*Biochimica et Biophysica Acta*, 979 (1989) 69–76
Elsevier



BBA 74313

# The role of the positively charged N-terminus of the signal sequence of *E. coli* outer membrane protein PhoE in export

Dirk Bosch, Paul de Boer, Wilbert Bitter and Jan Tommassen

*Department of Molecular Cell Biology and Institute of Molecular Biology, State University of Utrecht, Utrecht (The Netherlands)*

(Received 26 July 1988)

Key words: PhoE, export; Signal sequence; Charge distribution; (*E. coli*)

**Signal sequences of prokaryotic exported proteins have a dipolar character due to positively charged amino-acid residues at the N-terminus and to a preferentially negatively charged region around the cleavage site. The role of the two lysine residues at the N-terminus of the signal sequence of outer membrane protein PhoE of *E. coli*-K12 was investigated. Replacement of both of these residues by aspartic acid slightly affected the kinetics of protein translocation in vivo. This export defect, which was observed only when PhoE was overproduced, could not be suppressed by the *prlA4* mutation, which has been shown to restore export defects caused by alterations in the hydrophobic core of the signal sequences of various exported proteins. In an in vitro translocation assay, the export defect was more pronounced. Replacement of both lysines by uncharged residues did not disturb the kinetics of protein export in vivo. In the in vitro assay, an extraordinarily efficient processing was detected upon incubation of this precursor with inverted cytoplasmic membrane vesicles. However, this efficient processing was not accompanied by more efficient translocation of the protein. We conclude that the positively charged residues at the N-terminus of the signal sequence are not essential for protein export, but contribute to the efficiency of the process.**

## Introduction

Proteins which are exported from the cytoplasm of *E. coli* K-12 are synthesized as precursors with an N-terminal 15–30 amino-acid-long extension called the signal sequence. These signal sequences are essential for translocation of the protein across the cytoplasmic membrane [1,2]. During or shortly after translocation the signal sequence is proteolytically removed by one of two distinct leader peptidases, which are located in the cytoplasmic membrane. Leader peptidase II removes the signal sequence from lipoprotein molecules, whereas leader peptidase I removes the signal sequence from other precursors.

Structural similarities among signal sequences can be used to divide the signal sequence into three segments [3]: (i) an N-terminal positively charged segment containing 1–3 basic amino-acid residues, (ii) a hydrophobic core of 10–15 amino-acid residues and (iii) a less hydrophobic C-terminal segment containing the signal-sequence cleavage site. The region around the cleavage site seems to be preferentially negatively charged [4]. Especially the amino acid at position +2 in the mature protein is strongly biased towards negatively charged residues, whereas neither an Arg nor a Lys has been observed in this position.

Of these three segments, the functioning of the hydrophobic core is best-documented. Mutations which reduce the length or hydrophobicity of this segment in the signal sequences of several proteins have been described [1,5,6]. In general, these mutations have a pronounced effect on the translocation efficiency. The effects of these mutations can extragenically be suppressed by mutations in the *prlA* (*secY*) gene [6–8].

We have recently studied the role of the negatively charged residues in the region around the processing site. Replacement of the Glu residue in position +2 of the mature outer membrane PhoE protein by Lys resulted in a weak export defect when studied in vivo or in vitro (unpublished data). Reduced cleavage efficiency by purified leader peptidase I suggested a conformational alteration in the mutant precursor. It was argued that this negatively charged residue might function in

Abbreviations: DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Correspondence: J. Tommassen, Department of Molecular Cell Biology, State University Utrecht, Transitorium 3, Padualaan 8, 3584 CH Utrecht, The Netherlands.

connection with the positively charged residues at the N-terminus of the signal sequence to stabilize a looped structure of the signal sequence [9], which may be required for export.

The role of the positively charged residues at the N-terminus of the signal sequence is not well understood. In case of the major outer membrane lipoprotein, the total charge at the N-terminus has systematically been reduced from +2 to +1, 0, −1 and −2 [10,11]. Reduction to 0 had an effect on protein synthesis but hardly or not on translocation. However, it should be noted that the second residue of the signal sequence in this construct was Ala. It seems likely that the initiator fMet is cleaved off when followed by Ala [12,13], thus creating a positive charge at the N-terminus. When the charge was further reduced to −1 or −2, there was a pronounced effect on translocation in vivo. However, when the functioning of these mutant signal sequences was tested in an in vitro system using inverted microsomal membranes, no translocation defect was observed [14].

Since the situation in the signal sequence of the lipoprotein is not completely clear, and also since the lipoprotein follows at least partly a different translocation pathway, as evidenced by the dependency on a distinct leader peptidase and as suggested by the distinctive properties of lipoprotein signal sequences [15], we wanted to assess the functioning of the positive charges at the N-terminus of the signal sequence of PhoE protein. In the present study, we replaced the basic amino-acid residues in the PhoE signal sequence by neutral and acidic residues. We studied the influence of these mutations on translocation and processing in vivo as well as in vitro.

## Material and Methods

*Bacterial strains, plasmids and growth conditions*

Bacterial strains used were all derivatives of *E. coli* K-12. CE1224 is deleted for *phoE* and does not produce the related OmpF and OmpC proteins as a result of an *ompR* mutation [16]. CE1248 also contains *phoE* and *ompR* mutations and carries in addition a *phoR* mutation resulting in constitutive expression of the *pho* regulon [17]. Strain NT1004 [7] is a derivative of MC4100 and carries the *prlA4* allele. This mutation suppresses the effect of defective signal sequences. Strains IQ85 [18] and MM52 [19] are also derivatives of MC4100 and contain the temperature-sensitive mutant alleles *secY24* and *secA51*, respectively. At the restrictive temperature, these strains are defective in protein export. Plasmid pHG329 is a derivative of pUC18 and carries the *LacZα* expression region with a multiple cloning site and the *rop* gene for controlled plasmid replication [20]. Unless stated otherwise, bacteria were grown under aeration at 37°C in L-broth [16] or in a medium described by Levinthal et al. [21] in which the phosphate concentration is limiting for growth. Where necessary, the medium was supplemented with chloramphenicol (25 μg/ml) or ampicillin (50 μg/ml).

*DNA manipulations*

Plasmid DNA was prepared as described in Ref. 22 by Birnboim and Doly, followed by CsCl-ethidium bromide isopycnic centrifugation. Recombinant DNA techniques were performed essentially as described by Maniatis et al. [23]. Restriction endonuclease reactions and bacteriophage T4 DNA ligase treatments were performed as proposed by the manufacturers of these enzymes. The synthetic oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer. DNA sequencing was performed as described by Sanger et al. [24].

The construction of the recombinant plasmids is outlined in Fig. 1. Plasmid pJP315 was constructed previously [25] and carries a deletion which removes the complete promotor region and the codons for the first three amino-acid residues (Met-Lys-Lys) of the PhoE signal sequence. A *SalI* recognition site overlapping with the fourth codon of the signal sequence is present. The plasmid was digested with *Eco*RV and *Bam*HI linkers were ligated to the DNA. Subsequently, a *SalI*-*Bam*HI restriction fragment was isolated and ligated into plasmid pHG329. Due to an in-frame fusion with the DNA coding for *LacZα*, the resulting plasmid pLAC-200 encodes an N-terminally extended PhoE precursor as indicating in Fig. 1. Mutagenic cassettes were synthesized containing a ribosome binding site (SD) and three codons for the first three amino-acid residues of the signal sequence. To terminate possible translation of a fusion protein the cassettes contained a stopcodon in the PhoE reading frame upstream from the ribosome binding site. The cassettes are flanked by *Hin*dIII and *Sal*I cohesive ends. The mutagenic cassettes 1, 2 and 4 were ligated into *Hin*dIII- and *Sal*I-digested pLAC-200, resulting in the plasmids pLAC-201, pLAC-202 and pLAC-204. These *phoE* constructs were subcloned to obtain protein expression under control of the *phoE* promoter. To this purpose, plasmid pJP360 (unpublished data), a derivative of pJP29 of which the DNA coding for the PhoE signal sequence is deleted but which still contains a functional *phoE* promoter, was digested with *Pst*I. The sticky ends of the linearized plasmid were digested with S1 exonuclease and *Hin*dIII linkers were ligated to the DNA. The *Hin*dIII-*Bgl*II fragment of this plasmid was subsequently substituted for the *Hin*dIII-*Bgl*II fragment of plasmids pLAC-201, pLAC-202 and pLAC-204, resulting in the plasmids pPHO-201, pPHO-202 and pPHO-204, respectively (Fig. 1). To obtain SP6 RNA polymerase, directed mRNA synthesis of the *phoE* mutants for use in the in vitro translation, translocation assay, *Hin*dIII-*Bam*HI re-

striction fragments of pLAC-201, pLAC-202 and pLAC-204 were subcloned into the *Hin*dIII-*Bam*HI-digested vector pGEM-blue (Promega Biotec), resulting in the plasmids pGEM-201, pGEM-202 and pGEM-204, respectively.

*In vivo pulse label and trypsin accessibility experiments*

Cells induced for the synthesis of PhoE protein by phosphate starvation were labeled for 30 s at 30 °C with [$^{35}$S]methionine and subsequently chased with an excess of non-radioactive methionine as described [26].

To test the accessibility of periplasmic proteins for trypsin, cells were radiolabelled for 30 s at 30 °C, chased for 20 s and put on ice. The cells were resuspended in 1 ml ice-cold 100 mM Tris-HCl/0.25 mM sucrose (pH 8.0) with either 10 mM $MgCl_2$ or 5 mM EDTA. The presence of EDTA makes the outer membrane leaky, thus allowing the entrance of trypsin into the periplasm. 50 μg of trypsin (Serva) was added and the suspension was kept on ice for 20 min prior to the addition of a 3-fold excess of trypsin inhibitor (Serva). The cells were washed with 100 mM Tris-HCl/0.25 mM sucrose/10 mM $MgCl_2$ and resuspended in 15 μl hot 1% SDS/50 mM Tris-HCl/1 mM EDTA (pH 8.0) and PhoE proteins were subsequently immunoprecipitated.

*In vitro translocation and processing*

In vitro transcription, translation and translocation reactions were carried out essentially as described [27], except that SP6 RNA polymerase was used to catalyse mRNA synthesis. 1 μg of *Eco*RI linearized plasmid was transcribed with 3 units SP6 RNA polymerase for 1 h at 40 °C in a buffer containing 20 mM Hepes, 100 mM magnesium acetate, 2 mM spermidine, 10 mM DTT, 0.5 mM NTP and 15 U RNAase inhibitor (RNA guard; Pharmacia) (pH 7.3). The mRNA was used to direct the translation of PhoE protein. The translation was carried out for 25 min at 37 °C using an S-135 extract of *E. coli* strain MRE600. Co-translational translocation was investigated by adding inverted vesicles of the cytoplasmic membrane of strain MRE600 5 min after protein synthesis was initiated. The susceptibility of precursors to leader peptidase I was tested by adding purified leader peptidase in 0.2% β-glucoside to the translation mix 5 min after protein synthesis was initiated.

*Immunoprecipitation*

For immunoprecipitation of [$^{35}$S]methionine-labelled proteins, cell pellets were resuspended in 15 μl 1% SDS/50 mM Tris-HCl/1 mM EDTA (pH 8.0) and incubated for 5 min at 100 °C. Then, 750 μl Triton buffer containing 2% Triton X-100, 50 mM Tris-HCl, 0.15 M NaCl, 0.1 mM EDTA and 1 mg/ml BSA (pH 8.0) was added. The suspension was centrifuged for 10 min and 1 μl of monoclonal antibody mE1, which recognizes denatured PhoE, was added to the supernatant. After overnight incubation at 4 °C, 3 mg of protein A-Sepharose CL-4B (Pharmacia) was added and incubated for 1 h at room temperature under gentle shaking. The pellet obtained after centrifugation for 4 s in an Eppendorf centrifuge was washed twice in Triton buffer, resuspended in sample buffer and incubated for 5 min at 100 °C. The sampled were analysed by SDS-PAGE and autoradiography.

## Results

*Expression of the mutant proteins*

A series of plasmids was constructed encoding PhoE protein under *lac* promotor control as described in Materials and Methods. The signal sequence of the PhoE precursor encoded by pLAC-201 is similar to the wild-type PhoE signal sequence with a net charge at the N-terminus of +2. This net charge is altered in the PhoE precursors encoded by pLAC-202(−2) and pLAC-204(0). The pLAC-200 encoded precursor is expected to carry 2 N-terminal positive charges. One is caused by Arg(-22) and one by the N-terminal α-amino group. As is the case with β-galactosidase, the fMet of the N-terminus, consisting of *LacZ*-α-amino-acid residues, is expected to be removed from this precursor (Fig. 1).

When cell envelopes of CE1224 cells carrying the plasmids pLAC-200, pLAC-201, pLAC-202 and pLAC-204 grown in TK medium with 2 mM IPTG were analysed by SDS-PAGE, a protein migrating with the same electrophoretic mobility as PhoE could be detected (data not shown). When no IPTG was added to the medium a residual amount of PhoE protein was still synthesized, probably because the amount of Lac repressor, encoded by the chromosomal *lacI* gene is not sufficient to inhibit the *lac* operators presented by the multicopy plasmids. The cell envelopes of the four CE1224 derivatives contained approximately equal amounts of PhoE protein, although this amount was lower than we usually observed when PhoE is expressed from its own promoter on a multicopy plasmid. Apparently, the synthetic oligonucleotides used for the cassette mutagenesis encode functional translation initiation signals and the amino-acid substitutions in the PhoE precursors do not influence the efficiency of protein synthesis.

*Translocation and processing of the mutant proteins in vivo*

CE1224 cells containing any of the four pLAC plasmids grown in the presence of IPTG were sensitive to the PhoE-specific phage TC45, showing that the proteins are translocated and normally incorporated into the outer membrane. To determine whether the kinetics of translocation and/or processing were af-

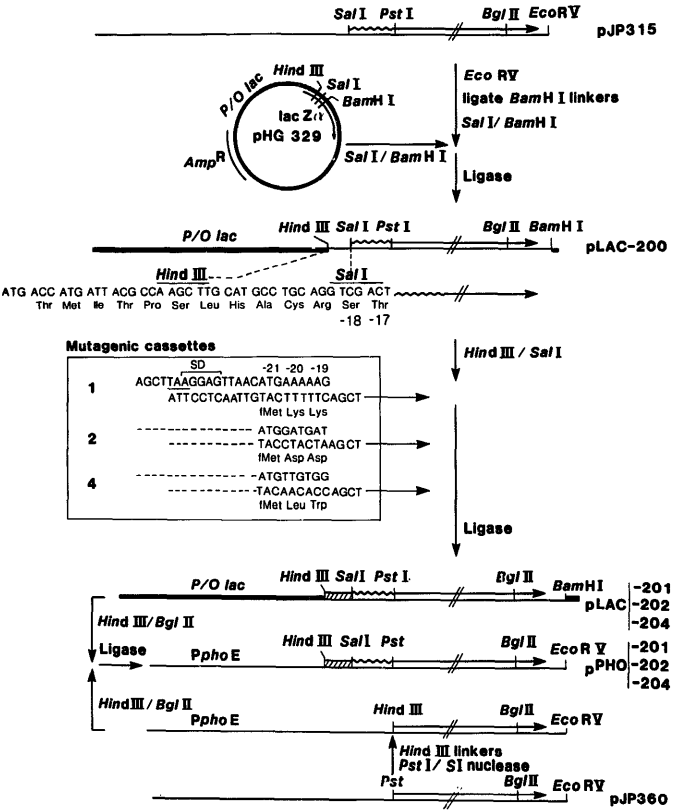


Fig. 1. Construction of recombinant plasmids encoding N-terminally mutated signal sequences. The *phoE* gene lacking the N-terminal three codons of the signal sequence is ligated into expression vector pHG329, resulting in plasmid pLAC-200. The mutagenic cassettes 1, 2 and 4 were subsequently inserted into plasmid pLAC-200. Insertion of cassette 1 results in plasmid pLAC-201, which encodes a completely wild-type PhoE precursor. Insertion of the cassettes 2 and 4 results in plasmids pLAC-202 and pLAC-204, respectively. Only the coding nucleotides of these cassettes are shown. The amino-acid substitutions created by these cassettes in the PhoE precursor are indicated. In the mutagenic cassettes, a stopcodon present in the PhoE reading frame upstream from the ribosome binding site (SD) is underlined. The mutations were subcloned to obtain expression under control of the *phoE* promotor (P*phoE*), resulting in the plasmids pPHO-201, pPHO-202 and pPHO-204.

fected by the mutations, pulse-label and pulse-chase experiments were performed followed by PhoE immunoprecipitation, SDS-PAGE and autoradiography. However, no PhoE precursors could be detected, showing that translocation and processing were very rapid in all cases (not shown).

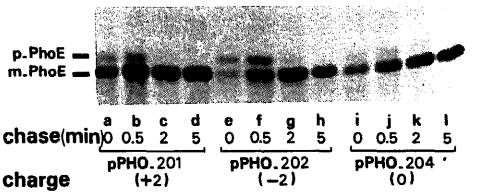


Fig. 2. Autoradiogram of [$^{35}$S]methionine-labeled proteins of CE1224 cells immunoprecipitated with antibodies directed against PhoE and separated by SDS-polyacrylamide gel electrophoresis. The cells contained pPHO-201 (wt) and the mutant plasmids pPHO-202 and pPHO-204 encoding PhoE precursors with an N-terminal charge as indicated between brackets. Cells were starved for phosphate and pulse-labeled with [$^{35}$S]methionine for 30 s. The pulse was followed by the indicated chase period (min). The positions of precursor PhoE (p-PhoE) and mature PhoE (m-PhoE) are shown.

Previously, in similar pulse-label experiments we could observe the precursor of PhoE protein when it was expressed from its own promoter on a multicopy plasmid [26]. Apparently, translocation and processing are retarded under such overproduction conditions. Therefore, we wanted to assess the effect of the signal-sequence mutations under such overproduction conditions and the plasmids pPHO-201, pPHO-202 and pPHO-204 were constructed as described in Materials and methods. In these plasmids the mutant alleles of pLAC-201, pLAC-202 and pLAC-204 are placed under control of the *phoE* promoter. When pPHO-201-containing CE1224 cells, expressing the wild-type PhoE protein, were labelled for 30 s, most of the PhoE protein was present in the mature form (Fig. 2, lane a) and after a 30 s chase period only minor amounts of precursor could be detected (lane b). The pPHO-204 encoded precursor showed similar kinetics of processing (lanes i and j, respectively). However, when pPHO-202-containing CE1224 cells were labelled for 30 s, most of the protein was present in the precursor form (lane e) and after a 30 s chase period a large amount of the protein was still not converted to mature PhoE (lane f). Apparently, the translocation and/or processing of this mutant precursor is slightly retarded, as compared to the wild-type precursor under similar conditions.

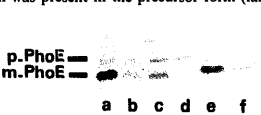


Fig. 3. Trypsin accessibility of proteins in whole cells. Cells contained the plasmids pPHO-201 (lanes a and b), pPHO-202 (lanes c and d) and pPHO-204 (lanes e and f) encoding PhoE precursors with an N-terminal charge as indicated. Cells were pulse-labeled with [$^{35}$S]methionine for 30 s followed by a chase period of 20 s with non-radioactive methionine. The cells were then incubated with trypsin in the presence of 10 mM $MgCl_2$ (M) or 5 mM EDTA (E) and PhoE proteins were immunoprecipitated and analysed by SDS-PAGE and autoradiography. The positions of the mature protein (m-PhoE) and the precursor (p-PhoE) as well as the N-terminal charge of the precursors are indicated.

To determine whether the accumulated precursors are translocated across the cytoplasmic membrane, trypsin-accessibility experiments were performed. Cells carrying pPHO-201, pPHO-202 and pPHO-204 were radiolabelled for 30 s and subsequently chased for 20 s with non-radioactive methionine. The cells were incubated with trypsin in the presence of 10 mM $Mg^{2+}$ or 5 mM EDTA. EDTA permeabilizes the outer membrane, thus allowing the entry of trypsin into the periplasm. PhoE proteins were subsequently immunoprecipitated and analysed by SDS-PAGE and autoradiography (Fig. 3). The precursor is protected against trypsin/EDTA treatment (lanes b, d and f) and is apparently not translocated across the cytoplasmic membrane. Interestingly, after the 20 s chase period, mature PhoE is accessible for trypsin when this proteinase is present in the periplasm (Fig. 2, lanes b, d and f), whereas the protein is fully protected after a 5 min chase period, as was shown previously [28]. Apparently, during the 20 s chase period, the processed PhoE is not yet normally incorporated into the outer membrane.

Since the effects on protein export of the N-terminal mutations were only minor, we wondered whether the mutant precursors cross the cytoplasmic membrane via an abnormal protein-export pathway. It is assumed that normal protein export is dependent on expression of functional SecY and SecA proteins; expression of the temperature-sensitive mutations *secY24* and *secA51* results in the accumulation of precursors of exported proteins [18,19]. The processing of the mutant PhoE precursors in the *secY24* and *secA51* strains was studied in pulse-label and pulse-chase experiments, as described in Materials and Methods, except that 2.5 h prior to the labelling, part of the cell cultures were shifted from

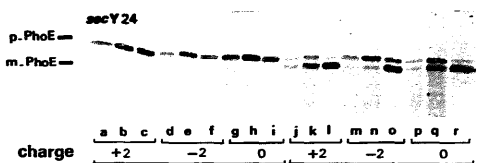


Fig. 4. Autoradiogram of [$^{35}$S]methionine-labeled proteins of *secY24* strain IQ85 immunoprecipitated with antibodies directed against PhoE and separated by SDS-polyacrylamide gel electrophoresis. The cells contained the plasmids pPHO-201 (+2), pPHO-202 (−2) and pPHO−204 (0). Cells were starved for phosphate at the non-permissive (42°C) or the permissive temperature (30°C) and pulse-labeled with [$^{35}$S]methionine for 30 s. The pulse was followed by a chase period of 0 s (a, d, g, j, m and p), 20 s (b, e, h, k, n and q) and 2 min (c, f, i, l, o and r). The PhoE precursor (p-PhoE) and the mature protein (m-PhoE) are indicated.

30°C to 42°C, the non-permissive temperature. At the permissive temperature, as is the case in strain CE1224 (Fig. 2), processing of the pPHO-204-encoded precursor showed similar kinetics as the wild-type precursor, whereas processing kinetics of the pPHO-202 encoded precursor was reduced (Fig. 4, lanes j–r). However, no processing could be detected of either the wild-type PhoE precursor or of the mutant precursors at the non-permissive temperature (Fig. 4, lanes a–i). Similar results were obtained in case of the *secA51* mutation (not shown). Apparently, all precursors follow the general SecA- and SecY-dependent translocation pathway.

The *prlA4* mutation in the *secY* gene has been shown to restore the export of LamB [7], MalE [8] and PhoA [6] precursors, which carry mutations in the hydrophobic core of the signal sequences. The delay in processing of the pPHO-202-encoded precursor was, however, not restored by the *prlA4* mutation, as was revealed by pulse-label and pulse-chase experiments. On the contrary, the processing kinetics of this precursor seem even more retarded than in strain CE1224; after a 2 min chase period approx. 50% of the protein is still present in its precursor form (Fig. 5, lane f). The *prlA4* mutation is apparently unable to suppress the N-terminal mutations in the signal sequence.

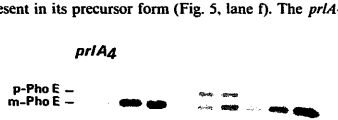


Fig. 5. Autoradiogram of [$^{35}$S]methionine-labeled protiens of *prlA4* strain NT1004 immunoprecipitated with antibodies directed against PhoE and separated by SDS-polyacrylamide gel electrophoresis. The cells contained plasmids pPHO-201 (lanes a, b and c), pPHO-202 (lanes d, e and f) and pPHO-204 lanes (g, h and i) encoding PhoE precursors with an N-terminal charge as indicated. Cells were pulse-labeled with [$^{35}$S]methionine for 30 s. The pulse was followed by a chase period of 1, 15 and 120 s, as indicated. The PhoE precursor (p-PhoE) and the mature protein m-PhoE are indicated.

*Translocation and processing of the proteins in vitro*

The translocation of the proteins was also studied in an in vitro transcription–translation and translocation system [28]. Co-translational import of in vitro synthesized protein into inverted vesicles of the cytoplasmic membrane can be demonstrated by their protection against externally added proteinase K. As shown previously [28], the wild-type PhoE precursor, now encoded by pGEM-201, was partly processed into the mature form when cytoplasmic membrane vesicles were added 5 min after initiation of protein synthesis (Fig. 6, lane b). Incubation of this suspension with proteinase K revealed that part of the precursor and most of the mature PhoE were protected against proteinase activity (lane c). Hardly any processing (lane g) and protection (lane b) of pGEM-202-encoded proteins could be detected. As compared to the wild-type precursor, a large amount of the pGEM-2-4-encoded precursor was converted to mature protein when incubated with the vesicles (lane 1). Surprisingly, however, only a small fraction of the mature protein was protected against proteinase K activity (lane m). The amount of protein

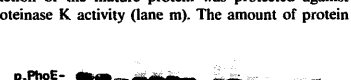


Fig. 6. In vitro cotranslational translocation of PhoE precursors, containing an N-terminal charge as indicated into vesicles of the cytoplasmic membrane. Translation products of pGEM-201, pGEM-202 and pGEM-204 (lanes a, f and k) were incubated with vesicles (lanes b, g and l) and subsequently with proteinase K (lanes c, h and m). Processing of the in vitro translation products was done with 40 μg/ml (lanes d, i and n) or 4 μg/ml (lanes e, j and o) purified leader peptidase I. The positions of the PhoE precursor (p-PhoE) and the mature protein (m-PhoE) are indicated.

protected against proteinase activity was less than in the case of the wild-type protein. The relatively efficient conversion of the pGEM-202-encoded precursor to mature protein (lane 1) does not seem to be caused by an improved interaction with leader peptidase, as was shown by the processing of the precursors by purified leader peptidase I in detergent. At a concentration of 40 μg/ml of leader peptidase I, comparable amounts of the wild-type precursor and the pGEM-204-encoded precursor are converted to mature protein (Fig. 6, lanes d and n, respectively). The pGEM-202-encoded precursor is more efficiently processed (lane i), suggesting a conformational alteration in this precursor, making it a better substrate for leader peptidase. When less leader peptidase is added (4 μg/ml), only processing of a small amount of the pGEM-202-encoded precursor could be detected (lane j).

## Discussion

Signal sequences of prokaryotic exported proteins have a dipolar character due to a positively charged N-terminus and a region around the processing site, which is preferentially negatively charged [4]. Substitution in mature PhoE protein of the favorable negatively charged Glu(+2) to the unfavorable Lys(+2) affected the kinetics of protein export (unpublished data). Since the in vitro interaction of this precursor with purified leader peptidase I was also disturbed, the export defect was ascribed to an aberrant conformation of the precursor. Possibly, the formation of a postulated looped orientation of the signal sequence in the cytoplasmic membrane, in which the N- and C-termini of the signal sequence face the cytoplasm [9], is disturbed by an electrostatic repulsion between positive charges at the N-terminus and the Lys in position +2 of this mutant. Here we describe the influence on protein export of substitutions of the positively charged N-terminal amino-acid residues in the PhoE precursor. The amino-acid residues introduced in the mutants were chosen such that the removal of the formylated initiation methionine, which depends on the adjacent amino-acid residues [12,13], is not to be expected. Consequently, like in the case of the wild-type precursor, the N-terminal α-amino group of the mutant precursors is expected not to contribute to the charge at the N-terminus.

Substitution of the two N-terminal positively charged amino-acid residues into Asp residues affected the kinetics of protein export in vivo as well as in vitro. This could, in analogy to the Lys(+2) for Glu(+2) substitution, be partly explained by a distrubed formation of the looped structure of the signal sequence due to an electrostatic repulsion of the N-terminal negative charges and Glu(+2). Indeed, the conformation of this precursor seems to be changed, since the in vitro interaction with purified leader peptidase I is improved. However, the effects on protein export of the N-terminal charge substitutions are especially in the in vitro assay more pronounced than the effects on export of the Lys(+2) for Glu(+2) substitution. The export defect of the N-terminal negatively larged precursor may therefore be better explained by a reduced initial interaction of this mutant precursor with the cytoplasmic membrane if one assumes that, for export, an interaction between the positively charged N-terminus of the signal sequence and the negatively charged phosphatidylglycerol (PG) in the cytoplasmic membrane is required. In this respect, it is interesting to note that mutants with a severely reduced PG content have recently been shown to be partly disturbed in protein export [29].

The consequences of the N-terminal charge substitution from +2 to −2 on PhoE export are not as pronounced as the consequences on export of similar mutations in the *E. coli* outer membrane lipoprotein [10,11]. This can be explained by considering that the lipoprotein uses at least partly a different export pathway. The export in *E. coli* of mutant staphylokinase precursors having a negatively charged N-terminus is even more hampered [30], as in the case of the mutant lipoproteins. Here, the export defect was observed in a heterologous system in which the interaction between the precursor and the cellular export apparatus may not be optimal. On the other hand, other characteristics of the signal sequence or the mature part of the exported protein could determine the relative influence of the charge substitutions on protein export. It has previously been shown that a mutation in the hydrophobic core of a signal sequence fused to the mature regions of β-lactamase and *Staphylococcus aureus* Nuclease A affects export of these proteins differently [31].

No export defect in vivo could be detected of the precursor carrying the Leu(-20) Trp(-19) for Lys(-20) Lys(-19) substitution. Apparently, the presence of N-terminal positive charges is not required for protein export. Incubation of this N-terminally uncharged precursor with inverted cytoplasmic membrane vesicles revealed that substantial amounts of this precursor were processed but not protected against proteinase activity. This can be explained by a very efficient insertion of this more hydrophobic signal sequence into the inverted membrane vesicles in a way independent of the normal pathway, but such that the cleavage site is presented correctly to the endogenous leader peptidase I. However, this interaction does not lead to further translocation of the protein. This phenomenon does not seem to occur in vivo since in such a case one would expect processing of the accumulated mutant precursor in the SecY and SecA background.

Differences between the in vivo and in vitro experiments may be explained by the presence of cytoplasmic factors in vivo, which could partly restore aberrant

export. A similar conclusion was drawn from experiments on the translocation of the wild-type PhoE precursor across membranes from a mutant *E. coli* strain with a reduced amount of phosphatidylglycerol in the membranes [29]. In vivo, hardly any delay in translocation was observed, whereas the translocation in vitro was disturbed significantly. It was shown that a cytoplasmic factor present in the mutant strain could improve translocation of PhoE in vitro. The expression of these factors may be specifically induced when for one reason or another export of precursors is disturbed. For example, it has been shown that the synthesis of SecA is derepressed when protein export is disturbed because of mutations in the *secA*, *secD* and *secY* genes or because of expression of a MalE-LacZ fusion [32].

Export defects of various exported proteins, caused by mutations in the hydrophobic core of the signal sequences, could be suppressed by a mutation in the *prlA* gene. Although the export of the wild-type PhoE precursor and the N-terminally mutated precursors was dependent on a functional product of *secY*, which is allelic with *prlA*, the export defect of the negatively charged precursor could not be suppressed by the *prlA4* mutation. Apparently, the *prlA4* allele is not completely unspecific in suppressing signal sequence mutations.

**Acknowledgements**

We wish to thank J. Beckwith, K. Ito and T. Silhavy for strains MM52, IQ85 and NT1004, respectively, and G. Stuart for plasmid pHG329. Purified leader peptidase was kindly provided by W. Wickner. The contributions of H. de Cock and T. de Vrije with the in vitro assays are very appreciated.

**References**

1 Bedouelle, H., Bassford, Jr., P.J., Fowler, A.V., Zabin, I., Beckwith, J. and Hofnung, M. (1980) Nature 285, 78–81.
2 Emr, S.D., Hedgpeth, J., Clément, J.M., Silhavy, T.J. and Hofnung, M. (1980) Nature 285, 82–85.
3 Von Heijne, G. (1985) J. Mol. Biol. 184, 99–105.
4 Von Heijne, G. (1986) J. Mol. Biol. 192, 287–290.
5 Emr, S.D. and Silhavy, T.J. (1980) J. Mol. Biol. 141, 63–90.
6 Michaelis, S., Inouye, H., Oliver, D. and Beckwith, J. (1983) J. Bacteriol. 154, 366–374.
7 Emr, S.D., Hanley-Way, S. and Silhavy, T.J. (1981) Cell 23, 79–88.
8 Emr, S.D. and Bassford, P.J. (1982) J. Biol. Chem. 257, 5852–5860.
9 Batenburg, A.M., Brasseur, R., Ruysschaert, J.M., Van Scharrenburg, G.J.M., Slotboom, A.J., Demel, R.A. and De Kruijff, B. (1988) J. Biol. Chem. 263, 4202–4207.
10 Inouye, S., Soberon, X., Franceschini, T., Nakamusa, K., Itakura, K. and Inouye, M. (1982) Proc. Natl. Acad. Sci. USA 79, 3438–3441.
11 Vlasuk, G.P., Inouye, S., Ito, H., Itakura, K. and Inouye, M. (1983) J. Biol. Chem. 258, 7141–7148.
12 Ben-Bassal, A., Bauer, K., Chang, S.H., Myambo, K., Boosman, A. and Chang, S. (1987) J. Bacteriol. 169, 751–757.
13 Tsunasawa, S., Stewart, J.W. and Sherman, F. (1985) J. Biol. Chem. 260, 5382–5391.
14 Garcia, P.D., Ghrayeb, J., Inouye, M. and Walter, P. (1987) J. Biol. Chem. 262, 9463–9468.
15 Klein, P., Somorjai, R.L. and Lau, P.C.K. (1988) Protein Eng. 2, 15–220.
16 Thommassen, J., Van Tol, H. and Lugtenberg, B. (1983) EMBO J. 2, 1275–1279.
17 Van der Ley, P., Amesz, H., Tommassen, J. and Lugtenberg, B. (1985) Eur. J. Biochem. 147, 401–407.
18 Shiba, K., Ito, K., Yura, K. and Corretti, D.P. (1984) EMBO J. 3, 631–635.
19 Oliver, D.B. and Beckwith, J. (1981) Cell 25, 765–772.
20 Stewart, G.S.A.B., Lubinsky-Mink, S., Jackson, C.G., Cassel, A. and Kuhn, J. (1986) Plasmid 15, 172–181.
21 Levinthal, C., Singer, E.R. and Fetherolf, K. (1962) Proc. Natl. Acad. Sci. USA 48, 1230–1237.
22 Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1524.
23 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
24 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
25 Tommassen, J., Koster, M., Overduin, P. (1987) J. Mol. Biol. 198, 633–641.
26 Bosch, D., Leunissen, J., Verbakel, J., De Jong, M., Van Erp, H. and Tommassen, J. (1986) J. Mol. Biol. 189, 449–455.
27 Bosch, D., Voorhout, W. and Tommassen, J. (1988) J. Biol. Chem. 263, 9952–9957.
28 De Vrije, T., Tommassen, J. and De Kruijff, B. (1987) Biochim. Biophys. Acta 900, 63–72.
29 De Vrije, T., De Swart, R.L., Dowhan, W., Tommassen, J. and De Kruijff, B. (1988) Nature 334, 173–175.
30 Iino, T., Takahashi, M. and Sako, T. (1987) J. Biol. Chem. 262, 7412–7417.
31 Lehnhardt, S., Pollitt, S. and Inouye, M. (1987) J. Biol. Chem. 262, 1716–1719.
32 Rollo, E.E. and Oliver, D.B. (1988) J. Bacteriol. 170, 3281–3282.