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Optimal posttranslational translocation of the precursor of PhoE protein across *Escherichia coli* membrane vesicles requires both ATP and the protonmotive force

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In order to reach their final destination, periplasmic and outer membrane proteins have to pass the cytoplasmic membrane of *Escherichia coli* cells. To study the transport of PhoE protein, we developed an in vitro transcription-translation and translocation system. In this in vitro system, the protein is synthesized as a larger precursor, which can be processed by purified leader peptidase. The precursor can be translocated into inverted inner membrane vesicles as judged by the protection against externally added protease. Only part of the translocated protein is in the processed mature form. Translocation can occur posttranslationally and requires both ATP and the protonmotive force for an optimal process. Upon incubation of vesicles with mature PhoE protein or precursor PhoE in the absence of ATP, the proteins are bound to the vesicles, but they are not translocated, since they are still sensitive to externally added protease.

Introduction

Most cellular proteins have to be transported to their final subcellular compartment. During this process the proteins often have to be inserted into or translocated across membranes. In eukaryotic cells the translocation of newly synthesized proteins across the endoplasmic reticulum was believed to be a strictly cotranslational process. But recently, evidence has been provided that many secretory and membrane proteins can be translocated in vitro late in their translation or after synthesis is completed [1–4]. This translocation process appeared to be dependent on a signal recognition particle-signal sequence-ribosome complex, probably necessary to maintain a translocation competent state of the nascent chains. In contrast, the translocation of mitochondrial precursor proteins is generally posttranslational and might be mediated by receptors on the cytoplasmic surface of the outer membrane [5–7]. However, in case of apocytochrome *c* recent studies strongly suggest that lipid molecules could be involved in the interaction of these proteins with the membrane and provide an insertion and translocation pathway [8–10]. In the prokaryotic *Escherichia coli* cells periplasmic and outer membrane proteins have to pass the inner membrane. Some of these proteins cross the membrane late in their synthesis after approx. 80% is completed,

Abbreviations: ACMA, 9-amino-7-chloro-2-methoxyacridine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; PMSF, phenylmethylsulfonyl fluoride.

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others are posttranslationally transported [11–14]. Until now, very little is known about the actual molecular components involved in the transport of a protein through the membrane. In analogy to the eukaryotic case the use of in vitro systems is likely to give valuable insight in this matter. Such systems have been recently described for some periplasmic and outer membrane proteins, which can be processed and translocated in the presence of inverted membrane vesicles. It was shown that in such systems posttranslational translocation can take place [15,16]. Chen and Tai [17] demonstrated a requirement for ATP during protein translocation across the inner membrane. Furthermore, it was reported that an energized membrane may play a more or less important role in an efficient translocation process [18,19].

In this paper, we describe an in vitro translation-translocation system for PhoE protein, one of the major outer membrane proteins. In vivo, the synthesis of PhoE protein is derepressed by growth in limiting phosphate concentrations [20]. It functions in a trimeric form as a pore for P_i and P_i-containing solutes [21] and other negatively charged solutes [22]. In the in vitro system, PhoE protein is synthesized as a larger precursor, which can be processed into mature PhoE by leader peptidase in detergent or by the addition of inverted inner membrane vesicles. PhoE protein could be translocated posttranslationally into these vesicles and translocation appeared to be dependent on the protonmotive force and ATP.

Materials and Methods

Cell extract

As S-135 cell extract was prepared according to Müller and Blobel [15]. *Escherichia coli* strain MRE600 [23] was grown at 37 °C in a medium containing 0.1% $Na_2HPO_4 \cdot 2H_2O$, 0.5% NaCl, 1% peptone, 0.5% yeast extract, 0.2% glucose and 0.002% guanine, thymine, uracil and adenine. Cells were rapidly chilled by the addition of ice to a culture of exponentially growing cells (A_{660} 0.25–0.3). All subsequent steps were carried out at 4 °C. The cells were washed and resuspended (0.5 g/ml) in buffer A (50 mM triethanolamine acetate/50 mM potassium acetate/15 mM magnesium acetate/1 mM dithiothreitol (pH 7.5)) containing 0.5 mM PMSF. For cell lysis, the suspension was passed twice through a French pressure cell at 4000 lb/inch^2. Immediately after lysis, 1 mM dithiothreitol was added to the lysate. After a low speed centrifugation (5 min at 14500 × *g*) the cell extract was centrifuged twice at 30000 × *g* (30 min). The supernatant was incubated for 90 min at 37 °C to decrease the amount of endogenous mRNA and centrifuged twice at 135000 × *g* for 20 min to pellet the remaining polysomes and membrane vesicles. The cell extract was dialyzed overnight against buffer B (10 mM triethanolamine acetate/10 mM magnesium acetate/22 mM ammonium acetate/1 mM dithiothreitol (pH 7.5)) with two changes. The S-135 lysate was rapidly frozen in liquid N_2 and stored in aliquots at −80 °C. The translation activity of the lysate did not change on storage for up to 6 months.

Preparation of membrane vesicles

Inverted vesicles of the inner membrane were prepared according to Müller and Blobel [25] with some modifications. MRE600 cells were grown in L-broth [24] to an absorbance at 660 nm of 1.0–1.2 absorbance units whereafter they were chilled on ice. Spheroplasts were made by the lysozyme-EDTA treatment [25]. Both whole cells and spheroplasts were used for the preparation of membrane vesicles. The cells or spheroplasts were resuspended (0.2 g/ml) in buffer K (50 mM triethanolamine acetate/250 mM sucrose/1 mM EDTA/1 mM dithiothreitol/0.5 mM PMSF (pH 7.5)) and passed twice through a French press at 8000 lb/inch^2. Cell debris was removed by low speed centrifugation (5 min at 6000 × *g*) and the membrane vesicles were pelleted at 165000 × *g* for 1.5 h. The vesicles were resuspended in buffer L (buffer K without EDTA and PMSF). Part of this suspension was centrifuged (5 min at 10000 × *g*) to remove some aggregated material. The supernatant consist of a crude fraction of inner and outer membrane vesicles. The other part was layered on top of a sucrose-step gradient in buffer M (buffer K without sucrose) according to Osborn et al. [26] to separate inner and outer membrane vesicles.

Centrifugation was carried out for 18 h at 25000 rpm (SW27 rotor). Three bands were collected, called the inner membrane 1, inner mem-

brane 2 and outer membrane fraction. Both inner membrane fractions contained more than 90 percent inner membrane material (based on protein content), as measured by scanning with a Vitatron densitometer at λ_{610} the intensity of the protein bands on a polyacrylamide gel stained with Fast green. The inner membrane 2 fraction contained about 1.4-times more outer membrane proteins than the inner membrane 1 fraction. The fractions were diluted in buffer K to lower the sucrose concentration, pelleted and resuspended in buffer L as described above. Aggregated material was removed and the membrane preparations were diluted to a concentration of 20–30 A_{280} U/ml. The crude, inner membrane 1 and inner membrane 2 fractions contained 0.5 mg protein/A_{280} unit and 0.4 μmol phospholipid/mg protein. The outer membrane fraction contained 0.7 mg protein/A_{280} unit and 0.2 μmol phospholipid/mg protein. The vesicle preparations were quickly frozen in liquid N_2 and stored at −80 °C. They were used with identical results after storage for up to 4 months. Protein was determined according to the method of Lowry et al. [27] and lipid phosphate by measuring the total phosphate content after perchloric acid destruction according to the method of Fiske and SubbaRow [28]. This value was corrected for the presence of some residual free phosphate and in case of the outer membrane fraction for the lipopolysaccharide phosphate content.

In vitro transcription and translation

Plasmid pJP29 [29] contains the structural gene for PhoE protein and the *cat* gene, coding for the cytoplasmic protein chloramphenicol acetyltransferase. The plasmid was transcribed by RNA-polymerase in a transcription mix [30] containing per 100 μl 4.4 U of RNA-polymerase and 25 μg of plasmid DNA. Incubations were carried out at 37 °C for 15 min and the mixture was directly used in the translation system or stored at −80 °C. Both methods gave comparable results in the translation assay.

A 25 μl translation assay contained 40 mM Tris-acetate, 60 mM potassium acetate, 11 mM magnesium acetate, 20 mM ammonium acetate (pH 8.0), 2 mM dithiothreitol, 8 mM creatine phosphate, 1 μg of creatine phosphokinase, 2.0 mM ATP, 0.5 mM CTP, GTP and UTP, 5 μCi of L-[^{35}S]methionine, 100 μM each of 19 amino acids, 2.5 μg of tRNA, 0.3 mM folinic acid, 4 μl of transcription mix and 3 μl of S-135 lysate. Translation was carried out in all cases at 37 °C. Incorporation of [^{35}S]methionine into the total protein fraction was measured by hot trichloroacetic acid precipitation of 2-μl samples on filter discs. The rest of the samples were prepared for polyacrylamide gel electrophoresis according to Lugtenberg et al. [31]. Individual proteins synthesized were analysed by overnight fluorography of 11% SDS-polyacrylamide gels.

In vitro translocation

In order to investigate cotranslational translocation a membrane fraction was added to a final concentration of 1 unit per ml (absorbance at 280 nm) 5 min after protein synthesis had been started. Incubation was continued for 20 min. To study posttranslational translocation the translation system was incubated for 25 min, after which protein synthesis was inhibited by the antibiotic puromycin (1 mM). To the system an equal volume of translocation mix was added, consisting of the translation buffer and folinic acid in the same concentrations as in the translation system, ATP and a membrane fraction (final concentrations 4 mM and 1 A_{280} unit/ml, respectively), puromycin (1 mM) and non-radioactive L-methionine (100 μM). The incubation was continued for 20 min at 37 °C.

Translocation of proteins was demonstrated by their protection against externally added proteinase K. The concentration of the protease was 200 μg/ml and the samples were incubated for 15 min at 37 °C. The protease activity was stopped by the addition of PMSF (4 mM).

Association of proteins with membrane vesicles

To examine the association of PhoE protein with membrane vesicles the translation/translocation samples were layered on a sucrose cushion (100 μl of 0.5 M in translation buffer) and centrifuged for 30 min in a Beckman Airfuge (30° A-100 rotor, 148 000 × *g*). The pellet and supernatant fractions were collected and analysed by fluorography of SDS-polyacrylamide gels.

In vivo labelling

The *ompR* mutant strain CE1224 [24] containing plasmid pJP29 was used for in vivo labelling of PhoE. The cells were grown at 37°C in a synthetic medium in which the P_i-concentration can be varied [32]. Derepression of PhoE protein synthesis was achieved as described [33]. Induced cells were pulse-labelled for 30 s with [^{35}S]methionine (12.5 μCi/0.5 ml culture) followed by cold trichloroacetic acid precipitation (5%).

Immunoprecipitation

Immunoprecipitation was performed as described by Ito et al. [34] except that the immunocomplexes were precipitated by Protein A coupled to Sepharose. Rabbit antiserum against SDS-denatured PhoE protein has been described [35].

ATP determination

The ATP content of the translation/translocation mixtures was enzymatically determined with phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase as described by Jaworek et al. [36].

ACMA fluorescence

The electrochemical membrane potential was determined by measuring the fluorescence quenching of 9-amino-7-chloro-2-methoxyacridine (ACMA) at 500 nm after excitation at 420 nm using a Perkin-Elmer LS-5 Luminescence spectrometer. The initial fluorescence of inner membrane vesicles in buffer (130 μg protein per 3 ml) (50 mM sodium morpholinopropane sulfonate/10 mM $MgSO_4$ (pH 7.3)) and the fluorescence level after addition of 1 μM ACMA was taken as 0% and 100%, respectively.

Quantitation of proteins

Protein bands on polyacrylamide gels were scanned for their radioactivity with a thin-layer scanner (Pannax equipment Ltd.). The resolution of the scanner was insufficient to measure the prePhoE and PhoE protein bands separately.

Chemicals

Creatine phosphokinase, puromycin dihydrochloride, proteinase K, β-octyl glucoside, hexokinase, nucleotides (sodium salt), phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and RNA-polymerase and tRNA from *E. coli* MRE600 were obtained from Boehringer (F.R.G.). Folinic acid (citrovorum factor, calcium salt) was from Serva (F.R.G.), creatine phosphate (di-Tris salt) and FCCP from Sigma (U.S.A.), PMSF from Merck (F.R.G.) and DCCD from Fluka (Switzerland). [^{35}S]Methionine (1000 Ci/mmol) was obtained from Amersham International (U.K.). ACMA was a gift of Dr. Klaas Krab (Free University, Amsterdam).

Results

In vitro synthesis and characterization of proteins

A first insight in protein synthesis in a translation assay containing transcripts derived from plasmid pJP29 was obtained by measuring the incorporation of [^{35}S]methionine into trichloroacetic acid precipitable material. After a lag phase of approximately 5 min, protein synthesis started with a rate which was constant for up to 20 min (data not shown). Thereafter, the rate of protein synthesis decreased and prolonged incubation (25–60 min) resulted in a loss of incorporated [^{35}S]methionine, presumably caused by proteolytic degradation of the polypeptides. Therefore, we decided to use 25-min incubation times in subsequent protein synthesis experiments. For analysis

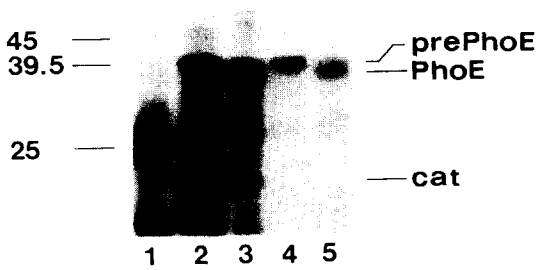

Fig. 1. Comparison of in vitro and in vivo synthesized proteins from transcripts of plasmid pJP29. Lanes 1 and 2: [^{35}S]methionine-labelled proteins of in vitro translation assays in the absence or presence of pJP29 transcripts, respectively. Lane 3: proteins from in vivo pulse-labelled CE1224 cells containing plasmid pJP29 induced for PhoE synthesis. Lanes 4 and 5: samples as in lane 2 and 3, respectively, were immunoprecipitated by rabbit antiserum against SDS-denatured PhoE protein. The positions of the molecular weight standard proteins (kDa) are shown on the left.

of the synthesized proteins, the translation products were separated on a polyacrylamide gel in SDS and visualized by fluorography (Fig. 1). A translation system without transcripts added, resulted in a low background synthesis caused by some translation of undegraded endogenous mRNA in the cell lysate (lane 1). The addition of transcripts from plasmid pJP29 yielded two major bands with apparent molecular weights of 40000 and 22000 and a minor band (M_r 20000) (lane 2). The 22000 protein represents the cytosolic chloramphenicol acetyl transferase (cat) and the origin of the minor band is unknown. To compare the 40000 product with the PhoE protein synthesized in vivo from the same plasmid, cells induced for PhoE protein synthesis were pulse-labelled for 30 s with [^{35}S]methionine. Under these conditions the precursor form of PhoE protein (prePhoE) with an apparent molecular weight of 40000 and the processed mature PhoE protein (M_r 37500) can readily be distinguished (lane 3) [29]. Antibodies specific for denatured PhoE protein were able to precipitate both PhoE and prePhoE synthesized in whole cells (lane 5) and the 40000 product from the cell-free system (lane 4). Thus, based on the co-migration of the 40000 product with prePhoE and the precipitation of this protein by PhoE-specific antibodies, the 40000 protein synthesized in the cell-free system is considered to be the precursor form of PhoE protein. Apparently, the product of the regulatory gene *phoB,* which is required for PhoE protein synthesis in vivo [32], is not necessary for the in vitro transcription of the *phoE* gene.

In vitro processing of prePhoE by leader peptidase

It has been shown that pure leader peptidase in detergent is able to process in vitro synthesized precursor proteins such as M13 procoat and pre-OmpA into their mature forms [37,38]. When increasing amounts of leader peptidase in 0.2% β-octyl glucoside were added during the translation of PhoE transcripts a new band of a lower molecular weight (37500) appeared (Fig. 2, lanes 1–3). The electrophoretic mobility of this product corresponded to the mature form of PhoE. After synthesis of prePhoE was completed and translation was inhibited by puromycin, the precursor could also be processed (lane 4).

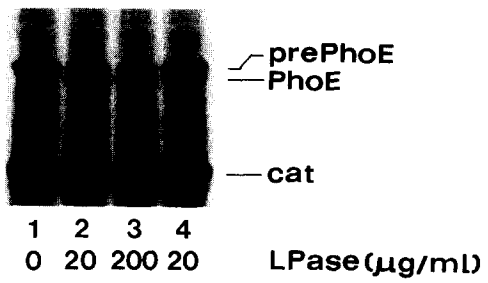

Fig. 2. In vitro processing of prePhoE by pure leader peptidase. Leader peptidase (LPase) was added at the indicated concentrations in the presence of 0.2% β-octyl glucoside during the translation of prePhoE (lanes 1–3). Lane 4: after synthesis of prePhoE was completed during an incubation of 25 min of the cell-free system and translation was inhibited by puromycin (1 mM) leader peptidase was added posttranslationally.

In vitro translocation

In order to obtain the same direction of translocation as in whole cells, inverted membrane vesicles isolated from the cytoplasmic membrane of *E. coli* have to be used in the in vitro system. In our experiments translocated proteins are defined as those proteins which are protected against externally added proteinase K. In the absence of

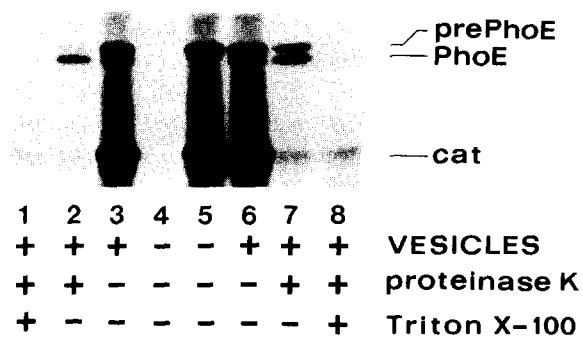

Fig. 3. In vitro cotranslational translocation of PhoE protein into inner membrane vesicles. Lanes 4 and 5: 5 and 25 min incubation of the cell-free system without vesicles added. A crude fraction, containing inner and outer membrane vesicles, isolated from spheroplasts (lanes 1–3) or from whole cells (lanes 6–8) was added in a concentration of 1 A_{280} U/ml 5 min after protein synthesis was started. The incubation was continued for 20 min. Samples shown in lanes 3 and 6 were directly prepared for gel electrophoresis. Samples in lanes 1, 2, 7 and 8 were incubated with proteinase K during 15 min at 37 °C (200 µg/ml) in the presence (lanes 1 and 8) or absence (lanes 2 and 7) of 1% Triton X-100.

vesicles the precursor was not converted to the mature form (Fig. 3, lane 5) and was completely digestible by proteinase K (results not shown). To study processing (conversion of prePhoE into PhoE) and translocation membrane vesicles were added to the cell-free system. The presence of the vesicles at the start of protein synthesis impaired the incorporation of [^{35}S]methionine into total protein for about 70%. Therefore, the vesicles were added 5 min after protein synthesis was initiated. At that time there was no detectable synthesis of fully elongated prePhoE yet (lane 4). After 20 min of continued incubation with a crude vesicle fraction, containing inner and outer membrane vesicles isolated from whole cells, part of the precursor was processed into the mature form (lane 6). Digestion of the unprotected proteins by proteinase K revealed that part of the precursor and most of mature PhoE remained (lane 7).

The translocation activity of these vesicles, defined as the percentage of the total PhoE protein (prePhoE + PhoE) synthesized in the presence of vesicles which was insensitive to proteinase K, was 23–33% under these conditions. Approximately 50% of the protected PhoE protein appears to be processed. When the membranes were disrupted by 1% Triton X-100 also the protected proteins became sensitive to the protease (lane 8). The same concentration of a crude fraction of vesicles isolated from spheroplasts (1 A_{280} U/ml) showed a slightly lower translocation activity (lanes 1–3), when compared to the vesicles isolated from whole cells. Despite this lower translocation activity these vesicles gave a relative higher processing of the translocated proteins or less protection of the precursor form (compare lanes 2 and 7).

The absolute amount of translocated proteins was constant for a concentration range of vesicles of 0.5–1.5 A_{280} U/ml, indicating that the amount of vesicles is not the rate limiting step of the translocation activity. Higher concentrations of 2.5–5.0 A_{280} U/ml impaired PhoE protein synthesis. Sucrose gradient purified inner membrane 1 and inner membrane 2 fractions gave similar results as crude fractions of vesicles concerning the processing of prePhoE and the translocation activity.

In vivo, PhoE protein is present in the outer membrane in a trimeric form. To investigate the possible formation of these trimers in vitro after translocation into vesicles derived from spheroplasts, the samples were incubated in the presence of SDS for 30 min at 37 °C instead of at 95 °C prior to application on a gel. Under these conditions the trimers are stable and migrate in the 70 000–80 000 range [39]. However, treating the samples in this manner resulted in two protein bands with the same electrophoretic mobility as the monomeric forms of prePhoE and PhoE.

Posttranslational translocation of PhoE protein can only be studied when protein synthesis is completely blocked. We observed that the addition of puromycin after a 25 min translation assay was sufficient to completely inhibit any protein synthesis during a following posttranslational translocation experiment (data not shown). With this posttranslational translocation assay it was possible to show that prePhoE was partly processed and part of the precursor and most of the mature form were protected against proteinase K, similar as in a cotranslational translocation assay. The translocation activity during the posttranslational incubation of membrane vesicles was 8–9%.

Energy requirements for translocation

We next studied the requirement of the protonmotive force and ATP for translocation and processing of prePhoE. The posttranslational translocation assay was used because protein synthesis is an ATP-dependent process. The results of some typical experiments are shown in Table I. Omission of ATP in the translocation mixture and preincubation of the vesicles for 5 min at 37 °C in the presence of the uncoupler FCCP (5 μM) resulted in a low level of translocation of prePhoE (11–17%) compared to a control incubation with untreated vesicles (taken as 100%). Depletion of residual ATP in the translation mixture with glucose and hexokinase (10 mM and 20 μg/ml, respectively) completely inhibited translocation.

In order to distinguish between the influence of the protonmotive force and ATP on prePhoE translocation and processing, ATP was depleted in the system and an electrochemical membrane potential was generated by the addition of succinate, measured as the quenching of ACMA fluo-

TABLE I

IMPORTANCE OF ATP AND PROTONMOTIVE FORCE (pmf) FOR THE POSTTRANSLATIONAL TRANSLOCATION OF PhoE PROTEIN INTO MEMBRANE VESICLES

Concentrations of the additions: ATP (4 mM), FCCP (5 μM), DCCD (100 μM), hexokinase (20 μg/ml), glucose (10 mM), ethanol (1.3%), succinate (20 mM) and $(NH_4)_2SO_4$ (64 mM).

ATP	pmf	Additions during		Relative translocation activity (%) [a]
		preincubation	translocation	
++	++	–	ATP	100
±	–	FCCP [b]	–	11–17
–	–	hexokinase + glucose [c], FCCP [b]	–	0
–	++	hexokinase + glucose [c]	succinate	0
++	–	FCCP, DCCD [b]	ATP	17–30
++	+	DCCD [b]	ATP	179
++	+	ethanol [b]	ATP	112
++	++	$(NH_4)_2SO_4$ + glucose [c]	ATP, succinate	39

[a] Translocation activity of a control posttranslational translocation was taken as 100%.

[b] Preincubation of the vesicles for 5 min at 37 °C.

[c] Preincubation of the puromycin-treated translation system for 10 min at 25 °C before the addition to the translocation mixture.

TABLE II

GENERATION OF A PROTONMOTIVE FORCE BY SUCCINATE AND ATP

Membranes	Relative fluorescence (%)				
	ACMA	succinate	KCN	ATP	FCCP
No membranes	100	97	94	–	–
Control membranes	100	0.4	106	–	–
No membranes	100	–	–	87	81
Control membranes	100	–	–	11	93
DCCD [a]	100	–	–	62	78
DCCD, FCCP [a]	100	–	–	86	–
Ethanol [a]	100	–	–	49	86

[a] Inner membrane vesicles were preincubated for 5 min at 37 °C with the following addition: DCCD (100 μM), FCCP (5 μM) or ethanol (1.3%). Fluorescence quenching was measured after the sequential addition of succinate (5 mM) and KCN (2 mM) or ATP (1 mM) and FCCP (5 μM).

rescence (Table II). Again, translocation did not occur, which proved the essential role of ATP in this process. To check if the presence of ATP alone is sufficient, the vesicles were first incubated with FCCP and DCCD (100 μM) to inhibit the hydrolysis of ATP by the endogenous H^+-ATPase. These preincubated vesicles were unable to generate a protonmotive force in the presence of ATP (Table II). A translocation mixture containing these vesicles and ATP (4 mM) was able to translocate 17–30% as compared to the control system. The ATP concentration measured after the incubation was 3.3 mM. This means that ATP was not limiting in this system, since after a control incubation 2.3 mM ATP was measured. Thus, the presence of a protonmotive force is not essential, but it increased the translocation of prePhoE strongly. Control experiments were carried out to verify the effect of the different additives in the translocation assay. In one control experiment, comparable to an experiment to deplete ATP concerning the incubation for 10 min at 25 °C and the additions except for hexokinase (a suspension in ammonium sulfate solution), a lower level of translocation was shown (39%). This decrease in translocation activity in this system did not account for the observed total inhibition of translocation in the absence of ATP. Preincubation of the vesicles with DCCD caused less quenching of the ACMA fluorescence induced by ATP (Table II), while the translocation of prePhoE across these vesicles is markedly increased (179%). The increase was not caused by an inhibition of the ATP hydrolysis, since ATP is in excess concentrations available, nor by the presence of ethanol in which DCCD is dissolved, which did not stimulate the translocation significantly. Thus, ATP and the protonmotive force were both required to give an optimal translocation of prePhoE into inner membrane vesicles.

Association of PhoE protein with membrane vesicles

To determine whether association between PhoE or its precursor with membrane vesicles exist in the absence of translocation and processing, we performed sedimentation experiments. The results of these experiments are shown in Table III. In the absence of vesicles most of the precursor remained in the supernatant, indicating

TABLE III

ASSOCIATION OF PROTEINS WITH MEMBRANE VESICLES IS INDEPENDENT OF TRANSLOCATION

Distribution of proteins between the pellet and supernatant fraction as a percentage of the total protein synthesized. cat, cytoplasmic protein chloramphenicol acetyltransferase.

Incubation conditions of proteins		Distribution of protein (%)	
		super-natant	pellet
Without vesicles	prePhoE	80	20
	PhoE [b]	94	6
	cat	97	3
Cotranslational translocation	(pre)PhoE	12	88
	cat	80	20
Inhibited translocation [a]	prePhoE	8	92
Posttranslational incubation	PhoE [b]	22	78

[a] Translocation was inhibited in an ATP-depleted system.
[b] PhoE protein was synthesized during a cotranslational incubation with leader peptidase.

that prePhoE does not aggregate into larger particles under these conditions. After a cotranslational incubation 88% of (pre)PhoE sedimented with the vesicles, while most of the cytoplasmic protein chloramphenicol acetyltransferase remained in the supernatant fraction (80%). Part of the pelleted (pre)PhoE protein consist of the translocated proteins (97% of the protected proteins were pelleted with the vesicles, data not shown). The other part could represent incompletely translocated proteins still sensitive to proteinase K. This kind of association is probably not the only possibility as we demonstrated that in the absence of translocation (in an ATP-depleted system) more than 90% of the formed precursor was bound to the membranes.

We also studied the possible association of in situ synthesized mature PhoE with the vesicles. First the precursor was converted into the mature form during a cotranslational incubation with leader peptidase (200 μg/ml). The mature PhoE was then incubated with the vesicles in a translocation assay where posttranslational translocation of prePhoE was demonstrated. Also PhoE became associated with the membranes (Table III), but was still sensitive to proteinase K (data not shown). In a control experiment, it was shown that a low concentration of β-octyl glucoside (0.2%) did not influence the protection of translocated (pre)PhoE during incubation with proteinase K. In the absence of vesicles, PhoE remained in the supernatant.

Discussion

In this paper a cell-free system from *E. coli* capable of translation and translocation of PhoE protein is described. Transcripts from plasmid pJP29 direct the synthesis of the precursor of PhoE protein. PrePhoE can be processed into mature PhoE by pure leader peptidase in detergent or in the presence of plasma membrane vesicles. In the latter case PhoE protein is translocated into the vesicles as judged by the protection of PhoE against externally added proteinase K.

PrePhoE is most likely correctly processed by pure leader peptidase as only one defined cleavage product with the same electrophoretic mobility as mature PhoE has been found. Although we could not exactly quantify the relative amounts of precursor and mature form separately, the data suggest that processing of prePhoE in the presence of vesicles only occurred for translocated proteins. However, one cannot conclude from our results that processing and translocation are strictly coupled, because also some precursor was found to be translocated without cleavage of the leader peptide. In vivo, it was earlier demonstrated that translocation was not necessarily coupled to processing [40,41]. The presence of translocated prePhoE in our in vitro system could not be due to a low concentration or activity of the leader peptidase, because the ratio of protected prePhoE and PhoE does not change during a 4 to 25 min cotranslational incubation or after a 20 min chase period (with 100 μM non-radioactive methionine added to the system) in which the total amount of translocated proteins remained the same (results not shown). Therefore, it appears that in vitro two types of protected PhoE protein exist. One that is translocated and processed in the normal way, and another which becomes protected by the vesicles but cannot be cleaved by the leader peptidase. In both cases ATP and the protonmo-

tive force appear to be required for the translocation step.

During a cotranslational incubation only 23–33% of the total PhoE protein synthesized becomes insensitive to proteinase K. This relative low amount was not due to a limiting vesicle concentration as higher concentrations did not stimulate the translocation activity. In similar in vitro systems, other outer membrane proteins, such as LamB and OmpA [15,16] were also synthesized as a larger precursor and only partially translocated (25–30%) into inner membrane vesicles. Müller and Blobel [42] showed that a partially purified soluble factor was able to stimulate the translocation activity of LamB protein and two periplasmic proteins. It thus could be that the amount of such a factor is limited in these in vitro systems.

The translocation activity during a posttranslational incubation was lower (8–9%) as compared with a cotranslational incubation (23–33%). This does not mean that posttranslational translocation is less efficient, because one cannot distinguish between co- and posttranslational translocation of prePhoE in a cotranslational assay. The possibility exist that the involvement of a soluble factor for translocation of prePhoE is cotranslationally required.

Our experiments suggests that ATP is absolutely essential for transport and the protonmotive force greatly contributes to an optimal translocation process of PhoE protein in vitro. These results correspond with recent published experiments for alkaline phosphatase and OmpA protein performed with H^+-ATPase deficient membrane vesicles isolated from mutant strains [18,19]. Chen and Tai [18] reported an even less important role for the electrochemical membrane potential. Membrane vesicles devoid of H^+-ATPase could use ATP for protein translocation as efficiently as H^+-ATPase containing vesicles, generating a membrane potential. But generation of a protonmotive force by a substrate of the respiratory chain in these H^+-ATPase deficient vesicles could stimulate protein translocation, even when an excess ATP concentration was present, indicating that the protonmotive force was able to facilitate the process.

In vivo experiments clearly showed that upon dissipation of the protonmotive force by uncouplers, the processing of precursor proteins and translocation across the plasma membrane is slowed down without altering the intracellular level of ATP [43,44]. Recently it became possible to uncouple the synthesis and the translocation of a secretory protein into mammalian rough endoplasmic reticulum. This allowed to investigate the energy requirement for this process and it appeared that the posttranslational translocation into these microsomes was energy dependent [1].

We studied the membrane association properties of PhoE protein and found that even in the absence of translocation (thus without ATP) the precursor form became spontaneously associated to the vesicles. Association of a precursor protein with the membrane is necessary to initiate the translocation of that protein. Therefore, the transport of PhoE protein across the inner membrane of *E. coli* could consist of a process of three sequential events: (1) association, (2) translocation and (3) processing.

Although proteins certainly are involved in the processing step and possibly in the translocation of the precursor, membrane lipids could very well play a role in the association and translocation step. Such lipid involvement in protein translocation has already been shown for M13 procoat and apocytochrome *c* which do not require any membrane proteins for both the association and insertion and (partial) translocation into model membranes [45,46,10]. The requirement of lipids for the translocation process of PhoE will now be investigated with the in vitro system described in this paper.

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