluble, protease-sensitive form was able to form a soluble complex with OMPs. Here, we demonstrated that full-length PhoE protein, imported into IM vesicles, interacts with Skp that is co-purified with IM vesicles, most likely due to association to the membrane. Similar results have been reported previously for OmpA (15). Interestingly, we were not able to detect an interaction of membrane-associated Skp with the PhoE-DHFR hybrid. We had to add additional periplasmic extract, containing soluble Skp, to obtain cross-linking. In contrast, cross-linking of Skp to full-length PhoE protein is possible without the addition of additional periplasmic proteins but the efficiency of cross-linking does increase when periplasm is added. These results do not necessarily mean that the membrane-associated form of Skp is not able to interact with the N-terminal portion of the PhoE protein in the fusion protein *per se*. It might be that this part has no cross-linking targets in close proximity of Skp, but it is also possible that it has a reduced affinity for binding to membrane-associated Skp, thereby decreasing the possibility for detection. In addition, it is possible that the protease-sensitive Skp that

was added with the periplasmic extract is converted to the resistant form in the spheroplast preparations. This would raise the Skp concentration to higher levels at the membrane and allow detection of interaction with the fusion protein by cross-linking. We propose that the initial interaction of PhoE translocation intermediates occurs with the membrane-associated form of Skp.

Schäfer *et al.* (15) suggested that Skp is involved in generating and maintaining the solubility of early folding intermediates of OMPs in the periplasm. In contrast to OmpA, however, release of the PhoE protein is not dependent on Skp. PhoE remains associated to the membrane to a large extent even in wild type spheroplasts. The release of PhoE from the spheroplasts was not significantly influenced by bivalent cations or the addition of addition of periplasmic proteins. Possibly, *de novo* synthesis of another or an additional component is necessary to get release of PhoE or the PhoE:Skp complex from the cytoplasmic membrane. It is not clear why the porin protein PhoE behaves differently as compared with OmpA in this respect. One possibility is that the assembly of the trimeric porins requires a different assembly route as compared with the monomeric protein OmpA. In this context it is interesting to mention that assembly of porins but not OmpA requires *de novo* synthesis of lipidic components (either lipopolysaccharide and/or phospholipids) (37-40). Possibly, the observed differences in the release of OmpA *versus* that of the PhoE protein from spheroplasts is a reflection of this difference in the assembly route.

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