Requirement for Conformational Flexibility in the Signal Sequence of Precursor Protein*

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According to the “unlooping” model (de Vries, T., Batenburg, A. M., Killian, J. A., and de Kruijff, B. (1990) Mol. Microbiol. 4, 143-150), proposed to explain how signal sequences serve to target proteins into the secretory pathway, the initial interaction of the signal sequence with the membrane in a helix-turn-helix conformation (spanning half of the bilayer) plays an important role in the initiation of the translocation reaction. To test this model we have introduced 2 cysteines (at positions -5 and -19) in the signal sequence of the Escherichia coli outer membrane protein PhoE. The mutations did not influence the translocation of precursor PhoE in vitro or in vitro. The 2 cysteines were oxidized to form a disulfide bridge. In vitro translocation of the looped precursor into inner membrane vesicles was disturbed. The looped precursor competed with translocation of wild type precursor PhoE, and looped precursor that was first bound to inner membrane vesicles could be translocated after the addition of dithiothreitol. Apparently, the mutant precursor with a disulfide bridge in the signal sequence is arrested as a very early intermediate in the translocation process. All of these results are consistent with the proposed unlooping model and show that, besides the primary structure characteristics of a signal sequence, conformational flexibility is needed to initiate the translocation reaction.

Signal sequences serve to target proteins into the secretory pathway both in prokaryotic and in eukaryotic cells. Their exact role in the export mechanism is still far from understood. Signal sequences appear to interact with proteins of the export apparatus such as SecA (1, 2), SecE and SecY (3), or the Ffh protein (4), which seems to be a component of the bacterial analogue of the signal recognition particle. Other lines of evidence, both in biophysical and biological systems, point to a direct interaction between signal sequences and membrane lipids (5-8). In agreement with these data, biophysical studies revealed a strong interaction between a synthetic copy of the signal peptide of the outer membrane protein PhoE of Escherichia coli and lipids in model membrane systems (9, 10). Experiments with lipid monolayers resulted in the proposal of a mechanistic model for the involvement of signal sequences in the translocation process, i.e., the “unlooping” model (11). A similar kind of model was proposed on the basis of the statistically frequent occurrence of alpha-helix destabilizing amino acids in the central region of the signal sequence (12) and can be extrapolated from nuclear magnetic resonance data on the conformation of the signal sequence of OmpA and LamB in a membrane mimetic environment (13, 14). The essential parts of the unlooping model are as follows. First, the positively charged NH2 terminus of the signal sequence interacts with the head groups of negatively charged phospholipids in the membrane. Subsequently, the signal sequence interacts as a loop in the core of the bilayer leaving the NH2 and COOH termini at the cytoplasmic face of the membrane. This helix-turn-helix motif, spanning half of the bilayer, could serve as a recognition motif for proteinaceous components of the translocation machinery and/or might give rise to local changes in the lipid organization, which possibly could form a transient state in the translocation process. After this stage, the signal sequence unloops and moves the NH2 terminus of the mature sequence across the bilayer. This unlooping of the signal sequence is thought to be the initiation of the translocation process. To test this model in the context of a complete precursor protein, we have introduced cysteines at strategic positions in the signal sequence of PhoE to enable the stabilization of the putative looped signal sequence by the formation of an intramolecular disulfide bridge. The behavior of the looped precursor was investigated in a well defined in vitro translocation system. The results show that conformational flexibility in the signal sequence is needed for the initiation of the translocation process.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The E. coli K12 strains used were CE1344 (F-, ΔlacU169 araD139 rpsL thi rela ropT::TnlO) (15), K003 (F-, Δun-cBC::TnlO) (16), CE1224 (F-, thr leu ΔproA-B-phen-gpt his thi arg Y galK xyl rpsL supE supR) (17), and MM52 (F-, ΔlacU169 araD139 rpsL thi relA secA51) (18).

DNA Manipulations and Plasmids—Plasmid DNA was purified as described (19), followed by anion-exchange chromatography on QIagen columns (Diagen, Dusseldorf, Germany). Recombinant DNA techniques were performed essentially as described (20). Restriction endonuclease reactions and T4 DNA ligase treatments were performed as described by the manufacturers of the enzymes (Pharmacia Biotech Inc. and New England Biolabs Inc.). The mutagenic oligonucleotides d(5'GGCCAGGTGCTA-CATTCCATT-3') and d(5'GCCAGGTTGCTGTA-CATTCCATT-3'), which were used for site-specific mutagenesis, were synthesized on a Biosearch 8600 DNA synthesizer. Mutagenesis was performed via a two-step polymerase chain reaction (21). Mutations were confirmed by double-stranded DNA sequencing using the T7 DNA polymerase sequencing kit (Pharmacia). Plasmids pJP29 (22) and pJP320 (23) contain the phosph gene. Plasmid pNN100 was created by cloning the BamHI-EcoRV fragment of pJP320 containing phosph into the expression vector pJF118 E/H (24), digested with HindIII and (after filling in the protruding ends with Klenow fragment of E. coli DNA polymerase) with BamHI. In the resulting plasmid, PhoE is expressed under control of the tac promoter. Plasmids pNN1 and pNN3 were created by site-specific mutagenesis of pJP29, and the double mutant plasmid pNN4 was subsequently obtained from pNN3. The corresponding mutations are listed in Fig. 1. To achieve overproduction of the mutant PhoE proteins, the PacI-ClaI fragments containing the mutations were substituted for the corresponding fragment of plasmid pNN100, creating plasmids pNN101, pNN103, and pNN104 and placing the mutant phosph genes under control of the tac promoter.

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Pulse Labeling Experiments—Cells, grown with aubie under phosphate limitation to induce the expression of PhoE protein, were labeled with 2.5 \( \mu \)Ci of \( \left[^{35}S\right]\)methionine for 30 s at 30 °C and subsequently chased with an excess of nonradioactive methionine as described (22). Radiolabeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (25), followed by autoradiography.

Proteins—Wild type and mutant forms of prePhoE were purified as described (26) with some modifications. Overexpression of prePhoE was achieved in an mutant strain MM52 containing plasmids with phoE under control of the tac promoter. Cells were grown at 30 °C in Luria broth until an optical density at 660 nm of 0.7 was reached. Then, PhoE was pelleted by centrifugation for 30 min at 140,000 g, and the pellet was dissolved in translocation buffer and incubated for 1 h at 37 °C.

For analysis, the samples were repurified by chromatography on a MonoQ column as described (26). After proteinase K treatment to digest nontranslocated precursors, the samples were analyzed with the modification that urea-stripped inner membrane vesicles from strain TLA100.2 rotor. The pellet was dissolved in translocation buffer and centrifuged for 30 min at 80,000 g. The pellet was dissolved in 8 M urea, 50 mM Tris/HCl, pH 8.0. The material was repurified by chromatography on a MonoQ column as described (26).

Ferricyanide Treatment of PrephoE—PrephoE was purified (1 mg/ml in 8 mM urea, 50 mM Tris/HCl, pH 8.0, 2 mM DTT) diluted 8-fold into 10 mM potassium ferricyanide and incubated for 1 h at room temperature. Trichloroacetic acid was added to a final concentration of 10%. After a 30-min incubation on ice, the mixture was centrifuged at 12,000 \( \times g \). The resulting precipitate was washed twice with acetone and dissolved in 8 mM urea, 50 mM Tris/HCl, pH 8.0. The material was repurified by chromatography on a MonoQ column as described (26).

Translocation of prePhoE—Translocation of purified prePhoE into inner membrane vesicles was carried out as described (26) with the modification that DTT (10 mM end concentration) was only present when reducing circumstances were essential. After proteinase K treatment to digest nontranslocated precursors, the samples were analyzed with SDS-PAGE under nonreducing conditions. prePhoE and PhoE proteins were detected with an alkaline phosphatase-conjugated secondary antibody (Tago, Inc.). The alkaline phosphatase activity was visualized with a solution of 0.1 mM diazotized diazoaminoen (pH 8.0, 1.1 mM MgCl\(_2\), 0.35 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.37 mM nitro blue tetrazolium.

Uncoupling of Binding and Translocation of prePhoE into Inner Membrane Vesicles—prePhoE was mixed with SecA, SecB, ATP, and inner membrane vesicles in a standard translocation reaction, the mixture was layered on a sucrose cushion (0.5 mM sucrose, 40 mM Tris acetate, pH 8.0, 4 mM magnesium acetate, 28 mM potassium acetate), and vesicles were pelleted by centrifugation for 30 min at 140,000 \( \times g \) in a TLA100.2 rotor. The pellet was dissolved in translocation buffer and split into three fractions. One was used to determine the amount of pelleted material. To the other two fractions, ATP (4 mM end concentration) was added, and a translocation reaction was performed in the presence or absence of DTT. After a 20-min incubation at 37 °C, proteinase K was added, and the protected material was analyzed with SDS-PAGE and Western immunoblotting (29) using a polyclonal antiserum directed against PhoE and an alkaline phosphatase-conjugated secondary antibody (Tago, Inc.). The alkaline phosphatase activity was visualized with a solution of 0.1 mM diazotized diazoaminoen (pH 8.0, 1.1 mM MgCl\(_2\), 0.35 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.37 mM nitro blue tetrazolium.

Translocation ATPase Activity—To assay translocation ATPase activity, the same conditions were applied as in the translocation reaction, with the modification that urea-stripped inner membrane vesicles from strain TLA100.2 were incubated at 0 °C. The reactions were incubated at 37 °C for 10 min in a water bath. The reactions were stopped by the subsequent additions of 800 \( \mu \)l of color reagent (0.68% malachite green (Sigma) and 10.5 g/liter ammonium molybdate in 1 N HCl and 0.1% Triton X-100) and 100 µl of 34% citric acid. After 30 min at room temperature, the amount of released inorganic phosphate was photometrically determined (30) and compared with a standard curve prepared with potassium hydrogen phosphate. In a typical experiment, the reactions were performed in triplicate, and, after subtraction of the ATPase activity without the addition of precursor protein, the results were compared with the translocation ATPase activity of wild type PhoE in the presence of DTT. The standard deviation was approximately 10%.

Translocation Competition Experiment—[\(^{35}S\)]Methionine-labeled wild type prePhoE (0.5 pg/µl) was mixed with an equal amount of nonradioactive mutant prePhoE. As controls, radiolabeled prePhoE was mixed with an equal amount of bovine serum albumin, which was dissolved in 8 mM urea, 50 mM Tris/HCl, pH 8.0, or wild type PhoE. These different mixtures were used in a translocation reaction. The translocated and, after inactivation of the protease with phenylmethylsulfonyl fluoride and trichloroacetic acid precipitation, the samples were analyzed by SDS-PAGE.

RESULTS

Translocation of Cysteine-containing Mutant Precursors in Vivo—To examine the role of the signal sequence in the translocation process, we decided to introduce cysteines in the signal sequence of PhoE by site-directed mutagenesis (Fig. 1). The selective introduction of cysteine residues gives unique properties to the mutant precursor due to the high chemical reactivity of the aminoacyl side chain and to the fact that wild type prePhoE does not contain any cysteine. The positions of the cysteines in the double mutant protein were chosen such that the side chains are in close proximity when the signal sequence is in a helix-turn-helix conformation according to the computer model for the PhoE signal sequence (31). To test whether the introduced cysteines influence the translocation of PhoE in vivo, pulse-chase experiments were performed. All cysteine-containing mutant proteins were processed with similar kinetics as the wild type protein (Fig. 2). Since processing takes place at the periplasmic face of the membrane, these mutations apparently had no deleterious effect on the translocation of PhoE in vivo.

Translocation of Oxidized Double Mutant Precursor in Vitro—The purified mutant precursors translocated with wild type efficiency into inner membrane vesicles in a standard in vitro translocation reaction (results not shown). To introduce an intramolecular disulfide bridge in the signal sequence, the purified double mutant precursor containing 2 cysteines was oxidized with potassium ferricyanide and, after resolation, used in an in vitro translocation reaction. The oxidized precursor did not translocate into inner membrane vesicles under reducing circumstances (Fig. 3D, lane 7). The precursor was translocated when DTT was added (lane 8). To exclude the possibility that a nonspecific modification, which might occur during the ferricyanide treatment, directly abolished the translocation reaction, the same oxidation procedure was carried out with the wild type precursor. Translocation of wild type prePhoE was not affected by the ferricyanide treat-

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1. The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; prePhoE, precursor PhoE.
Pulse was followed by the indicated chase periods. Precursor PhoE; mature PhoE.

A-5CVC-19 proteins, respectively. DTT could reverse this effect, and DTT did not enhance the translocation efficiency of the different oxidized mutant prePhoE forms solubilized in 8 M urea material; DTT, and this binding was functional. Furthermore, the data suggest that upon oxidation of the double mutant precursor an intramolecular disulfide bridge was formed that prevented translocation.

**Binding of Looping Precursor to Inner Membrane Vesicles**—At what stage is the precursor with an intramolecular disulfide bridge blocked in the translocation process? First, we studied the interaction of the precursor with SecA. It has been shown that the precursor of OmpA stimulates the SecA ATPase activity (1) and increases the sensitivity of SecA to V8 protease (32) in a signal sequence-dependent way. However, even with wild type prePhoE, no similar effects on SecA could be detected. Therefore, the interactions of the looped precursor with the export apparatus had to be tested in a more indirect assay, i.e., the functional binding to inner membrane vesicles. The oxidized precursor was incubated under nonreducing circumstances with inner membrane vesicles at 0 °C. The mixture was centrifuged through a sucrose cushion. Approximately 20% of the total amount of precursor was found in the pellet fraction. A similar amount of precursor was found in the pellet when DTT was present during the incubation showing that the disulfide bridge formation did not inhibit the binding to the vesicles. To determine whether the binding to the vesicles was functional, the pellet was dissolved into translocation buffer, ATP was added, and translocation reactions were performed. In the case of the looped precursor, the addition of DTT to the translocation buffer gave a 4–6-fold stimulation of translocation (Fig. 4). The small amount of translocation in the absence of DTT is probably due to some reduction of the intramolecular disulfide bridge during the reisolation procedure. DTT had no similar effect on the translocation of wild type prePhoE. It could be, in the case of the double mutant, that the translocated material in the presence of DTT is derived from a precursor population that was specifically bound to the vesicles and released upon dissolving the vesicles in translocation buffer. However, a 10-fold dilution of the reisolated vesicles compared with the volume in the binding reaction had no effect on the efficiency of translocation (data not shown). Furthermore, since neither SecA nor SecB was added after reisolation of the vesicles, such precursors would not have been targeted efficiently to the translocation apparatus. Apparently, the translocation had occurred from a complex that was functionally bound to the inner membrane vesicles during the preincubation at 0 °C. In conclusion, the binding of the precursor to inner membrane vesicles was not disturbed by the disulfide bridge, and this binding was functional. Furthermore, the data suggest that the looped precursor is a very early intermediate in the translocation process.

**Fig. 3.** Translocation of mutant precursors *in vitro.* PrePhoE translocation into inner membrane vesicles was carried out by dilution of the different oxidized mutant prePhoE forms solubilized in 8 M urea into a translocation mixture without or with DTT as indicated. Panels A–D show the translocation of the wild type (WT), A-5C, V-C-19, and A-5CVC-19 proteins, respectively. St, 10% standard of the total added material; p, precursor PhoE; m, mature PhoE.

**Fig. 4.** Functional binding of looped precursor to inner membrane vesicles. Oxidized prePhoE was preincubated in a translocation mixture with inner membrane vesicles. The vesicles were then pelleted through a 0.5 M sucrose cushion, dissolved in translocation buffer, and, after addition of DTT (lanes 2 and 4), further incubated for 20 min at 37 °C. After proteinase K treatment, the amount of translocated material was analyzed by SDS-PAGE and Western immunoblotting. Lanes 1 and 3 show the amount of material that can be translocated without the addition of DTT. The efficiency of translocation after binding is also indicated. St, 25% standard of the pelleted material; p, precursor PhoE; m, mature PhoE.

![Image of plasmids](https://example.com/plasmids.png)

**Plasmid:** pJP29, pNN1, pNN3, pNN4

**Fig. 2.** Processing kinetics of the cysteine containing mutant proteins *in vivo.* Plasmid-containing derivatives of strain CE1224 were grown under phosphate limitation and pulse-labeled for 30 s. The pulse was followed by the indicated chase periods. p, precursor PhoE; m, mature PhoE.

![Image of gel electrophoresis](https://example.com/gel.png)

**A.** WT  
**B.** A-5C  
**C.** V-C-19  
**D.** A-5CVC-19

**Efficiency %**

**Performance:**

1. WT  
2. A-5C  
3. V-C-19  
4. A-5CVC-19

To analyze further whether the looped precursor is directed into the correct translocation pathway, a competition experiment was performed. A fixed amount of 35S-methionine-labeled wild type prePhoE was mixed with nonradioactive wild type or looped precursor, and the translocation of the labeled precursor was determined. To be sure that competition at the level of the membrane was determined and not at the level of SecA or SecB, the experiments were performed with a rate-limiting amount of vesicles and an excess of SecA and SecB. The results showed that nonradioactive wild type prePhoE competed with the radiolabeled precursor to the same extent as the looped precursor, whereas bovine serum albumin, as a non-translocatable control, had no effect (Fig. 6). Apparently, the looped precursor entered the same pathway as the wild type precursor.

DISCUSSION

In this study, we have shown that prePhoE with an intramolecular disulfide bridge in the signal sequence cannot be translocated into inner membrane vesicles. To gain insight in the stage at which the translocation is blocked, the interaction of the looped precursor with the export apparatus was tested. The looped prePhoE was still able to bind to inner membrane vesicles and could be translocated into these vesicles after reduction of the disulfide bridge. Furthermore, the looped precursor competed as efficiently as wild type nonradioactive precursor with radioactive wild type prePhoE in translocation experiments. These results imply that the looped precursor is targeted to the export apparatus and accumulates as an early translocation intermediate at the level of the membrane. This leads to the intriguing question of what stage is inhibited after this initial binding step. The possibilities are (i) unlooping of the signal sequence in the lipid bilayer is prevented by the disulfide bridge, or (ii) interaction of the signal sequence with SecA, SecY, or SecE is disturbed by the disulfide bridge. The accumulation of a translocation intermediate of proOmpA, of which a large portion of the mature sequence had been translocated into inner membrane vesicles, has been reported to enhance ATP hydrolysis by SecA (33). The looped precursor did not stimulate the ATPase activity, but the ATPase activity could be restored to wild type levels by the addition of DTT. This result implies that translocation is already blocked at a stage before the hydrolysis of ATP. Because SecA has ATPase activity and the precursor of OmpA stimulated both this activity (1) and sensitivity of SecA to V8 protease (32) in a signal sequence-dependent way, we attempted to investigate prePhoE-SecA interactions in a similar way. However, no similar effects of wild type prePhoE on SecA were detected. The reason for this discrepancy is unknown. The data obtained so far are consistent with the unlooping model where the disulfide bridge would prevent the unlooping of the signal sequence in the bilayer, but alternative interpretations, i.e. incorrect interaction of the looped precursor with SecA, SecY, or SecE, cannot be excluded. However, the results clearly show that, besides the primary structure characteristics of a signal sequence (positively charged NH2 terminus, hydrophobic core, and cleavage site), which are all present in the looped signal sequence, conformational flexibility is necessary to initiate the translocation process. That conformational flexibility is probably an important feature of signal sequences was previously suggested by the statistically frequent occurrence of α-helix destabilizing amino acids in the center of the hydrophobic core (12). Batenburg et al. (31) have proposed that the glycine in position -10 of prePhoE gives this conformational flexibility. Future experiments will be directed to determine whether this glycine is indeed giving the necessary flexibility to the signal sequence.

REFERENCES

Conformational Flexibility in Signal Sequences